JONATAN ERSCHING

**Estudo das bases celulares e moleculares do controle da resposta
imune mediada por linfócitos T CD8⁺ específicos durante a infecção
experimental por *Trypanosoma cruzi* e vacinação com vetor
adenoviral recombinante**

Tese apresentada ao Programa de Pós-Graduação em Imunologia do Instituto de Ciências Biomédicas da Universidade de São Paulo para obtenção do Título de Doutor em Ciências.

São Paulo
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Orientador: Prof. Dr. Maurício Martins Rodrigues
Coorientadora: Prof.^a Dr.^a Karina Ramalho Bortoluci

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Candidato(a): Jonatan Ersching.

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **110** nas fls. **92** do livro **02** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a) Maurício Martins Rodrigues, Coordenador(a) da Linha de pesquisa **Estudo das bases celulares e moleculares do controle da imunodominância da resposta imune de linfócitos T CD8+específicos induzidos pela infecção experimental por Trypanosoma cruzi ou imunização com adenovírus recombinantes** do qual participam o(s) alunos **Jonatan Ersching**, está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA) em **21.09.2010, com validade de 3 anos.**

São Paulo, 22 de setembro de 2010.

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Prezado Professor,

Sua licença para uso de animais em experimentação, constante no protocolo em epígrafe, foi prorrogada até 21.09.2016.

Reitero que havendo alteração de metodologia e inserção de novos alunos ao projeto de pesquisa vinculado à referida licença a CEUA/ICB deverá ser informada.

Cordialmente,

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador - CEUA-ICB/ /USP

Ilmo. Sr.
Prof. Dr. Maurício Martins Rodrigues
Departamento de Imunologia
Instituto de Ciências Biomédicas - USP

Trabalho realizado no Laboratório de Desenvolvimento de Vacinas Recombinantes contra Protozoários Patogênicos do Centro de Terapia Celular e Molecular da Universidade Federal de São Paulo – Escola Paulista de Medicina sob orientação do Prof. Dr. Maurício Martins Rodrigues. Projeto financiado pela CAPES, INCTV/CNPq e FAPESP e realizado com bolsa de doutorado direto Processo FAPESP 2010/09361-8.

"Aqui estão os loucos. Os desajustados. Os rebeldes. Os encenqueiros. Os que fogem ao padrão. Aqueles que veem as coisas de um jeito diferente. Eles não se adaptam às regras, nem respeitam o *status quo*. Você pode citá-los ou achá-los desagradáveis, glorificá-los ou desprezá-los. Mas a única coisa que você não pode fazer é ignorá-los. Porque eles mudam as coisas. Eles empurram adiante a raça humana. E enquanto alguns os veem como loucos, nós os vemos como gênios. Porque as pessoas que são loucas o bastante para pensarem que podem mudar o mundo são as únicas que realmente podem fazê-lo."

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Gautama Buddha

"Desaprender oito horas por dia ensina os princípios."

Manoel de Barros

Para Larissa.
Para meus pais.
Para meus Professores.

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RESUMO

ERSCHING, J. **Estudo das bases celulares e moleculares do controle da resposta imune mediada por linfócitos T CD8⁺ específicos durante a infecção experimental por *Trypanosoma cruzi* e vacinação com vetor adenoviral recombinante.** 2015 237 f. Tese (Doutorado em Imunologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015

O reconhecimento por linfócitos T CD8⁺ de antígenos expostos no contexto de MHC classe I é fundamental para o controle de tumores e de infecções intracelulares virais, fúngicas, bacterianas e parasitárias. No entanto, o controle da indução destes linfócitos é ainda incompletamente conhecido. *Trypanosoma cruzi* é o protozoário intracelular causador da doença de Chagas, uma patologia crônica cardíaca e/ou digestiva largamente negligenciada. O controle da infecção pelo *T. cruzi* depende da resposta de linfócitos T CD8⁺. Porém, o parasito evade a imunidade do hospedeiro através da indução subótima destes linfócitos e alteração do seu fenótipo para um perfil pró-apoptótico. Esta resposta subótima pode ser corrigida pela vacinação genética com adenovírus recombinantes. No entanto, não se sabe como *T. cruzi* induz uma resposta defeituosa dos linfócitos T CD8⁺, nem como o vírus reverte esta resposta. Estas questões são importantes para o entendimento geral da imunidade mediada por linfócitos T CD8⁺ e foram objeto de estudo da presente tese. Inicialmente, foi caracterizada *in vitro* a via de processamento dos epítomos de *T. cruzi* restritos a MHC classe I, que é citosólica: dependente do proteassomo, TAP e realçada pelo imunoproteassomo, um complexo molecular análogo ao proteassomo convencional, porém com subunidades catalíticas alteradas. O envolvimento do imunoproteassomo na indução de imunidade dos linfócitos T CD8⁺ foi investigado *in vivo* utilizando animais triplamente deficientes das subunidades β1i, β2i e β5i (TKO). Estes animais exibiram uma resposta de linfócitos T CD8⁺ drasticamente reduzida após a infecção por *T. cruzi* ou vacinação genética, avaliada pela frequência e número de linfócitos específicos, bem como pela diferenciação *in vivo* e função efetora destas células. Entretanto, a resposta dos linfócitos T CD4⁺ não foi alterada. Além disso, os clones de linfócitos T CD8⁺ ativados pela infecção ou vacinação foram diferentes nos animais TKO em comparação aos normais. Surpreendentemente, ainda, a resistência à infecção na ausência do imunoproteassomo foi seriamente comprometida e a proteção induzida pela vacinação foi completamente abolida, indicando que o imunoproteassomo é essencial para o processamento dos epítomos de *T. cruzi* relacionados à imunidade protetora gerada tanto durante a infecção como na vacinação. Na segunda parte desta tese, foi observado que *T. cruzi*, mas não adenovírus, é capaz de tornar uma resposta ótima de linfócitos transgênicos induzidos *in vivo* em subótima. Esta interferência reproduziu o fenótipo pró-apoptótico descrito para os clones de linfócitos T CD8⁺ que naturalmente reconhecem o parasito, mas foi independente da molécula CD95. A interferência do parasito envolveu a supressão ativa do *priming* dos linfócitos T CD8⁺ mediada por linfócitos T CD4⁺ CD25⁻ Foxp3⁺ de modo independente de IL-10, mas parcialmente dependente de TGF-β e CTLA-4. Animais depletados de células Foxp3⁺ infectados com *T. cruzi* controlaram melhor a parasitemia e tiveram uma resposta mais intensa de linfócitos T CD8⁺ específicos. Estes dados sugerem pela primeira vez a participação de linfócitos T CD4⁺ CD25⁻ Foxp3⁺ na supressão do *priming* de linfócitos T CD8⁺ e representam um mecanismo importante para a evasão da resposta imune pelo *T. cruzi*, o que não ocorre durante a vacinação genética.

Palavras-chave: Linfócitos T CD8⁺. *Trypanosoma cruzi*. Vacina. Adenovírus. Foxp3. Imunoproteassomo.

ABSTRACT

ERSCHING, J. **Study of the cellular and molecular basis of specific CD8⁺ T lymphocyte immune response control during experimental infection by *Trypanosoma cruzi* and vaccination with recombinant adenoviral vector.** 2015 237 p. Ph.D. Thesis (Immunology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015

The recognition by CD8⁺ T lymphocytes of antigens displayed on the context of MHC class I molecules is a fundamental requirement for the control of tumors and intracellular infections by viruses, fungi, bacteria and parasites. However, the control of the induction of these lymphocytes is yet ill defined. *Trypanosoma cruzi* is the intracellular protozoan that causes Chagas disease, a highly neglected cardiac and/or digestive chronic pathology. The control of *T. cruzi* infection is dependent on CD8⁺ T cell response. However, the parasite evades host immunity through the suboptimal induction of these lymphocytes and shift of their phenotype into a proapoptotic profile. This suboptimal response can be corrected by genetic vaccination with recombinant adenoviruses. Although, it is unknown how *T. cruzi* induces the poor response of CD8⁺ T cells, and how adenovirus circumvents it. These questions are important for the general understanding of CD8⁺ T lymphocytes-mediated immunity and were subject of investigation in this thesis. Initially, the processing pathway of MHC class I-restricted epitopes from *T. cruzi* was characterized *in vitro*, which is cytosolic: dependent on proteasome, TAP, and enhanced by the immunoproteasome, a molecular complex analogous to the conventional proteasome, but featuring altered catalytic subunits. The involvement of the immunoproteasome in the induction of CD8⁺ T lymphocyte immunity was investigated *in vivo* using triply deficient animals in the subunits $\beta 1i$, $\beta 2i$ and $\beta 5i$ (TKO). These mice exhibited a drastically reduced response of CD8⁺ T cells after *T. cruzi* infection or genetic vaccination, assessed by the frequency and numbers of epitope-specific lymphocytes, as well as their differentiation and function *in vivo*. Despite, the response of CD4⁺ T cells was unaltered. Moreover, CD8⁺ T cell clones activated by infection or vaccination were different in TKO animals compared to normal mice. Surprisingly, yet, resistance to infection in the absence of immunoproteasomes was seriously compromised and the protection induced by vaccination completely abolished, indicating that the immunoproteasome is essential for the processing of *T. cruzi* epitopes related to protective immunity generated during infection or vaccination. In the second part of this thesis, was observed that *T. cruzi*, rather than adenovirus, is capable of modifying an optimal response of *in vivo*-induced transgenic CD8⁺ T cells to suboptimal. This interference resembled the proapoptotic phenotype of naturally occurring CD8⁺ T cell clones that recognize the parasite, although it was independent of CD95 molecule. Parasite interference involved the active suppression of CD8⁺ T cell priming mediated by CD4⁺ CD25⁻ Foxp3⁺ T cells in an IL-10-independent, but TGF- β and CTLA-4 partially dependent manner. Foxp3⁺ cells-depleted animals infected with *T. cruzi* controlled better the parasitemia and presented a stronger response of specific CD8⁺ T cells. These data suggest for the first time the involvement of CD4⁺ CD25⁻ Foxp3⁺ T cells in the suppression of CD8⁺ T cell priming and represent an important mechanism for immunity evasion by *T. cruzi*, which does not occur during genetic vaccination.

Keywords: CD8⁺ T lymphocytes. *Trypanosoma cruzi*. Vaccine. Adenovirus. Foxp3. Immunoproteasome.

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LISTA DE SÍMBOBLOS, ABREVIATURAS E SIGLAS

⁻ - negativo

^{-/-} - geneticamente deficiente em homozigose

^{-/+} - geneticamente deficiente em hemizigose

% - por cento

°C - graus Celsius

⁺ - positivo

x - vezes

7-AAD – do inglês, *7-aminoactinomycin D*

ACK – do inglês, *amonium-chloride-potassium lysing buffer*

Actb – gene da beta actina

AdASP-2 – vetor adenoviral inserido de *Asp2*

Adβ-gal – vetor adenoviral inserido de *Glb1*

ANOVA – do inglês, *analysis of variance*

APC – do inglês, *allophycocyanin*

ASC – do inglês, *apoptosis-associated speak-like protein containing CARD*

ASP-2 – do inglês, *amastigote surface protein 2*

Asp2 – gene da ASP-2

ATPases – adenilpirofosfatase

Bcl-2 – do inglês, *B cell lymphoma 2*

BMDC – do inglês, *bone marrow-derived dendritic cell*

BrdU – 5-bromo-2-deoxiuridina

BSA – do inglês, *bovine serum albumin*

BTLA – do inglês, B and T lymphocyte attenuator precursor

Ca²⁺ - cálcio

CBA – do inglês, *cytometric bead array*

CCR – do inglês, *C-C chemokine receptor*

CD – do inglês, *cluster of differentiation*

cDNA – do inglês, *complementary deoxyribonucleic acid*

Cia. - companhia

CpG ODN – do inglês, *CpG oligodeoxynucleotides*

CTLA-4 – do inglês, *cytotoxic T-lymphocyte associated protein 4*

CXCR – do inglês, *C-X-C chemokine receptor*

Cy7 – do inglês, *cyano 7*

CZ – cruzi

DAB – 3,3-diaminobenzidina

Dapl1 – do inglês, *death associated protein-like 1*

DC – do inglês, *dendritic cell*

DMSO – dimetilsulfóxido

DNA – do inglês, *deoxyribonucleic acid*

DRiPs – do inglês, *defective ribosomal products*

DTR – do inglês, *diphtheria toxin receptor*

EDTA – do inglês, *ethylenediamine tetraacetic acid*

ELISA – do inglês, *enzyme-linked immuno sorbent assay*

ELISPOT – do inglês, *enzyme-linked immuno-spot*

Eomes – eomesodermina

EPO – epoxomicina

F – do inglês, *forward*

FACS – do inglês, *fluorescence-activated cell sorting*

Fas – membro 6 da superfamília de receptores de TNF

FasL – ligante de Fas

FITC – do inglês, *fluorescein isothiocyanate*

Foxp3 – do inglês, *forkhead box P3*

FoxP3 – gene da Foxp3

g – aceleração gravitacional

GAPDH – do inglês, *glyceraldehyde-3-phosphate-dehydrogenase*

Gapdh – gene da GAPDH

GATA-3 – do inglês, *GATA-binding protein 3*

GFP – do inglês, *green fluorescent protein*

GITR – do inglês, *glucocorticoid-induced TNF receptor-related protein*

Glb1 – gene da beta galactosidase 1

GM-CSF – do inglês, *granulocyte macrophage colony stimulating factor*

gMFI – do inglês, *geometric median of fluorescence intensity*

GP- glicoproteína

Gr. – grupo

h – hora

H-2K^b-SIINFEKL – tetrâmero de moléculas H-2 ligadas ao epítopo SIINFEKL

HeLa – linhagem celular retirada da Henrietta Lacks
HIV – do inglês, *human immunodeficiency virus*
HPRT – do inglês, *hypoxanthine-guanine phosphoribosyltransferase*
Hprt – gene da HPRT
i.m. – intramuscular
i.p. – intraperitoneal
i.v. – intravenoso
ICS – do inglês, *intracellular staining*
IFN – interferon
Ig – imunoglobulina
Igfbp4 – do inglês, *insulin-like growth factor-binding protein 4*
IL – interleucina
KLRG1 – do inglês, *killer cell lectin-like receptor G1*
L – litro
LAG-3 – do inglês, *lymphocyte activation gene 3*
LCMV – do inglês, *lymphocytic choriomeningitis virus*
LFA-1 – do inglês, *lymphocyte function-associated antigen 1*
LMP – do inglês, *large multifunctional proteasome*
lpr – mutação que causa linfoproliferação por perda da função de CD95
LPS – lipopolissacarídeo
m – mili
M – molar
MECL1 – do inglês, *multicatalytic endopeptidase complex-like 1*
MHC – do inglês, *major histocompatibility complex*
mRNA – do inglês, *messenger ribonucleic acid*
Myd88 – do inglês, *myeloid differentiation protein 88*
n – nano
NaCl – cloreto de sódio
NLRP3 – do inglês, *NOD-like receptor pyrin domain containing 3*
NOD1 – do inglês, *nucleotide binding oligomerization domain containing protein 1*
NP – nucleoproteína
Nrp1 – neuropilina 1
ns – não significativo

OTI – TCR transgênico específico para o epítipo da ovalbumina restrito a MHC classe I SIINFEKL

p – pico

P – probabilidade estatística de se obter o mesmo resultado ao acaso

PA28 $\alpha\beta$ – do inglês, *proteasome activator 28 $\alpha\beta$*

PB – do inglês, *pacific blue*

PBS – do inglês, *phosphate buffered saline*

PCR – do inglês, *polymerase chain reaction*

PD-1 – do inglês, *programmed cell death 1*

PD-L1 – do inglês, *programmed cell death ligand 1*

pDC – do inglês, *plasmacytoid dendritic cell*

PE – do inglês, *phycoerythrin*

pep - peptídeo

PerCP – do inglês, *peridinin chlorophyll protein*

PFU – do inglês, *plaque forming units*

Psmb – gene *proteasome subunit beta-type*

R – do inglês, *reverse*

RA – do inglês, *retinoic acid*

ROR γ -t – do inglês, *RAR-related orphan receptor gamma t*

RPMI-1640 – meio de cultura *Roswell Park Memorial Institute -1640*

RT – do inglês, *reverse transcription*

S – valor de sedimentação

s.c. – subcutâneo

SDS – do inglês, *sodium dodecyl sulfate*

SFC – do inglês, *spot forming colony*

SIV – do inglês, *simian immunodeficiency virus*

Swap70 – do inglês, *switch B-cell complex 70kDa subunit*

T-bet – do inglês, *T-box expressed in T cells*

TAP – do inglês, *transporter associated with antigen processing*

Tap1 – gene da TAP-1

TCR – do inglês, *T cell receptor*

TGF- β – do inglês, *transforming growth factor beta*

Tim-3 – do inglês, *T cell immunoglobulin mucin 3*

TKO – do inglês, *triple knockout*

TLR – do inglês, *Toll like receptor*

TNF – do inglês, *tumor necrosis factor*

TNFR-1 – do inglês, *tumor necrosis factor receptor 1*

TRAIL-R2 – do inglês, *TNF-related apoptosis-inducing ligand receptor 2*

TRANCE – do inglês, *TNF-related activation-induced cytokine receptor*

Treg – célula T reguladora

TRIF – do inglês, *TIR-domain-containing adapter-inducing interferon beta*

Tris-HCl – trisaminometano com pH corrigido com ácido clorídrico

TS – trans-sialidase

TSA – do inglês, *trypomastigote surface antigen 1*

U.V. – ultravioleta

V α - região variável da cadeia alfa do TCR

V β – região variável da cadeia beta do TCR

WT – do inglês, *wild type*

α – alfa

β – beta

γ - gama

$\Delta \Delta$ Ct – do inglês, delta delta cycle threshold

μ – micro

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1 INTRODUÇÃO

1.1 Papel dos linfócitos T CD8⁺ no controle de infecções

Os linfócitos T CD8⁺ são células do sistema imune adaptativo que possuem a elegante habilidade de reconhecer o ambiente intracelular de outras células e responder às perturbações deste meio, eliminando células anômalas. Este reconhecimento decorre da ubíqua exposição de peptídeos citosólicos ligados a moléculas de MHC classe Ia na superfície celular. O complexo peptídeo-MHC classe Ia é capaz de interagir com o TCR de linfócitos T CD8⁺, como revisado recentemente em detalhe (ROSSJOHN et al., 2015), e (no caso do peptídeo sinalizar uma condição de infecção ou anomalia) desencadear a proliferação, diferenciação e função citotóxica destes linfócitos. Este mecanismo de reconhecimento mediado pela interação MHC Ia-TCR sustenta o controle de tumores e infecções intracelulares virais, bacterianas, fúngicas e parasitárias, bem como a rejeição de transplantes; e seu descobrimento foi de tamanha importância para a Imunologia que rendeu o Prêmio Nobel de Medicina à Rolf Martin Zinkernagel e Peter Doherty em 1996 (HAMMERLING, 1997; ZINKERNAGEL, 1974; ZINKERNAGEL; DOHERTY, 1974).

A relevância dos linfócitos T CD8⁺ para os animais dotados de sistema imune adaptativo, *Gnathostomata*, também pode ser sugerida pelo sequenciamento do genoma do bacalhau do Atlântico (*Gadus morhua*). Evolutivamente, este peixe mandibulado perdeu todos os genes de MHC classe II, CD4 e cadeia invariante, o que não o tornou mais susceptível a infecções no ambiente natural. A imunidade deste animal depende de um número expandido de genes de MHC classe I, sugerindo que o reforço da resposta por diferentes subtipos de linfócitos T CD8⁺ poderia compensar a falta de imunidade mediada por linfócitos T CD4⁺ (STAR et al., 2011). Nos mamíferos, os mecanismos específicos de controle de infecções intracelulares por linfócitos T CD8⁺ são múltiplos e a preponderância de cada um em particular varia de acordo com o patógeno (WONG; PAMER, 2003). Os patógenos mais amplamente utilizados pela comunidade científica para estudar a resposta de linfócitos T CD8⁺ em modelos murinos são o vírus *Lymphocytic Choriomeningitis* (LCMV) e a bactéria *Listeria monocytogenes*. Estes foram os primeiros patógenos utilizados nos trabalhos seminais de Zinkernagel e são bons modelos experimentais porque induzem uma resposta de linfócitos T CD8⁺ bastante conspícua. No entanto, inúmeros outros patógenos são estudados em modelos experimentais que

contribuem para o melhor entendimento de como se dá a resposta de linfócitos T CD8⁺. Estes incluem os vírus da influenza (SCHLIE et al., 2015), herpes (CUBURU et al., 2015), hepatite B (GUIDOTTI et al., 2015), HIV/SIV (MUDD et al., 2012), vírus sincicial respiratório (ROSSEY et al., 2014) e vaccinia (ARAKI et al., 2009); as bactérias *Mycobacterium tuberculosis* (TZELEPIS et al., 2015) e *Salmonella typhimurium* (BINDER et al., 2013); bem como os protozoários *Plasmodium spp* (RADTKE et al., 2015), *Leishmania spp* (GAUTAM et al., 2014), *Toxoplasma gondii* (GROVER et al., 2014) e *Trypanosoma cruzi* (VASCONCELOS; BRUNA-ROMERO; et al., 2012).

Os linfócitos T CD8⁺ podem secretar citocinas importantes para o controle de infecções, como IL-2, IFN- γ e TNF. Outro mecanismo relevante de efetuação da resposta imune mediada por linfócitos T CD8⁺ corresponde à citotoxicidade: indução de morte celular da célula-alvo infectada, a qual pode ser induzida por inúmeras vias (LIEBERMAN, 2010), como a interação de FasL (CD178) no linfócito T CD8⁺ com Fas (CD95) na célula-alvo, ou ainda pela liberação de vesículas contendo TNF e linfotoxina pelo linfócito, as quais ligam-se ao receptor TNFR-1 na célula-alvo. Tanto Fas como TNFR-1 possuem domínios citoplasmáticos de morte que recrutam as caspases 8 e 10 e levam à apoptose. Outra forma de induzir morte da célula-alvo consiste na liberação de grânulos citotóxicos pelos linfócitos T CD8⁺ (YANNELLI et al., 1986). Estes grânulos podem conter moléculas que abrem poros na membrana da célula-alvo e causam morte celular. Estas moléculas incluem perforinas, proteínas monoméricas que formam poros na membrana ao se polimerizarem de modo dependente de Ca²⁺; e os poros formados permitem a entrada de granzimas, que são serina esterases que podem interferir com vias intracelulares na célula-alvo, levando à degradação do DNA e apoptose (LIEBERMAN, 2010).

O reconhecimento de antígenos pelos linfócitos T CD8⁺ é necessário tanto para a ativação inicial destes linfócitos quanto para sua ação efetora e depende do processamento e apresentação de epítopos ligados a moléculas de MHC classe Ia. Enquanto qualquer célula que expressa MHC classe Ia é virtualmente capaz de se tornar uma célula-alvo da resposta efetora de linfócitos T CD8⁺, apenas as células apresentadoras de antígeno profissionais são postuladas como capazes de induzir a ativação inicial de linfócitos T *naïve* (STEINMAN; WITMER, 1978). Esta ativação inicial depende, além da presença de epítopos ligados a moléculas de MHC, do reconhecimento de padrões moleculares por receptores do sistema imune inato que

ativam vias nas células apresentadoras de antígenos que levam à secreção de citocinas e expressão de moléculas coestimuladoras, sinalizando assim um contexto inflamatório (JANEWAY, 1989). As células dendríticas (DC) são ótimas provedoras destes três sinais e acredita-se que sejam as melhores células capazes de induzir uma resposta primária de linfócitos T CD8⁺. Esta ativação pode ocorrer tanto pela apresentação direta de antígenos presentes no citosol que são processados via proteassomo e transportadas por TAP para o retículo endoplasmático, onde se ligam a moléculas de MHC classe I recém-sintetizadas, quanto pela apresentação cruzada de antígenos de origem exógena que, por diferentes vias, citosólicas ou endossomais, acessam as moléculas de MHC classe I (JOFFRE et al., 2012; NAIR-GUPTA; BLANDER, 2013). Corroborando a importância da apresentação cruzada para o controle de infecções, inúmeros modelos experimentais utilizando microscopia intravital sugerem que subtipos específicos de DC capturam antígenos no tecido infectado, migram e transferem estes antígenos para as DC residentes nos linfonodos drenantes, onde ocorre a indução primária da resposta dos linfócitos T CD8⁺ (JOHN et al., 2009; ROTHFUCHS et al., 2009), enquanto outros modelos sugerem que as DC diretamente infectadas são as mais relevantes para induzir respostas primárias de linfócitos T CD8⁺ (DUPONT et al., 2014; RADTKE et al., 2015). Estes linfócitos T CD8⁺ *naïve* podem se diferenciar em inúmeros subtipos, controlados por citocinas, fatores de transcrição e modificações epigenéticas (GRAY; KAECH; STARON, 2014). De modo simplificado, estes subtipos podem ser agrupados em células efetoras (com fenótipo CD62L^{baixo}, CD44^{alto}, KLRG1^{alto}, CD127^{baixo}), que recirculam e controlam a infecção; em células de memória efetora (com fenótipo CD62L^{baixo}, CD44^{alto}, KLRG1^{alto}, CD127^{alto}), residentes no tecido; ou ainda em células de memória central (com fenótipo CD62L^{baixo}, CD44^{baixo}, KLRG1^{baixo}, CD127^{alto}), residentes no linfonodo e que sustentam respostas anamnéticas quando de uma infecção secundária (BELZ; KALLIES, 2010; OBAR; LEFRANCOIS, 2010; SALLUSTO; GEGINAT; LANZAVECCHIA, 2004). Ainda, os linfócitos T CD8⁺ podem ser regulados e induzidos a estados de tolerância, anergia ou ignorância imunológica (AYRES; SCHNEIDER, 2012; BILATE; LAFAILLE, 2012; MAEDA et al., 2014).

Em todas as etapas de ativação primária ou secundária de linfócitos T CD8⁺ descritas acima já foram relatados mecanismos de evasão dos patógenos, levando a um controle subótimo da infecção, que comumente progride para uma fase crônica.

Estes mecanismos incluem a indução de morte de células apresentadoras de antígeno (AMARAL et al., 2014), falta ou inibição ativa do reconhecimento pelos receptores do sistema imune inato (PADILLA; SIMPSON; TARLETON, 2009; YEN et al., 2014), produção de sinais anti-inflamatórios pelas células apresentadoras de antígenos (FU et al., 2015; NIESEN et al., 2015; RUFFELL et al., 2014), a modulação negativa do processamento de epítomos e da expressão de MHC classe I (SMED-SORENSEN et al., 2012; STEERS et al., 2009), a alteração funcional das DC por citocinas (HAQUE et al., 2014), a indução de morte dos linfócitos T CD8⁺ (CHEN et al., 2015; DAY et al., 2014; GUILLERMO et al., 2007), a alteração da capacidade de migração das células apresentadoras de antígenos ou dos linfócitos T CD8⁺ (COLEMAN et al., 2015; HERMIDA et al., 2014; LEE et al., 2014), bem como inúmeros mecanismos de regulação ativa desengatilhada pelas DC (CARRANZA et al., 2012; LUO et al., 2014; MASCANFRONI et al., 2013; NORRIS et al., 2013).

Em muitos casos, ainda, a indução subótima dos linfócitos T CD8⁺ pode ser acompanhada de constante estimulação antigênica e a progressão para uma fase crônica de infecção pode levar a um estado de exaustão destes linfócitos (GAUTAM et al., 2014), ou ainda de erosão da resposta imune (GRUJIC et al., 2010). Em outros casos, os linfócitos T CD8⁺ cronicamente estimulados podem se ativar e passar a responder contra o tecido do hospedeiro, levando a quadros de imunopatologia mediada por linfócitos citotóxicos (MARTIN et al., 2007).

Por outro lado, a utilização de vetores que são sabidamente ótimos na indução de resposta de linfócitos T CD8⁺ pode ser cooptada para a entrega de antígenos de patógenos que naturalmente evadem esta resposta. Esta estratégia tem sido amplamente estudada para o desenvolvimento de vacinas recombinantes contra os três patógenos de maior impacto no mundo: HIV, *Mycobacterium tuberculosis* e *Plasmodium spp* (BOURZAC, 2014), além de outros agentes infecciosos importantes (ERSCHING; PINTO, 2009; KOUP; DOUEK, 2011; RODRIGUES; ERSCHING, 2015). Apesar de atualmente nenhuma vacina baseada na indução de linfócitos T CD8⁺ estar disponível no mercado, diversos estudos de prova de conceito sustentam sua possibilidade teórico-científica. Especialmente, o emprego de vetores genéticos virais, como vaccínia, adenovírus, influenza, ou o vírus da febre amarela figuram entre as estratégias mais promissoras para a indução de linfócitos T CD8⁺ por vacinação.

1.2 Imunoproteassomo

A degradação de proteínas celulares maduras, sejam próprias ou derivadas de patógenos, bem como a degradação de produtos defectivos do ribossomo (DRiPs), gerados durante a síntese de proteínas novas, estão intimamente associadas à produção de peptídeos restritos a MHC classe Ia. A cinética e eficiência desta produção é estreitamente ligada à indução de linfócitos T CD8⁺ (ANTON; YEWDELL, 2014; DE GRAAF et al., 2011).

A degradação de proteínas e DRiPs é mediada por estruturas em forma de barril, denominadas proteassomos, que se localizam no citoplasma e núcleo. As proteínas que são endereçadas ao proteassomo normalmente contêm resíduos poliubiquitinados onde cada monômero de ubiquitina está ligado ao outro pelo resíduo de lisina da posição 48. O proteassomo é formado por uma partícula catalítica 20S cilíndrica constituída de quatro anéis empilhados, cada um formado de sete subunidades (BLUM; WEARSCH; CRESSWELL, 2013; COUX; TANAKA; GOLDBERG, 1996). Os dois anéis internos são formados por subunidades β , das quais três ($\beta 1$, $\beta 2$ e $\beta 5$) possuem atividade catalítica de caspase, quimotripsina ou tripsina. Portanto, cada proteassomo possui seis sítios com atividade catalítica que são expostos à face interna do cilindro 20S. Os dois anéis externos da partícula 20S são formados por subunidades α que formam um poro de 13 Ångström, o que exige a denaturação parcial prévia do substrato para entrada no cilindro catalítico. A partícula 20S é flanqueada por uma ou duas tampas, as partículas reguladoras 19S, formando o complexo 26S do proteassomo (19S-20S-19S). As partículas 19S controlam a entrada das proteínas na partícula 20S catalítica, normalmente via reconhecimento dos resíduos poliubiquitinados. A partícula 19S contém ATPases que auxiliam no desdobramento e denaturação das proteínas-substrato e auxiliam sua entrada na partícula 20S. Em resposta a IFN- γ , a subunidade 19S pode ser substituída pela subunidade ativadora do proteassomo, 11S (também denominada PA28 $\alpha\beta$), que recruta proteínas para degradação mais rapidamente (BASLER; KIRK; GROETTRUP, 2013; DE GRAAF et al., 2011).

As subunidades catalíticas do proteassomo convencional denominadas $\beta 1$, $\beta 2$ e $\beta 5$ são codificadas pelos genes *Psmb6*, *Psmb7* e *Psmb5*, respectivamente. No entanto, algumas células hematopoiéticas, incluindo DC, expressam formas alternativas das subunidades catalíticas $\beta 1$, $\beta 2$ e $\beta 5$ do proteassomo convencional.

Estas subunidades alternativas são codificadas pelos genes *Psmb9*, *Psmb10* e *Psmb8* e são denominadas $\beta 1i$ (ou LMP2), $\beta 2i$ (ou MECL1) e $\beta 5i$ (ou LMP7), respectivamente. Em outras células, estas subunidades catalíticas alternativas do proteassomo só são expressas quando induzidas principalmente pela exposição a IFN- γ (BOES et al., 1994; NANDI; JIANG; MONACO, 1996), mas também TNF (HALLERMALM et al., 2001) ou interferons do tipo 1 (SHIN et al., 2006). Os complexos proteolíticos contendo estas subunidades alternativas indutíveis são denominados imunoproteassomo (Figura 1).

Figura 1 – Representação esquemática da estrutura molecular do imunoproteassomo.

O núcleo catalítico 20S é formado por dois anéis externos α e dois anéis internos β . As subunidades com atividade proteolítica $\beta 1$, $\beta 2$ e $\beta 5$ podem, constitutivamente em algumas células ou em resposta a estímulos como IFN- γ , ser trocadas pelas subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$, caracterizando o imunoproteassomo (a). A unidade 20S do imunoproteassomo pode se associar às partículas reguladora 19S e ativadora 11S em diferentes combinações (b). Adaptado de um trabalho de revisão recente (MCCARTHY; WEINBERG, 2015).

Durante uma infecção um mesmo proteossomo pode ainda exibir subunidades catalíticas mistas, expressas por genes do proteossomo convencional e do imunoproteossomo (ZANKER et al., 2013). Em oposição ao proteossomo convencional, o imunoproteossomo cliva preferencialmente resíduos de aminoácidos hidrofóbicos e básicos, o que leva à geração mais eficiente de peptídeos que se ligam a moléculas de MHC classe Ia.

Por conseguinte, especula-se que o imunoproteossomo tenha evoluído em resposta a patógenos, pois é induzido num contexto inflamatório característico de infecções, sinalizado pela presença de citocinas, e assumiu um papel importante na geração de peptídeos antigênicos reconhecidos por linfócitos T CD8⁺ os quais controlam estes patógenos. No entanto, alguns autores defendem a ideia de que a indução do imunoproteossomo seja uma resposta fisiológica ao estresse oxidativo e tem por função primária evitar o acúmulo de proteínas defectivas no citoplasma, com a geração de epítomos restritos a MHC classe I ocupando um papel secundário nesta resposta (SEIFERT et al., 2010). Neste trabalho, foi sugerido que a exposição celular ao IFN- γ induz acúmulo de proteínas defeituosas, e que as proteínas poliubiquitinadas são preferencialmente reconhecidas e também degradadas mais rapidamente pelo imunoproteossomo, quando comparado ao proteossomo convencional. No entanto, esta hipótese foi bastante questionada, e inclusive refutada experimentalmente (NATHAN et al., 2013). A sugestão de uma maior eficiência no recrutamento de proteínas poliubiquitinadas pelo imunoproteossomo não poderia ser deduzida, tampouco explicada pela ação exclusiva das subunidades $\beta 1i$, $\beta 2i$ ou $\beta 5i$, já que estas estão no interior da partícula 20S, que não tem papel no recrutamento de substrato. Mas sim, a aceleração na degradação de proteínas pelo proteossomo poderia ser explicada pela troca da subunidade 19S por PA28 $\alpha\beta$, que sabidamente ocorre em resposta ao IFN- γ . No entanto, tanto o proteossomo convencional quanto o imunoproteossomo podem apresentar configurações que exibem a subunidade ativadora PA28 $\alpha\beta$, o que portanto não atribuiria o reconhecimento preferencial e a aceleração do processamento de epítomos exclusivamente ao imunoproteossomo.

Também já foi sugerido que em decorrência de infecções virais a produção de IFN- β poderia levar à indução da expressão de genes do imunoproteossomo em células pancreáticas, e isto estaria associado à geração de epítomos que levam a

auto-reatividade de linfócitos T CD8⁺ e desenvolvimento de diabetes tipo I (FREUDENBURG et al., 2013).

No que tange às subunidades catalíticas indutíveis, diversos trabalhos que utilizaram camundongos deficientes nas cadeias $\beta 1i$, $\beta 2i$ ou $\beta 5i$ sugeriram um papel do imunoproteassomo no incremento da geração de epítomos antigênicos restritos a MHC classe I e na formação da hierarquia de imunodominância destes epítomos (BASLER et al., 2011; DE GRAAF et al., 2011; FEHLING et al., 1994; HENSLEY et al., 2010; PANG et al., 2006; TU et al., 2009; VAN KAER et al., 1994). No entanto, apenas mais recentemente foi possível gerar um camundongo concomitantemente deficiente nas três subunidades catalíticas indutíveis do imunoproteassomo, o que lançou mais clareza no entendimento de se e quão diferente é o conjunto de peptídeos antigênicos gerados pelo imunoproteassomo em comparação ao proteassomo convencional (KINCAID et al., 2012).

Os camundongos deficientes das três subunidades catalíticas que formam o imunoproteassomo (TKO) gerados neste estudo exibiram níveis de proteínas poliubiquitinadas e expressão de subunidades α do proteassomo comparáveis a animais normais, enquanto a expressão de TAP-1 e MHC classe I era menor nos animais triplamente deficientes. Ainda, o número de células T CD8⁺ no timo e baço dos animais TKO correspondeu a aproximadamente 50% do encontrado em animais normais. Os autores atribuíram a falta de TAP-1 a uma questão metodológica, uma vez que o gene *Tap1* é flanqueado por *Psm8* e *Psm9* e sua expressão poderia ser comprometida pela excisão dos exons necessária para a geração do animal TKO. Porém os níveis de MHC classe I dos animais TKO eram ainda mais baixos do que de animais TAP-1^{+/-} controles, justificando a sugestão dos autores de que não a falta de TAP

1, mas principalmente a provisão deficiente de peptídeos na ausência do imunoproteassomo poderia comprometer a estabilidade e exportação de moléculas de MHC classe I para a superfície celular. Na mesma direção, o menor número de linfócitos T CD8⁺ seria decorrente da menor estimulação antigênica oriunda da redução da quantidade de complexos peptídeo-MHC classe I (KINCAID et al., 2012).

Experimentos *in vitro* mostraram que em comparação a DC de animais C57BL/6, as DC de animais TKO eram menos capazes de estimular linfócitos T CD8⁺ com TCR transgênicos específicos para epítomos endógenos, derivados de influenza ou mesmo da proteína ovalbumina. Mas quando as células eram forçadas

a expressar um mini-gene que codificava um epítipo de ovalbumina que não precisava ser processado, as DC de ambos animais estimulavam igualmente os linfócitos T CD8⁺ transgênicos cognatos, a despeito das diferenças na expressão de TAP-1 e MHC classe I, corroborando a ideia de que a deficiência observada nos animais TKO deve-se ao processamento de antígenos (KINCAID et al., 2012).

In vivo, camundongos TKO infectados com o vírus LCMV foram menos capazes do que seus pares C57BL/6 de estimular linfócitos T CD8⁺ transgênicos que foram transferidos para os animais e eram específicos para o epítipo GP33 do vírus. Animais TKO que receberam transferência adotiva de linfócitos T CD8⁺ policlonais normais e foram infectados com LCMV também tiveram resposta reduzida para os epítopos GP34 e GP118, enquanto a resposta específica para NP396 não foi alterada, e no caso do epítipo GP276 a resposta foi aumentada em comparação aos animais C57BL/6, sugerindo que a falta de imunoproteassomo pode alterar a imunodominância dos linfócitos T CD8⁺. Em contraste, a resposta de linfócitos T CD4⁺ observada em animais TKO infectados com LCMV era indistinguível da observada em camundongos C57BL/6, indicando que as células T e as células apresentadoras de antígenos não estavam globalmente afetadas nos animais TKO, cuja deficiência restringiu-se à apresentação de antígenos restritos a MHC classe I. Apesar disto, os autores não relataram maior susceptibilidade ao LCMV na ausência de imunoproteassomo (KINCAID et al., 2012).

Notavelmente, e contrastando com trabalhos anteriores que empregaram animais deficientes em apenas uma ou duas das subunidades catalíticas indutíveis, os animais TKO rejeitaram células de animais C57BL/6 rapidamente após a transferência adotiva de esplenócitos, indicando que os peptídeos restritos a MHC classe I gerados pelo imunoproteassomo presente nos animais C57BL/6 são tão distintos dos peptídeos gerados pelo proteassomo convencional (única forma presente nos camundongos TKO) que são reconhecidos como estranhos. Já a transferência de esplenócitos de animais TKO para C57BL/6 não gerou rejeição, o que pode ser esperado e explicado pela presença das duas formas de proteassomo nos camundongos C57BL/6, os quais toleram o conjunto de peptídeos gerados pelos animais TKO (KINCAID et al., 2012).

Em conjunto, os dados apresentados neste estudo mostraram pela primeira vez de forma sistemática que a quantidade e especificidade dos peptídeos gerados pelas três subunidades catalíticas específicas do imunoproteassomo são

marcadamente distintas da quantidade e especificidade dos peptídeos gerados pelo proteassomo convencional.

Em humanos, ainda, um estudo clínico de Fase 2b correlacionou a expressão de genes do imunoproteassomo com a proteção conferida pela vacina RTS,S contra malária após desafio (VAHEY et al., 2010). Esta é uma vacina recombinante constituída da subunidade correspondente aos domínios repetidos e porção C-terminal da proteína do circumsporozoíto de *Plasmodium falciparum* covalentemente ligada ao antígeno S do vírus da hepatite B e representa o melhor candidato vacinal já desenvolvido contra malária. No referido estudo, 39 voluntários receberam a vacina juntamente com os adjuvantes AS01 ou AS02, dos quais 13 foram protegidos. Análises de enriquecimento de grupos em experimentos de microarranjo identificaram genes relacionados a vias do imunoproteassomo e processamento de epítomos restritos a MHC classe I como os genes associados com proteção após três doses da vacina, e foram sugeridos pelos autores como possíveis marcadores para avaliação da eficiência das imunizações (VAHEY et al., 2010).

O papel do imunoproteassomo na indução de resposta imune que medeia o controle de infecções intracelulares, como sugerido nos parágrafos anteriores, representaria uma forte pressão seletiva para os patógenos, que poderiam desenvolver mecanismos adaptativos de evasão. De fato, isto já foi sugerido em trabalhos que reportam infecções experimentais por HIV-1, onde a proteína viral Gag p24 foi capaz de suprimir a expressão das subunidades $\beta 2i$ e $\beta 5i$ e inibir a apresentação de antígenos para linfócitos T CD8⁺ *in vitro* (STEERS et al., 2009). Por outro lado, a proteína Tat do HIV-1 foi capaz de induzir a expressão de $\beta 2i$ e $\beta 5i$, enquanto inibiu a expressão de $\beta 1i$, o que em conjunto melhorou a geração e apresentação de epítomos subdominantes restritos a MHC classe I (GAVIOLI et al., 2004). A existência de mecanismos antagônicos de indução e inibição do imunoproteassomo induzidos pelo mesmo patógeno pode ser de difícil interpretação. Uma possibilidade é que tais mecanismos sirvam justamente para garantir a permanência do patógeno concomitante ao seu controle pelo hospedeiro, em um equilíbrio que viabilize a coexistência de ambos por longos períodos.

Portanto, cada vez mais tem sido mostrada a relevância do imunoproteassomo para a geração, magnitude e diversidade da resposta de linfócitos T CD8⁺ que controlam infecções.

1.3 Regulação da resposta de linfócitos T CD8⁺

Para que os linfócitos T promovam a homeostase, eles devem ser adequadamente induzidos, e definir o tom da resposta imune envolve a integração de sinais intrincados e complexos. Respostas insuficientes são incapazes de controlar infecções e podem permitir a progressão de diversos tipos de câncer, enquanto respostas exacerbadas podem gerar danos por excesso de inflamação, bem como levar a quadros de alergia e autoimunidade, como ilustram os casos de asma, doença celíaca, lupus, diabetes autoimune, vitiligo e esclerose múltipla.

Um dos mecanismos mais importantes para o silenciamento da resposta de linfócitos T envolve a participação de células T reguladoras (Treg), originalmente descritas como CD4⁺ CD25⁺ (SAKAGUCHI et al., 1995). Mais tarde, ainda, o fator de transcrição Foxp3 foi proposto como um marcador relacionado à ontogenia e função das Treg (BENNETT et al., 2001; KASPROWICZ et al., 2003; ZIEGLER, 2006). Além de CD25 e Foxp3, outros marcadores têm sido propostos para caracterizar subpopulações destas células, incluindo Helios, Nrp1, PD-1, Swap70, Dapl1, Igfbp4, GITR, galectina-1, CD38, OX40 e CTLA-4, entre outros, como revisto recentemente (BILATE; LAFAILLE, 2012). A indução de Treg pode ocorrer no timo (nTreg) ou na periferia (iTreg), e envolve sinalização via TCR e CD28, mas sua função efetora não é necessariamente antígeno-específica. A indução de iTreg na periferia pode ocorrer num contexto inflamatório ou não inflamatório, e pode envolver a participação de IL-2, TGF- β , ácido retinóico, bem como ligantes do receptor de aril-hidrocarboneto (Ahr) (MUCIDA et al., 2007; QUINTANA et al., 2008).

Inúmeros mecanismos distintos de regulação mediados por Treg são conhecidos, como o consumo de IL-2, a produção de IL-35, TGF- β , IL-10, bem como a expressão de indolamina-2,3-dioxigenase (IDO) e mecanismos dependentes de contato mediados pela interação com LAG-3, CD39, CTLA-4 e GITR, ou ainda a citotoxicidade mediada por Treg expressando granzima B e perforina.

No entanto, a maioria dos mecanismos descritos para a função das Treg relata a regulação de linfócitos T CD4⁺ e nunca foram observados, ou sabidamente não se aplicam, durante o silenciamento de linfócitos T CD8⁺. Apenas muito recentemente, foi observado a indução de anergia em linfócitos T CD8⁺ humanos mediada por Treg (MAEDA et al., 2014). Neste trabalho, os autores identificaram em indivíduos saudáveis linfócitos T CD8⁺ anérgicos específicos para um antígeno da

pele relacionado ao desenvolvimento de vitiligo que por efeito de Treg tornam-se hiporresponsivos à reestimulação *in vitro*, quantificada pela capacidade dos linfócitos T CD8⁺ específicos proliferarem e produzirem citocinas.

Em um modelo murino de infecção por LCMV, a depleção de Treg levou a um aumento de até 100 vezes no número de linfócitos T CD8⁺ efetores, rompendo o estado de exaustão destas células. Porém, este aumento não teve nenhum efeito na redução da carga viral (PENALOZA-MACMASTER et al., 2014). Por outro lado, o bloqueio concomitante de PD-L1 e da sinalização por prostaglandina E2 durante a infecção por LCMV melhorou a função e sobrevivência dos linfócitos T CD8⁺ citotóxicos, contribuindo para o controle do vírus (CHEN et al., 2015).

Outro mecanismo de regulação dos linfócitos T CD8⁺ descrito num modelo de infecção pelo parasito *Encephalitozoon cuniculum* sugere a participação de TGF- β na apoptose e redução da funcionalidade destes linfócitos (BHADRA et al., 2014).

Portanto, muito pouco se sabe ainda sobre a participação de Treg na indução e efetuação da resposta mediada por linfócitos T CD8⁺, tampouco se a indução de Treg por patógenos pode representar um mecanismo de evasão do controle por linfócitos T CD8⁺.

1.4 Doença de Chagas

A Doença de Chagas, também conhecida como tripanosomíase americana, é uma condição cardíaca e/ou digestiva crônica, ou eventualmente aguda, causada pelo protozoário intracelular *Trypanosoma cruzi*, o qual infecta estimadamente 10 milhões de pessoas no mundo, principalmente nas regiões tropicais pobres (HOTEZ et al., 2008; RASSI; RASSI; MARIN-NETO, 2010). Esta é apontada pela Organização Mundial da Saúde como uma das 13 doenças tropicais negligenciadas mais importantes, e também é a principal doença cardíaca por causa infecciosa (KIRCHHOFF et al., 2004; WHO, 2009).

O ciclo de vida do *T. cruzi* foi originalmente descrito no Brasil por Carlos Chagas em 1909 (CHAGAS, 1909), o que talvez explique a tradição no estudo deste parasito entre a comunidade científica brasileira. Os parasitos são transmitidos para humanos pelos vetores *Triatoma infestans*, *Rhodnius prolixus* e *Triatoma dimidiata*, normalmente através das fezes contaminadas deixadas sobre a pele enquanto o inseto se alimenta de sangue humano. A transmissão não vetorial também é importante, ocorrendo principalmente por transfusão de sangue ou órgãos sólidos,

por via congênita, ou mesmo pela ingestão de alimentos contaminados (BERN et al., 2008; CENTERS FOR DISEASE; PREVENTION, 2006; PEREIRA et al., 2009; TORRICO et al., 2004).

O desenvolvimento de patologia crônica característica da Doença de Chagas ocorre anos após a infecção, em aproximadamente 30% dos indivíduos infectados. Os sintomas cardíacos e/ou digestivos são bastante variados e os fatores que levam à patologia são ainda pouco claros (RASSI; RASSI; MARIN-NETO, 2010). O tratamento é quimioterápico e baseia-se principalmente nas drogas benzonidazol e nifurtimox. Estes tratamentos provocam efeitos colaterais adversos e sua eficácia é limitada e controversa, com os melhores resultados obtidos na fase aguda (BERN, 2011; MATTA GUEDES et al., 2012; MUNOZ et al., 2013).

A prevenção, por outro lado, baseia-se no controle vetorial, uma vez que não existe ainda uma vacina contra *T. cruzi*. Contudo, o fato de pacientes imunossuprimidos reativarem a Doença de Chagas, com ressurgimento de parasitemia no sangue, é um indicativo de que o sistema imune confere algum grau de controle da infecção em humanos (BESTETTI et al., 2012; BRAZ; AMATO NETO; OKAY, 2008). Além disso, evidências experimentais pré-clínicas sugerem que é possível induzir uma resposta imune capaz de controlar a infecção por *T. cruzi*, sustentando a hipótese de que uma vacina para prevenir a Doença de Chagas é possível. No entanto, o desenvolvimento de vacinas contra protozoários permanece um desafio bastante complexo e no caso de doenças negligenciadas, como Chagas, restringe-se à pesquisa acadêmica básica, evidenciando o hiato entre a possibilidade teórico-científica e a existência real de uma vacina.

1.5 Papel dos linfócitos T CD8⁺ no controle da infecção por *T. cruzi*

Na infecção experimental de camundongos C57BL/6 com *T. cruzi* observa-se o controle da infecção concomitante ao aparecimento de uma resposta mediada por linfócitos T CD8⁺. Notavelmente, a depleção nestes animais de linfócitos T CD8⁺ com anticorpos ou o uso de linhagens deficientes em CD8 ou β -2 microglobulina, mas de mesmo fundo genético, levam a um aumento da parasitemia e mortalidade em resposta à infecção por *T. cruzi*, sugerindo a relevância dos linfócitos T CD8⁺ para o controle do parasito (ROTTENBERG et al., 1993; TARLETON, 1990; TARLETON et al., 1992). Os mecanismos responsáveis por este controle são múltiplos, incluindo, principalmente, a secreção de IFN- γ e a citotoxicidade direta

contra células infectadas, dependente de perforina (DE ALENCAR et al., 2009; MARTIN; TARLETON, 2004; MULLER et al., 2003; TZELEPIS et al., 2006).

Apesar do parasito expressar mais de 12 mil genes, os epítomos de *T. cruzi* reconhecidos por linfócitos T CD8⁺ durante a infecção experimental são poucos e encontrados em proteínas de superfície da família das *trans*-sialidases, incluindo a própria *trans*-sialidase (TS) e a proteína 2 da superfície de amastigotas (ASP-2) (MARTIN et al., 2006). Animais C57BL/6 infectados com a cepa Y de *T. cruzi* reconhecem o epítipo imunodominante VNHRFTLV (PA8) da ASP-2, o qual pode corresponder a até 30% da resposta de linfócitos T CD8⁺. Os epítomos subdominantes reconhecidos por estes animais são ANYDFTLV (TsKb-18) e ANYKFTLV (TsKb-20) do antígeno TSA (TZELEPIS et al., 2008; VASCONCELOS et al., 2004). Os mecanismos responsáveis pelo controle da imunodominância destes linfócitos T CD8⁺ permanecem desconhecidos, mas evidências prévias de nosso grupo sugerem a competição de linfócitos por células apresentadoras de antígenos como um fator preponderante (TZELEPIS et al., 2008). Ainda, é possível que a forte imunodominância de linfócitos T CD8⁺ consista em um mecanismo de evasão do controle mediado pelo hospedeiro, já que o reconhecimento de epítomos subdominantes de *T. cruzi* em um modelo experimental incrementou a proteção mediada por estes linfócitos (DOMINGUEZ et al., 2011).

Enquanto na maioria das infecções por diferentes patógenos a indução de linfócitos T CD8⁺ ocorre em aproximadamente 5 dias, o aparecimento destas células produtoras de IFN- γ é atrasado na infecção experimental por *T. cruzi*, sendo primeiramente observado em torno do décimo dia pós-infecção (TZELEPIS et al., 2006). Este atraso permite uma intensa replicação inicial do parasito, o que potencialmente pode levar ao estabelecimento precoce de reservatórios no hospedeiro que favoreceriam a progressão para a fase crônica.

Além do forte perfil de imunodominância e do atraso na indução de linfócitos T CD8⁺ em resposta à infecção por *T. cruzi*, estas células exibem baixa viabilidade, com um fenótipo caracterizado pela expressão de altos níveis do receptor pró-apoptótico Fas (CD95) (GUILLERMO et al., 2007; VASCONCELOS; BRUNA-ROMERO; et al., 2012). Assim, é plausível sugerir que a indução de apoptose utilizando a via de sinalização de CD95 nos linfócitos T CD8⁺ específicos para epítomos de *T. cruzi* poderia estar associada à incapacidade de um controle eficiente da infecção.

Ainda, a imunopatologia observada experimentalmente na fase crônica da infecção pelo *T. cruzi* pode estar associada à reativação dos linfócitos T CD8⁺ que permaneceram mas foram ineficazes em conferir imunidade estéril na fase aguda (GARZON et al., 2005).

Portanto, os estudos pré-clínicos sugerem que a resposta de linfócitos T CD8⁺ é ao mesmo tempo forte e essencial para o controle da fase aguda da infecção por *T. cruzi*, mas subótima e insuficiente para eliminar completamente o parasito, o que permitiria a progressão para a fase crônica da doença. Esta insuficiência da resposta pode ser correlacionada a um atraso na indução, forte padrão de imunodominância e perfil pró-apoptótico dos linfócitos T CD8⁺.

1.6 Indução de resposta de linfócitos T CD8⁺ contra *T. cruzi* através de vacinação com vetores adenovirais recombinantes

Para testar a hipótese de que é possível fortalecer a resposta de linfócitos T CD8⁺ para controlar a infecção por *T. cruzi*, nosso grupo desenvolveu um vetor adenoviral humano tipo 5 não replicativo que expressa ASP-2 (AdASP-2), o qual foi utilizado em um protocolo de imunização de uma linhagem de camundongos altamente susceptível (DE ALENCAR et al., 2009). Enquanto camundongos A/Sn imunizados com o vetor adenoviral que expressa beta-galactosidase (Adβ-gal) sucumbem rapidamente ao desafio subcutâneo com apenas 150 formas sanguíneas de *T. cruzi*, animais imunizados com AdASP-2 controlam a parasitemia e sobrevivem por anos após o desafio. Esta proteção, no entanto, é completamente abolida quando linfócitos T CD8⁺ são depletados com anticorpos, confirmando a hipótese de que a imunização com o vetor adenoviral é capaz de induzir uma resposta de linfócitos T CD8⁺ que, contrastando com a induzida naturalmente pelo *T. cruzi*, controla a infecção (DE ALENCAR et al., 2009; VASCONCELOS; BRUNA-ROMERO; et al., 2012).

A eficiência da resposta de linfócitos T CD8⁺ induzidos pela imunização experimental com AdASP-2 pode ser explicada, em parte, por uma cinética acelerada da indução destas células em comparação a animais *naïve* em resposta ao desafio (TZELEPIS et al., 2006). De fato, a imunização com vetor adenoviral induz uma resposta de longa duração, gerando um *pool* de linfócitos T CD8⁺ de memória efetora (CD127^{alto} CD62L^{baixo} CD44^{alto}) que persiste por mais de 98 dias

após a imunização e é resistente à erosão (RIGATO et al., 2011; VASCONCELOS; DOMINGUEZ; et al., 2012).

A presença de um *pool* de células específicas gerado antes da infecção explicam a proteção conferida pela vacinação de modo quantitativo e temporal. No entanto, a qualidade da resposta de linfócitos T CD8⁺ é também alterada pela imunização com AdASP-2. Um aspecto qualitativo divergente é a indução de resposta contra epítomos subdominantes observada durante a imunização com o vetor adenoviral, mas não durante a infecção com o parasito (DOMINGUEZ et al., 2011). Outro aspecto notável da imunização com o vetor adenoviral refere-se à indução de um *pool* de linfócitos TCD8⁺ de alta viabilidade, com baixa expressão de CD95, que predominam mesmo quando a imunização se dá no mesmo dia da infecção (VASCONCELOS; BRUNA-ROMERO; et al., 2012). Portanto, além da diferença quantitativa e temporal conferida pela vacinação, o vetor adenoviral é capaz de modificar a qualidade da resposta de linfócitos T CD8⁺ que medeiam o controle da infecção por *T. cruzi* (VASCONCELOS; DOMINGUEZ; et al., 2012). Evidências experimentais do nosso grupo sugerem que neste modelo as células T CD8⁺ que expandem e conferem proteção após o desafio com *T. cruzi* são mesmo aquelas induzidas pela imunização prévia, e não as células *naïve* recém-ativadas pela infecção. Ainda, a proliferação de linfócitos TCD8⁺ observada após o desafio é desnecessária à proteção mediada pela vacinação com AdASP-2, enquanto a recirculação dos linfócitos é, de fato, importante para a proteção (DOMINGUEZ et al., 2012).

1.7 Justificativa

O modelo murino experimental de infecção por *T. cruzi* e vacinação com vetor adenoviral recombinante evidencia a importância da resposta de linfócitos T CD8⁺ no controle de infecções intracelulares de maneira didática. Explorar o paralelo entre a resposta subótima destes linfócitos, induzida pela infecção por *T. cruzi*, e a resposta ótima, cooptada pela vacinação com vetor adenoviral, enseja a identificação precisa de bases celulares e moleculares que controlam a indução e efetuação da resposta de linfócitos T CD8⁺ em cada caso, o que pode ser extrapolado para o entendimento mais geral de mecanismos que levam à cronicidade de infecções intracelulares e o direcionamento para desenvolver estratégias vacinais ou terapêuticas mais eficientes contra estes patógenos.

Especificamente, não existem informações na literatura corrente acerca do papel do imunoproteassomo na indução e efetuação da resposta de linfócitos T CD8⁺ contra um patógeno intracelular que induz uma infecção crônica em que estes linfócitos podem ainda contribuir para a imunopatologia. Tampouco é sabido se o imunoproteassomo tem relevância para o sucesso de uma estratégia vacinal eficaz contra qualquer patógeno. Dada a importância basilar dos linfócitos T CD8⁺ e IFN- γ no modelo experimental de infecção por *T. cruzi* e vacinação com adenovírus recombinante, nós julgamos que este modelo poderia maximizar a possibilidade de observar algum papel do imunoproteassomo na ativação, especificidade e resposta efetora dos linfócitos T CD8⁺.

Outro ponto essencial relacionado à resposta de linfócitos T CD8⁺ contra *T. cruzi* que ainda é muito pouco entendido consiste nos mecanismos que levam a resposta contra este parasito ser subótima. A identificação destes mecanismos pode ser importante para compreender a progressão da infecção e eventualmente direcionar intervenções imunoterapêuticas. Para estudar o que leva a resposta de linfócitos T CD8⁺ ser subótima na presença de *T. cruzi* nós consideramos que seria interessante desenvolver um modelo no qual a célula apresentadora de antígeno, o linfócito T CD8⁺ e a quantidade de antígeno processado são normalizados. Com estas variáveis controladas, seria possível observar o impacto específico do *T. cruzi* na indução destes linfócitos *in vivo* e identificar mecanismos pelos quais o parasito interfere nesta indução.

2 OBJETIVOS

2.1 Objetivo geral

O objetivo geral desta tese foi caracterizar bases celulares e moleculares que controlam a expansão, fenótipo e função efetora de linfócitos T CD8⁺ específicos induzidos durante a infecção experimental por *T. cruzi* ou vacinação com um vetor adenoviral humano tipo 5 recombinante que expressa a proteína ASP-2 de *T. cruzi*.

2.2 Objetivos específicos

O primeiro objetivo específico desta tese foi testar a hipótese de que o imunoproteassomo é importante para a geração e efetuação da resposta de linfócitos T CD8⁺ específicos contra epítomos de *T. cruzi* em camundongos vacinados com um vetor adenoviral e/ou infectados com o parasito. Para testar esta hipótese foram investigados:

- i) as vias e a participação do imunoproteassomo no processamento de antígenos restritos a MHC classe I por DC e apresentação *in vitro* para linfócitos T CD8⁺ específicos;
- ii) o papel do imunoproteassomo na indução de linfócitos T CD8⁺ específicos durante a infecção *in vivo* por *T. cruzi*;
- iii) o papel do imunoproteassomo na indução de linfócitos T CD8⁺ específicos induzidos por vacinação de animais com adenovírus recombinante inseridos de *Asp2*;
- iv) a importância do imunoproteassomo para a proteção contra *T. cruzi* em animais geneticamente vacinados com *Asp2* e/ou infectados com *T. cruzi*.

O segundo objetivo específico desta tese foi testar a hipótese de que a resposta subótima de linfócitos T CD8⁺ observada na infecção por *T. cruzi* está relacionada com a indução por DC expostas ao parasito de mecanismos ativos de supressão do *priming* destes linfócitos *in vivo*. Para cumprir este objetivo, foram estudados:

- i) o impacto específico da exposição ao *T. cruzi* na proliferação, diferenciação, fenótipo e função efetora de linfócitos T CD8⁺ transgênicos induzidos *in vivo* por DC carregadas com o respectivo epítipo cognato;
- ii) o perfil de viabilidade e ativação das DC expostas a *T. cruzi* utilizadas para indução de linfócitos T CD8⁺ *in vivo*;
- iii) a capacidade de DC previamente expostas ao *T. cruzi* de induzirem ativamente a supressão do *priming* de linfócitos T CD8⁺ *in vivo*;
- iv) a participação de linfócitos T CD4⁺ na supressão da resposta de linfócitos T CD8⁺ induzidos por DC expostas a *T. cruzi*;
- v) a investigação do fenótipo e mecanismos envolvidos com a supressão do *priming in vivo* de linfócitos T CD8⁺ por linfócitos T CD4⁺ em resposta ao *T. cruzi*;
- vi) o fenótipo após a infecção pelo *T. cruzi* de camundongos cujas células CD4⁺ Foxp3⁺ são removidas.

3 ANIMAIS, MATERIAIS E MÉTODOS

3.1 Animais

Este projeto foi submetido à aprovação da Comissão de Ética no Uso de Animais da Universidade de São Paulo (protocolo 110/10). Durante os experimentos, os animais foram mantidos no biotério do Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia – CEDEME, da Universidade Federal de São Paulo – Escola Paulista de Medicina, seguindo as normas internas. Camundongos fêmeas de 8 semanas de idade foram utilizados nos experimentos. As linhagens C57BL/6J, C57BL/6-Tg(TcraTcrb)1100 Mjb/J (OTI) e B6.129P2(SJL)-Myd88^{tm1.1Defr}/J (Myd88^{-/-}) originalmente do *The Jackson Laboratory* foram adquiridas no CEDEME. Animais B6.129S2-Tap1^{tm1Arp}/J (TAP^{-/-}) originalmente comprados do *The Jackson Laboratory* foram gentilmente doados pela Professora Verônica Coelho da Faculdade de Medicina da Universidade de São Paulo. Animais B6.129S2-Cd4^{tm1Mak}/J (CD4^{-/-}) adquiridos do *The Jackson Laboratory* pelo Professor Alexandre de Castro Keller, da Universidade Federal de São Paulo-Escola Paulista de Medicina, foram gentilmente doados por ele para este trabalho. Animais TKO, geneticamente deficientes das subunidades catalíticas $\beta 1i$, $\beta 2i$ e $\beta 5i$ do imunoproteassomo, foram gerados e gentilmente doados pelo Professor Kenneth Rock, da *University of Massachusetts* (KINCAID et al., 2012). Camundongos B6.MRL-Fas^{lpr}/J (Fas^{lpr}) e B6.129P2-Il10^{tm1Cgn}/J (IL-10^{-/-}) originalmente comprados do *The Jackson Laboratory* foram gentilmente doados pelo Professor Gustavo Amarante Mendes da Universidade de São Paulo. Camundongos com uma inserção bicistrônica do gene repórter que codifica a proteína eGFP no locus *FoxP3* (Foxp3-GFP) foram gerados no laboratório do Professor Vijay Kuchroo, da *Harvard Medical School* (BETTELLI et al., 2006) e doados para o Professor Alexandre Salgado Basso, da Universidade Federal de São Paulo-Escola Paulista de Medicina, que realizou o cruzamento destes animais com a linhagem CBy.SJL(B6)-*Ptprc*^a/J (CD45.1), originalmente comparada do *The Jackson Laboratory*, deste modo produzindo a prole de animais Foxp3-GFP CD45.1 congênicos que foram gentilmente doados para uso nos experimentos deste estudo. Animais que expressam o receptor símio da toxina diftérica juntamente com a proteína eGFP (DTR-eGFP) sob controle do promotor de *FoxP3* (DEREG) foram adquiridos do *The Jackson Laboratory* pela Professora Vera Lucia Garcia Calich da Universidade de São Paulo e gentilmente doados para este trabalho.

3.2 Parasitos e infecções

Para uso em experimentos *in vitro*, tripomastigotas de *T. cruzi* da cepa Y foram incubados com células LLC-MK2 (ATCC CCL-7) e recolhidos do sobrenadante da cultura após 6 dias. Para uso em experimentos *in vivo*, formas sanguíneas de *T. cruzi* foram mantidas por passagens semanais em camundongos ICR no Laboratório de Xenodiagnóstico do Instituto Dante Pazzanese de Cardiologia. Camundongos foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* por via *s.c.* na base da cauda. A parasitemia foi realizada pela coleta de 5 μ L de sangue da cauda e contagem imediata em microscópio no aumento de 40 $\times$ com lamínulas de 18 mm $\times$ 18 mm.

3.3 Plasmídeos

Os plasmídeos utilizados para imunizações no sistema dose-reforço heterólogo foram o vetor vazio pcDNA3 (GenScript) e plgSPclone9, com inserção de *Asp2*, gerado previamente pelo nosso grupo (BOSCARDIN et al., 2003). Onde citado, foram injetados 100 μ g de DNA plasmidial por via *i.m.* no músculo *tibialis anterior*.

3.4 Vírus

Vetores adenovirais humanos do tipo 5 recombinantes não replicativos com inserção de *Asp2* (AdASP-2) ou *Glb1* (Ad β -gal) foram gerados, caracterizados, crescidos e purificados como descrito anteriormente (MACHADO et al., 2006). Quando indicado, animais receberam 2×10^8 PFU de adenovírus por via *i.m.* no músculo *tibialis anterior*.

3.5 Peptídeos

Os peptídeos utilizados neste projeto foram sintetizados pela Genscript e tiveram pureza superior a 95% e identidade confirmada por espectrometria de massa em equipamento Q-TOF Micro equipado com fonte ionizante *electrospray*. A

sequência do peptídeo correspondente ao epítopo imunodominante da ASP-2 restrito a H-2^b (PA8) é VNHRFTLV, e dos peptídeos correspondentes aos epítotos subdominantes (TsKb18 e TsKb20) são ANYDFTLV e ANYKFTLV, respectivamente. Também foram sintetizados os peptídeos de sequência similar a estes: VNDYTLV e ANYDNFTLV. O peptídeo correspondente ao epítopo imunodominante de ovalbumina restrito a H-2^b e reconhecido por linfócitos T CD8⁺ transgênicos OTI possui a sequência SIINFEKL. O peptídeo de sequência ICPMYARV corresponde ao epítopo imunodominante restrito a H-2^b contido na β -galactosidase.

3.6 Multímeros

Tetrâmeros biotinilados de moléculas H-2K^b conjugadas com o peptídeo SIINFEKL e pentâmeros biotinilados de moléculas H-2K^b conjugadas com o peptídeo VNHRFTLV foram adquiridas da companhia ProlImmune e utilizadas para marcação de linfócitos T CD8⁺ específicos após a incubação com streptavidina conjugada com APC, adquirida da companhia BD.

3.7 Anticorpos para citometria de fluxo

Os anticorpos utilizados em protocolos de marcação de superfície para citometria de fluxo foram: CD3 PE (clone 145-2C11, Cia. BD), NK1.1 PE (clone PK136, Cia. BD), CD19 PE (clone 1D3, Cia. BD), CD11c APCCy7 (clone HL3, Cia. BD), CD40 APC (clone 2/23, Cia. BD), CD80 PerCP (clone 16-10A1, Cia. BD), CD86 PECy7 (clone GL1, Cia. BD), H-2K^b FITC (clone AF6-88.5, Cia. BD), I-A^b PE (clone AF6-120.1, Cia. BD), CD8 PerCP ou PB ou FITC (clone 53-6.7, Cia. BD), CD4 PerCP ou PECy7 (clone GK1.5 ou RM4-5, respectivamente, Cia. BD), V β 2 FITC (clone B20.6, Cia. BD), V β 3 FITC (clone KJ25, Cia. BD), V β 4 FITC (clone KT4, Cia. BD), V β 5.1/5.2 FITC (clone MR9-4, Cia. BD), V β 6 FITC (clone RR4-7, Cia. BD), V β 7 FITC (clone TR310, Cia. BD), V β 8.1/8.2 FITC (clone MR5-2, Cia. BD), V β 8.3 FITC (clone 1B3.3, Cia. BD), V β 9 FITC (clone MR10-2, Cia. BD), V β 10b FITC (clone B21.5, Cia. BD), V β 11 FITC (clone RR3-15, Cia. BD), V β 12 FITC (Clone MR11-1, Cia. BD), V β 13 FITC (clone MR12-3, Cia. BD), V β 14 FITC (clone 14-2, Cia. BD), V α 2 APCCy7 (clone B20.1, Cia. BD), CD44 PE (clone IM7, Cia. BD), CD62L APC (clone MEL-14, Cia. BD), CD11a (LFA-1) FITC (clone 2D7, Cia. BD), CD49d FITC (clone

R1-2, Cia. BD), CD38 PE (clone 90, Cia. BD), CD27 FITC (clone LG3A10, Cia. BD), CD69 PerCP (clone H1.2F3, Cia. BD), KLRG-1 FITC (clone 2F1, Cia. eBioscience), CD25 FITC ou PE (clone 7D4 ou PC61, respectivamente, Cia. BD), CD122 FITC (clone TM- β 1, Cia. BD), CD43 PECy7 (clone 1B11, Cia. BioLegend), CD45 FITC (clone 30-F11, Cia. BD), CD70 PE (clone FR70, Cia. BioLegend), CD71 FITC (clone C2, Cia. BD), CD134 (OX40) PE (clone OX-86, Cia. BioLegend), CD127 PE (clone SB/199, Cia. BD), CD272 (BTLA) PE (clone 8F4, Cia. eBioscience), CD279 (PD-1) FITC (clone J43, Cia. eBioscience), CD274 (PD-L1) PE (clone MIH5, Cia. BD), CD366 (Tim-3) Alexa Fluor 647 (clone B8.2C12, Cia. BioLegend), CD223 (LAG-3) PE (clone C9B7W, Cia. BD), CD262 (TRAIL-R2) PE (clone MD5-1, Cia. BioLegend), CD254 (TRANCE) PE (clone IK22/5, Cia. BioLegend), CD95 (Fas) PECy7 (clone Jo2, Cia. BD), CD178 (FasL) PE (clone MFL3, Cia. BD), CD195 (CCR5) FITC (clone C34-3448, Cia. BD), CD197 (CCR7) Alexa Fluor 647 (clone 4B12, Cia. BD), CD199 (CCR9) Alexa Fluor 647 (clone 9B1, Cia. BioLegend), CD183 (CXCR3) PerCP/Cy5.5 (clone CXCR3-173, Cia. BioLegend), CD184 (CXCR4) PE (clone 2B11/CXCR4, Cia. BD), CXCR7 PE (clone 8F11-M16, Cia. BioLegend), β 7 PerCP (clone FIB27, Cia. BioLegend), H-2K^bSIINFEKL PE (clone 25-D1.16, Cia. eBioscience), CD357 (GITR) FITC (clone DTA-1, Cia. eBioscience), CD304 (Nrp1) PE (policlonal, referência FAB566P, Cia. R&D Systems), CD45.1 PECy7 (clone A20, Cia. BD), PDCA-1 FITC (clone JF05-1C2.4.1, Cia. Miltenyi Biotec), CD205 biotinilado (clone 205 yekta, Cia. eBioscience) e F4/80 PE (clone BM8, Cia. eBioscience).

Em etapas de marcação intracelular por ICS foram utilizados IL-2 PerCP (clone JES6-5H4, Cia. BD), TNF PE (clone MP6-XT22, Cia. BD), IFN- γ APC (clone XMG1.2, Cia. BD), IL-4 PE (clone 11B11, Cia. BD), IL-10 PE (clone JES5-16E3, Cia. BD), IL-17 PE (clone TC11-18H10, Cia. BD). O anticorpo CTLA-4 PE (clone UC10-4B9, Cia. eBioscience) foi utilizado em protocolo de marcação intracelular.

Nos protocolos com marcação intranuclear, a etapa correspondente utilizou os anticorpos T-bet PE (clone 4B10, Cia. BD), Eomes FITC (clone Dan11mag, Cia. eBioscience), GATA-3 PE (clone L50-823, Cia. BD), ROR γ -t PE (clone Q31-378, Cia. BD) e FOXP3 PE (clone R16-715, Cia. BD).

Como isotipos-controle dos anticorpos listados acima foram utilizados IgG1 kappa FITC ou PE ou PECy7 ou APC (clone R3-34), IgG2a kappa FITC ou PE ou APC (clone R35-95), IgG2b kappa FITC ou PE (clone A95-1), IgG2 kappa FITC

(clone B81-3), ou IgG1 lambda PE (clone G235-2356), todos adquiridos da companhia BD.

3.8 Marcação de células para análise por citometria de fluxo

A marcação com os multímeros biotinilados foi realizada utilizando 2×10^6 esplenócitos de cada animal, antes das demais marcações, seguindo instruções do fabricante.

Para marcar antígenos de superfície, 2×10^6 esplenócitos de cada amostra foram incubados a 4 °C por 30 minutos juntamente com 0,5 µL de cada anticorpo e/ou estreptavidina conjugada com APC no volume de 0,3 µL/amostra diluídos em um volume final de 50 µL de PBS com 0,5% BSA e 2 mM EDTA (tampão de FACS). Após esta etapa, as amostras foram lavadas em tampão de FACS e imediatamente adquiridas em um equipamento BD FACS-Cantoll, ou subsequentemente utilizadas em etapas de marcação intracelular e/ou intranuclear.

A marcação intracelular das citocinas mencionadas no texto foi realizada após 6 h de reestímulo de esplenócitos com 10 µM do peptídeo indicado, ou com uma curva de diluição seriada de base 3 do peptídeo SIINFEKL, com concentrações variando de 1×10^{-6} M até 3×10^{-11} M. O meio utilizado foi composto de RPMI 1640 (Sigma) suplementado com 10 mM Hepes (Gibco), 0,2% bicarbonato de sódio (Sigma), 59 mg/L penicilina (Sigma), 133 mg/L estreptomicina (Sigma), 10% soro fetal bovino Hyclone (Thermo-Scientific), 2 mM L-glutamina (Gibco), 1 mM piruvato de sódio (Gibco), 55 µM 2-mercaptoetanol (Gibco), vitamina 1:100 (Gibco), aminoácidos não essenciais 1:100 (Gibco), brefeldina (BD) 10 µg/mL e anti-CD28 (clone 37.51, Cia. BD). Esplenócitos estimulados com meio ou concanavalina A 2,5 µM (Sigma) serviram como controles negativo e positivo, respectivamente. A marcação intracelular dos anticorpos foi realizada com *BD Cytotfix/Cytoperm Fixation/Permeabilization Kit* (BD) seguindo instruções do fabricante.

Para marcar fatores de transcrição foi realizado um protocolo de permeabilização intranuclear utilizando *Foxp3 Fixation/Permeabilization Concentrate and Diluent and Permeabilization Buffer* (eBioscience) de acordo com o protocolo do fabricante.

As amostras foram adquiridas em um equipamento BD FACS Canto II e analisadas no programa Flowjo 8.7 (Treestar).

3.9 Iniciadores

Tabela 1 – Iniciadores utilizados para genotipagem de animais OTI.

Iniciador	Sequência 5'-3'
TCR α OTI F	CAGCAGCAGGTGAGACAAAGT
TCR α OTI R	GGCTTTATAATTAGCTTGGTCC
TCR β OTI F	AAGGTGGAGAGAGACAAAGGATTC
TCR β OTI R	TTGAGAGCTGTCTCC
Controle interno F	CAAATGTTGCTTGTCTGGTG
Controle interno R	GTCAGTCGAGTGCACAGTTT

Tabela 2 – Iniciadores utilizados para quantificação dos níveis de transcrição de mRNA de citocinas.

Iniciador	Sequência 5'-3'
IL-6 F	TACCACTTCACAAGTCGGAGGC
IL-6 R	CTGCAAGTGCATCATCGTTGTTC
IL-12 p35 F	ACGAGAGTTGCCTGGCTACTAG
IL-12 p35 R	CCTCATAGATGCTACCAAGGCAC
TNF F	GGTGCCTATGTCTCAGCCTCTT
TNF R	GCCATAGAACTGATGAGAGGGAG
IL-23 p19 F	AATAATGTGCCCCGTATCCAG
IL-23 p19 R	GCTCCCCTTTGAAGATGTCAG
IL-27 p28 F	TCTCGATTGCCAGGAGTGAACC
IL-27 p28 R	AGTGTGGTAGCGAGGAAGCAGA
IFN- γ F	ACAGCAAGGCGAAAAAGGAT
IFN- γ R	TGAGCTCATTGAATGCTTGG
IL-4 F	TGAACGAGGTCACAGGAGAA
IL-4 R	CGAGCTCACTCTCTGTGGTG
IL-10 F	ATCGATTTCTCCCCTGTGAA
IL-10 R	TGTCAAATTCATTCATGGCCT
TGF- β F	TGATACGCCTGAGTGGCTGTCT
TGF- β R	CACAAGAGCAGTGAGCGCTGAA
GAPDH F	AAATGGTGAAGGTCGGTGTG
GAPDH R	TGAAGGGGTCGTTGATGG

Tabela 3 – Iniciadores utilizados para quantificação dos níveis de transcrição de mRNA das subunidades catalíticas do proteassomo e imunoproteassomo.

Iniciador	Sequência 5'-3'
β 1 F	CAGTCCTTTGCCATCGGAGGCT
β 1 R	CGTCCTTGGTCATGCCTTCCC
β 2 F	ATCAAGGTTACATTGGTGCAGCCC
β 2 R	ATGCTGTAGAGATGAGGTCCAGTAACA
β 5 F	GCCTATGATCTGGCCCGCCG
β 5 R	CCAGCCATCCTCCCGCACG
β 1i F	ACAGCCCTTTACCATCGGCGG
β 1i R	CCGGCACTCCTCAGGGGTCA
β 2i F	GGGGCTGGAGTAGCTGCGGA
β 2i R	GCGCAGGATACGGGTGACCG
β 5i F	CCACTGGCAGCGGGAACACC
β 5i R	AGCTCTGCGGCCAAGGTCGT
HPRT F	GGCCATCACATTGTGGCCCT
HPRT R	TGTAATCCAGCAGGTCAGCAAAGAAC

Tabela 4- Iniciadores utilizados para quantificação de *T. cruzi* em tecidos de animais infectados.

Iniciador	Sequência 5'-3'
CZ1 F	ASTCGGCTGATCGTTTTCGA
CZ2 R	AATTCCTCCAAGCAGCGGATA
CZ3 P	CACACACTGGACACCAA
β -actina F	CGGTTCCGATGCCCTGAGGCTCTT
β -actina R	CGTCACACTTCATGATGGAATTGA

Todos os iniciadores foram sintetizados pela companhia Invitrogen.

3.10 Extração de ácidos nucléicos e PCR

A extração de DNA para genotipagem dos animais OTI foi realizada pela digestão de amostras da cauda com tampão contendo 100 mM Tris-HCl pH 8,5, 5 mM EDTA, 0,2% SDS, 200 mM NaCl e 1 mg/mL de proteinase K. Após a incubação por 12 h a 55 °C o DNA foi precipitado com acetato de sódio 3M pH 5,2 em etanol 100%, lavado com etanol 70% e ressuspendido em água. O DNA foi então utilizado em reações de PCR convencional utilizando os iniciadores descritos na Tabela 1. A reação foi realizada em equipamento *Veriti Thermal Cycler Applied Biosystems* com

35 ciclos de 94 °C por 30 segundos, 55 °C por 1 minuto e 72 °C por 1 minuto. Os produtos da reação foram submetidos a eletroforese em gel de agarose 2% contendo brometo de etídeo e visualizados em um transiluminador U.V.

O mRNA de BMDC ou do coração, fígado e baço de animais previamente infectados com *T. cruzi* foi extraído com Trizol (Invitrogen) e em seguida purificado em colunas do kit *Quick RNA Miniprep* (Zymo Research) seguindo os protocolos dos fabricantes. O RNA foi quantificado em aparelho NanoDrop (Thermo Scientific) e o cDNA foi imediatamente sintetizado utilizando o kit *SuperScript III* (Invitrogen) para as amostras de BMDC e o *High-capacity cDNA reverse transcription kit* (Applied Biosystems) para amostras de tecido, conforme indicações dos fabricantes. A ausência de DNA genômico no produto da reação foi confirmada pelo uso de amostras onde não foi adicionada a transcriptase reversa. O cDNA produzido foi então utilizado em reações de RT-PCR semi-quantitativo em equipamento *StepOne Plus* (Applied Biosystems) utilizando *SYBR Green* (Thermo Scientific) e pares de iniciadores específicos, descritos nas Tabela 2 (para as amostras de BMDC) e Tabela 3 (para as amostras de tecidos infectados) (FREUDENBURG et al., 2013). As reações seguiram 35 ciclos de 94 °C por 30 segundos, 55 °C por 1 minuto e 72 °C por 1 minuto. Como controle endógeno foi utilizado o gene *Gapdh* (para BMDC) e *Hprt* (para tecido) a expressão relativa foi calculada pelo método $\Delta \Delta C_t$ utilizando amostras não infectadas como referência (LIVAK; SCHMITTGEN, 2001).

Para extração de DNA de coração e baço de animais infectados com *T. cruzi*, as amostras foram coletadas e inicialmente armazenadas a -20 °C. No momento da extração, os tecidos foram submergidos em nitrogênio líquido por 5 minutos e processados com lâminas de bisturi, antes de serem lisados por 2 h a 55 °C em tampão contendo 300 µg/mL de proteinase K, 0,5% SDS, 10 mM EDTA, 0,1 M NaCl e 10 mM Tris com pH 7,6. O DNA das amostras foi então extraído com um volume da solução fenol-clorofórmio-álcool isoamílico (24:24:1) e após centrifugação por 20 minutos a 4.200 g foi precipitado com etanol 70% e ressuspendido em água, sendo então quantificado em aparelho Nanodrop. Em seguida, 50 ng de DNA genômico obtido por este protocolo de extração foram utilizados para amplificação por PCR quantitativo utilizando os iniciadores CZ1 F e CZ2 R descritos na Tabela 4, específicos para uma região satélite nuclear do genoma de *T. cruzi* (PIRON et al., 2007), além de uma sonda CZ3 P para reação utilizando *TaqMan Universal Master Mix II* (Invitrogen). Amplificação de *Actb* (β -actina) serviu para normalização.

Alternativamente, uma diluição seriada de base 10 de DNA extraído de amostras contendo números conhecidos de parasitos de *T. cruzi* foi utilizada para construção de uma curva à qual os resultados foram interpolados. A reação foi executada em equipamento *StepOne Plus* (Applied Biosystems) com 50 °C por 2 minutos, 95 °C por 10 minutos, 40 ciclos de 95 °C por 15 segundos e 60 °C por 1 minuto.

3.11 Diferenciação e estímulo de BMDC

Células dendríticas derivadas de medula óssea (BMDC) foram geradas a partir do cultivo do lavado medular retirado do fêmur de camundongos das linhagens indicadas. Após lise das hemácias com tampão ACK, as células progenitoras foram cultivadas na concentração de 2×10^5 células/mL em RPMI 1640 (Sigma) suplementado com 10 mM Hepes (Gibco), 0,2% bicarbonato de sódio (Sigma), 59 mg/L penicilina (Sigma), 133 mg/L estreptomicina (Sigma), 10% soro fetal bovino Hyclone (Thermo-Scientific), 2 mM L-glutamina (Gibco), 1 mM piruvato de sódio (Gibco), 55 µM 2-mercaptoetanol (Gibco) e 20 ng/mL de GM-CSF recombinante (R&D Systems). A cultura foi feita em placas de 6 cavidades (Corning) com troca da metade do meio após 4 dias em cultura. A partir do sexto dia, as células foram então incubadas por mais 24 h ou pelo tempo indicado em presença ou ausência de tripomastigotas de cultura da cepa Y de *T. cruzi* na proporção de 3 parasitos por BMDC ou de 50 PFU por BMDC de Adβ-gal ou AdASP-2, ou na proporção indicada em cada experimento. Tripomastigotas de cultura de *T. cruzi* foram, quando indicado, inativados por congelamento em nitrogênio líquido por 20 minutos seguido de incubação a 56 °C por 20 minutos. Onde mencionado, BMDC foram incubadas com 1 µg/mL de LPS (Sigma) por 4 a 6 h ou 10 µg/mL de CpG ODN 1826 (Oley) por 4 a 6 h. Quando células expostas a *T. cruzi* ou adenovírus foram também incubadas com estes estímulos, a infecção foi realizada primeiro e os estímulos foram colocados nas últimas horas em cultura. Para controle positivo da indução de morte celular, foi adicionado 5 µg/mL de actinomicina D (Sigma) por 5 h. Ainda, onde indicado, BMDC foram incubadas com 1 µM de epoxomicina (Sigma) por 12h. Neste caso, a adição de epoxomicina e as infecções foram concomitantes. Quando BMDC foram carregadas com peptídeos, isto se deu após a infecção e/ou estimulação com LPS, pela incubação das células por 1 h a 37 °C em RPMI 1640 (Sigma) contendo

10% de soro fetal bovino (LGC) e 2 μ M do peptídeo mencionado. Após a incubação com peptídeo, as BMDC foram lavadas e utilizadas nos ensaios.

3.12 Microscopia de BMDC infectadas com *T. cruzi*

BMDC foram incubadas por 24 h com *T. cruzi* na proporção de 3 parasitos/célula em placas de 24 cavidades (Corning) com lamínulas circulares (Glasscyto) no fundo de cada cavidade. Estas lamínulas foram recolhidas, lavadas em PBS, fixadas em solução Bouin (Sigma) por 5 minutos, lavadas novamente em PBS, incubadas por 1 h com Giemsa (Merck) diluído em água (1:5), lavadas novamente em PBS e montadas com glicerol antes de serem fotografadas em aumento de 40 $\times$ em microscópio invertido AxioVertA.1 (Zeiss) com sistema de captação de imagens Zen lite (Zeiss).

3.13 Ensaios de morte celular

As BMDC estimuladas conforme indicado em cada figura sofreram marcação de superfície para citometria de fluxo e foram em seguida marcadas com anexina V e 7-AAD conforme instruções do fabricante do *PE Annexin V Apoptosis Detection Kit I* (BD Biosciences). A forma ativa das caspases 3 e 7 foi marcada com uma sonda fluorescente conforme instruções do fabricante utilizando *Vybrant FAM Caspase-3 and -7 Assay Kit* (Invitrogen – Molecular Probes). A marcação intracelular de Bcl-2 foi realizada pelo emprego de *FITC Hamster anti-mouse Bcl-2 sets* (BD) conforme instruções do kit.

3.14 Quantificação de citocinas em sobrenadante de cultura

Para avaliar a presença de citocinas no sobrenadante da cultura de BMDC expostas a *T. cruzi*, AdASP-2, ou agonistas de TLR, estas células foram incubadas conforme apontado em cada experimento e a presença de IL-1 β ou IL-12 p70 no sobrenadante das culturas foi avaliada utilizando kits de ELISA (BD) seguindo recomendações do fabricante. A detecção de IL-2, IL-4, IL-6, IL-10, IL-17A, TNF e IFN- γ foi aferida com *BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17*

Cytokine Kit (BD) em equipamento BD FACS-Canto II, seguindo especificações do fabricante.

3.15 Indução do *priming in vivo* de linfócitos T CD8⁺ OTI

Camundongos C57BL/6 fêmeas de 8 semanas de idade receberam por via *i.v.* no plexo retro-orbital uma suspensão de esplenócitos de camundongos OTI, perfazendo 1×10^4 linfócitos T CD8⁺ transgênicos OTI para cada animal. Este número de células foi definido em uma padronização prévia com diferentes quantidades variando de 1×10^3 a 1×10^6 (não mostrado). No dia seguinte, os animais foram imunizados por via *i.v.* com 5×10^5 BMDC previamente expostas ou não por 24 h a *T. cruzi* na proporção de 3 parasitos por célula ou AdASP-2 na proporção de 50 PFU por célula ou conforme mencionado em cada experimento. Imediatamente antes da transferência, as BMDC foram incubadas com 1 µg/mL de LPS por 4 a 6 h, lavadas 5 vezes com meio RPMI enriquecido com 10% de soro fetal bovino, incubadas com 2 µM do peptídeo SIINFEKL por 1 h a 37 °C e novamente lavadas 3 vezes. No quinto dia após a imunização os animais foram sacrificados e os esplenócitos utilizados para ensaios de fenotipagem e avaliação da resposta imune específica para o epítipo SIINFEKL, conforme indicado.

3.16 Isolamento de células

Para utilização nos ensaios de apresentação de antígenos *in vitro*, a separação dos linfócitos T CD8⁺ respondedores do baço de animais *naïve* ou infectados ou imunizados 15 dias antes foi realizada com *MACS CD8⁺ T cell isolation KitII* (Miltenyi Biotec) conforme instruções do fabricante, seguido de marcação com anticorpo CD8 PE (clone 53-6.7, Cia. BD) e *sorting* em equipamento BD FACS ArianII. A eluição das colunas de MACS contendo a fração CD8⁺ foi marcada com CD4 PECy7 (clone RM4-5, Cia. BD) e utilizada para separação das células CD4⁺ por *sorting*.

Para separação de DC de camundongos, as células dos linfonodos drenantes (inguinais e popliteais) foram retiradas e marcadas com os anticorpos de superfície para citometria de fluxo citados na seção de resultados. As células CD3⁻ NK1.1⁻ CD19⁻ IA^{b+} foram subdivididas em três subpopulações, isoladas em

equipamento BD FACS Arian: CD11c^{baixo} PDCA-1⁺, representando as DC plasmacitóides, CD11c^{alto} CD8⁻, representando as DC CD8⁻ e CD11c^{alto} CD8⁺, representando as DC CD8⁺.

Para isolamento de linfócitos T CD4⁺ utilizados nos experimentos de transferência adotiva de células, foi realizada separação com o *MACS CD4⁺ T cell isolation KitII* (Miltenyi Biotec) conforme protocolo do fabricante, rendendo enriquecimento superior a 95%. Quando utilizados animais Foxp3-GFP, a separação das células CD4⁺ Foxp3⁺ foi realizada com o *MACS CD4⁺ T cell isolation KitII* (Miltenyi Biotec) sucedido de marcação com CD4 PECy7 (clone RM4-5, Cia. BD) e *sorting* em equipamento BD FACS Arian.

3.17 ELISPOT

Nos ensaios de ELISPOT para quantificação do número de células secretoras de IFN- γ foram utilizadas placas de nitrocelulose de 96 cavidades (Multiscreen HA, Milipore) sensibilizadas com 10 μ g/mL do anticorpo de captura anti-IFN- γ (clone R4-6A2, Cia Pharmingen). Em estudos de apresentação de antígenos *in vitro*, BMDC foram estimuladas conforme citado em cada experimento e incubadas nestas placas com células CD4⁺ ou CD8⁺ isoladas do baço dos camundongos descritos. Em cada cavidade foram adicionadas 20.000 BMDC (ou DC isoladas do linfonodo) e 100.000 linfócitos T CD4⁺ ou T CD8⁺. A capacidade destes linfócitos produzirem IFN- γ foi então avaliada pela detecção do número de *spots* com 1 μ g/mL do anticorpo anti-IFN- γ biotilado (clone XMG1.2, Cia. Pharmingen) e subsequente incubação com 0,1 μ g/mL de estreptavidina conjugada com peroxidase (KPL), seguido de revelação com tampão 50 mM Tris-HCl pH 7,5, 1 mg/mL DAB (Sigma) e 1 μ L/mL de peróxido de hidrogênio 30% (Sigma). Como controles positivos, foram utilizadas BMDC carregadas com os peptídeos indicados em cada experimento ou adicionado concanavalina A nos poços na concentração de 2,5 μ M.

A resposta imune induzida *in vivo* mediada por linfócitos TCD8⁺ específicos também foi avaliada por ensaios de ELISPOT após a reestimulação de esplenócitos (100.000 células/cavidade) por 24 h com peptídeos específicos na concentração de 10 μ M, como já descrito por nosso grupo em detalhe (MIYAHARA et al., 1995), ou com uma curva de diluição seriada de base 3 do peptídeo SIINFEKL, com concentrações variando de 1×10^{-6} M até 3×10^{-11} M. O meio utilizado foi composto

de RPMI 1640 (Sigma) suplementado com 10 mM Hepes (Gibco), 0,2% bicarbonato de sódio (Sigma), 59 mg/L penicilina (Sigma), 133 mg/L estreptomicina (Sigma), 10% soro fetal bovino Hyclone (Thermo-Scientific), 2 mM L-glutamina (Gibco), 1 mM piruvato de sódio (Gibco), 55 μ M 2-mercaptoetanol (Gibco), vitamina 1:100 (Gibco), aminoácidos não essenciais 1:100 (Gibco) e IL-2 recombinante 20 U/mL (Sigma). Esplenócitos estimulados com meio ou concanavalina A 2.5 μ M (Sigma) serviram como controles negativo e positivo, respectivamente.

3.18 Proliferação celular *in vivo*

Nos ensaios de proliferação celular *in vivo*, animais receberam injeções de 2 mg de BrdU (BD) diluído em 200 μ L de PBS por via *i.p.* a cada 48 h durante todo o período do experimento. Para marcação de BrdU incorporado ao DNA, foi empregado o *BD FITC BrdU Flow Kit* (BD) seguindo o protocolo do fabricante após marcações com tetrâmeros e anticorpos de marcação na superfície celular.

3.19 Anticorpos para depleção ou bloqueio *in vivo*

O hibridoma PC61 foi utilizado para geração *in vivo* de fluido de ascite e 0,5 mL deste fluido foi injetado por via *i.p.* para depleção das células CD25⁺. O tratamento foi feito a cada 48 h durante todo o período do experimento, totalizando 3 injeções/animal.

Para bloquear CTLA-4, foi utilizado o anticorpo purificado 9D9, adquirido da companhia BioXCell. A injeção de 1 mg/animal via *i.p.* foi feita a cada 48 h durante todo o período do experimento, totalizando 3 injeções/animal.

Para bloquear TGF- β , foi utilizado o anticorpo purificado 1D11, adquirido da companhia BioXCell. A injeção de 2 mg/animal via *i.p.* foi feita a cada 72 h durante todo o período do experimento, totalizando 2 injeções/animal.

Nos grupos-controle, foi injetado IgG policlonal de rato adquirido da companhia Sigma, seguindo o protocolo análogo ao grupo ao qual serviu de controle.

3.20 Tratamento com toxina diftérica

Para depleção de células Foxp3^+ , animais DEREK foram injetados por via *i.p.* com 200 μL de PBS contendo 0,5 μg de toxina diftérica (R&D Systems). Este tratamento foi feito nos dias 1 e 2 após infecção com *T. cruzi*.

3.21 Análise estatística

Comparações entre os grupos foram realizadas por análise ANOVA de dois fatores seguido do teste Tukey *post-hoc*. Valores de parasitemia sofreram transformação logarítmica antes da aplicação dos testes. As curvas de sobrevivência foram construídas pelo método de Kaplan-Meier e submetidas ao teste log-rank (Mantel-Cox). Diferenças foram consideradas significativas quando valores de P foram $< 0,05$. As análises foram realizadas com o programa *GraphPad Prism* versão 6.0d.

4 RESULTADOS

4.1 Processamento e apresentação de antígenos expressos por *T. cruzi* e adenovírus recombinantes para linfócitos T CD8⁺ *in vitro*

A geração de resposta imune mediada por linfócitos T contra patógenos depende da interação destes patógenos com DC, as quais são provedoras de moléculas coestimuladoras, citocinas e epítopos processados e apresentados por moléculas de MHC para os linfócitos T. Apesar da relevância deste processo, pouco é conhecido a respeito da interação do *T. cruzi* com DC e o consequente impacto desta interação na apresentação de antígenos para linfócitos T CD8⁺. Tampouco se sabe em detalhe a via de processamento dos epítopos de *T. cruzi* restritos a MHC classe I.

Devido à relevância citada acima, inicialmente foi estudada a interação de DC murinas derivadas de medula óssea (BMDC) com tripomastigotas de *T. cruzi* gerados *in vitro* em cultura de células LLC-MK2 (linhagem derivada de epitélio renal de *Macaca mulatta*) ou com o adenovírus humano do sorotipo 5 recombinante que expressa a proteína ASP-2 de *T. cruzi* (AdASP-2), utilizado como vetor para vacinação experimental. O protocolo utilizado gerou de 70 a 90% de células CD11c⁺, com fenótipo H-2K^{b+}, I-A^{b+}, CD11b^{baixo}, CD8⁻, PDCA-1⁻, CD205⁻ e F4/80⁻. Quando essas células foram incubadas por 24 h com tripomastigotas de cultura de *T. cruzi* na proporção de 3 parasitos por BMDC, foi possível observar a presença de amastigotas no seu citoplasma através de coloração com Giemsa (Figura 2).

Figura 2 – Infecção de BMDC por *T. cruzi*.

Células dendríticas foram derivadas de medula óssea de camundongos C57BL/6 e incubadas com *T. cruzi* por 24 h na proporção de 3 tripomastigotas/célula e analisadas por fotomicrografia após coloração com Giemsa.

As BMDC também modularam positivamente a expressão de H-2K^b, I-A^b e das moléculas coestimuladoras CD40, CD80 e CD86 na sua superfície em resposta a estímulo com *T. cruzi* (3 parasitos/BMDC) e/ou AdASP-2 (50 PFU/BMDC). Esta modulação foi dependente de MyD88, molécula adaptadora importante para a sinalização de receptores do sistema imune inato do tipo Toll (TLR) que reconhecem padrões moleculares associados a patógenos. No entanto, este reconhecimento não ocorreu quando os parasitos utilizados foram inativados por congelamento e descongelamento. Além disto, a exposição simultânea de BMDC a *T. cruzi* e AdASP-2 não causou efeito notoriamente somatório nem inibitório na expressão de moléculas coestimuladoras, quando comparado ao parasito ou ao vírus isolados. Como controles positivos foram utilizados o agonista do receptor TLR4, LPS, cuja sinalização se dá via MyD88 e TRIF; bem como o agonista do receptor TLR9, CpG, cuja sinalização se dá exclusivamente via MyD88 (Figura 3).

Ainda, BMDC expostas ao *T. cruzi* ou AdASP-2 secretaram IL-12 p70 em quantidades similares, como detectado por ELISA. Já a presença de IL1- β no sobrenadante foi detectada em alta concentração quando da exposição de BMDC a *T. cruzi*, mas não quando estimuladas com AdASP-2 (Figura 4a). Já através de PCR semi-quantitativo foram determinados os níveis de transcrição de mRNA de citocinas nas BMDC estimuladas com *T. cruzi* que resultaram em maior transcrição de IL-6, IL-12 p35, e IL-10 em comparação a AdASP-2, enquanto o vírus induziu maior transcrição de mRNA de IL-27 p28, IL23 p19 e TNF do que o parasito (Figura 4b).

Figura 3 - Expressão de MHC e moléculas coestimuladoras na superfície de BMDC expostas a *T. cruzi* e/ou adenovírus.

BMDC derivadas de camundongos C57BL/6 ou MyD88^{-/-} foram deixadas sem tratamento (sombreado) ou incubadas por 24 h (linha cheia) com LPS, CpG, *T. cruzi* viável ou inativado por congelamento e descongelamento e/ou AdASP-2 conforme descrito na metodologia. A expressão de CD40 (a), CD80 (b), CD86 (c), MHC classe I (H-2K^b) (d) e MHC classe II (I-A^b) (e) foi avaliada por citometria de fluxo. Resultados representativos de pelo menos dois experimentos independentes.

Figura 4 – Citocinas induzidas em BMDC expostas a *T. cruzi* ou adenovírus.

BMDC derivadas de camundongos C57BL/6 foram deixadas sem tratamento ou incubadas por 24 h com *T. cruzi* cepa Y e/ou AdASP-2 de *T. cruzi*. A presença no sobrenadante das citocinas indicadas foi avaliada por ELISA (a) e a transcrição de mRNA quantificada por PCR semi-quantitativo em relação ao controle sem estímulo (b), conforme descrito na metodologia. Em a as barras representam média $\pm$ erro padrão com amostras em duplicata e ** indica diferença significativa entre *T. cruzi* e AdASP-2 ($P < 0,01$ ANOVA de dois fatores e teste Tukey *post hoc*). Em b, as barras representam o cálculo de expressão relativa pelo método $\Delta \Delta Ct$ com amostras em duplicata. *Gapdh* foi utilizado como controle endógeno.

Para avaliar e comparar a capacidade de BMDC expostas ao *T. cruzi* ou AdASP-2 de apresentarem antígenos para linfócitos T CD8⁺ *in vitro*, foi realizado um ensaio onde BMDC previamente incubadas com diferentes números de tripomastigotas de cultura de *T. cruzi* ou PFU de AdASP-2 foram colocadas em cocultura com linfócitos T CD8⁺ isolados do baço de camundongos C57BL/6 infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. 15 dias antes. A capacidade destes linfócitos T CD8⁺ secretarem IFN- γ após contato com as BMDC foi avaliada por ELISPOT e interpretada como resultado de apresentação de antígenos. Como controles positivos, BMDC foram carregadas com o peptídeo VNHRFTLV que corresponde ao epítipo imunodominante da proteína ASP-2 de *T. cruzi*, ou com a mistura dos peptídeos VNHRFTLV, ANYKFTLV e ANYDFTLV, que representam os principais epítipos de *T. cruzi* cepa Y restritos a H-2K^b. Como controle negativo, BMDC não tratadas foram utilizadas para estimular os linfócitos T CD8⁺. Outro controle negativo consistiu na incubação das diferentes BMDC com linfócitos T CD8⁺ isolados de animais *naïve*, em que não se observou secreção de IFN- γ (dados não mostrados). Notavelmente, tanto BMDC expostas a *T. cruzi* quanto BMDC expostas a AdASP-2 foram capazes de apresentar antígenos para linfócitos T CD8⁺ *in vitro* (Figura 5). Esta capacidade aumentou de acordo com o aumento do número de tripomastigotas de *T. cruzi* ou PFU de AdASP-2 incubados com as BMDC. No entanto, a capacidade máxima de estimulação dos linfócitos T CD8⁺ observada para BMDC expostas ao parasito foi inferior à obtida no caso do vírus.

A possível interferência do *T. cruzi* na capacidade de apresentação de epítipos restritos a MHC classe I expressos pelo adenovírus foi testada em um experimento de coexposição de BMDC ao parasito e ao adenovírus humano sorotipo 5 que expressa β -galactosidase (Ad β -gal). Neste experimento, BMDC coinfectadas ou incubadas apenas com Ad β -gal foram colocadas em cocultura com linfócitos T CD8⁺ isolados do baço de camundongos C57BL/6 previamente injetados com 2×10^8 PFU de Ad β -gal via *i.m.* 15 dias antes. A apresentação de epítipos virais por BMDC para estes linfócitos T CD8⁺ foi então avaliada através de ELISPOT para detecção do número de células secretoras de IFN- γ . As BMDC expostas unicamente a Ad β -gal ou a Ad β -gal + *T. cruzi* foram igualmente capazes de apresentar epítipos virais para linfócitos T CD8⁺ cognatos, indicando que o parasito não atrapalha a apresentação de antígenos virais. Além disto, BMDC carregadas com o peptídeo ICPMYARV correspondente ao epítipo imunodominante da β -galactosidase restrito

a H-2^b foram tão capazes de apresentar o epítipo para os linfócitos T CD8⁺ quanto BMDC que foram carregadas com ICPMYARV e expostas ao *T. cruzi* (Figura 6). Novamente, na presença de BMDC não infectadas ou de linfócitos T CD8⁺ isolados de animais *naïve* não se observou secreção de IFN- γ (não mostrado).

Figura 5 – Apresentação de antígenos *in vitro* para linfócitos T CD8⁺ por BMDC expostas a *T. cruzi* ou adenovírus.

BMDC derivadas de camundongos C57BL/6 foram deixadas sem tratamento ou expostas a *T. cruzi* ou AdASP-2 nas proporções indicadas e utilizadas em um ensaio de ELISPOT para IFN- γ com células respondedoras CD8⁺ isoladas do baço camundongos infectados 15 dias antes com *T. cruzi*. Como controles positivos foram utilizados o peptídeo imunodominante restrito a H-2^b VNHRFTLV (1 pep) ou a mistura dos peptídeos VNHRFTLV, ANYKFTLV e ANYDFTLV (3 pep). SFC: células formadoras de *spots*. Resultados representativos de pelo menos três experimentos independentes. As barras representam média $\pm$ erro padrão de amostras em triplicata. Asteriscos indicam diferença significativa entre *T. cruzi* e AdASP-2 para a mesma multiplicidade de infecção (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*).

Figura 6 – Apresentação de antígenos *in vitro* por BMDC coinfectadas com *T. cruzi* e adenovírus para linfócitos T CD8⁺ previamente induzidos contra o vírus.

BMDC derivadas de animais C57BL/6 foram deixadas sem tratamento ou incubadas com Adβ-gal em presença ou ausência de *T. cruzi*, conforme indicado. Estas células foram então colocadas em cocultura com células respondedoras T CD8⁺ isoladas de baço de camundongos imunizados com Adβ-gal 15 dias antes. O número de células produtoras de IFN-γ foi determinado por ELISPOT. O peptídeo da β-galactosidase restrito a H-2^b ICPMYARV foi utilizado como controle positivo. Resultados representativos de pelo menos dois experimentos independentes. SFC: células formadoras de spots. Barras representam média ± erro padrão de amostras em triplicata. Entre os grupos indicados não houve diferença significativa (ANOVA de dois fatores e teste Tukey *post hoc*).

Em outra abordagem, BMDC foram incubadas com *T. cruzi* juntamente ou não com Adβ-gal e então colocadas em cocultura com linfócitos T CD8⁺ retirados do baço de animais C57BL/6 infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. 15 dias antes. Neste caso, os linfócitos T CD8⁺ induzidos *in vivo* pelo parasito foram igualmente reestimulados *in vitro* por BMDC incubadas com *T. cruzi* em presença ou ausência de Adβ-gal, mostrando que também o vírus não atrapalhou a apresentação de antígenos do parasito para linfócitos T CD8⁺ (Figura 7).

Em conjunto, os resultados descritos acima indicam que DC expostas tanto a *T. cruzi* como a adenovírus recombinantes são capazes de processar e apresentar epítomos ligados a MHC classe I, bem como expressar moléculas coestimuladoras e secretar citocinas importantes para a estimulação de linfócitos T CD8⁺.

Figura 7 – Apresentação de antígenos *in vitro* por BMDC coinfectadas com *T. cruzi* e adenovírus para linfócitos T CD8⁺ previamente induzidos contra o parasito.

BMDC derivadas de animais C57BL/6 foram deixadas sem tratamento ou expostas ao *T. cruzi* em presença ou ausência de Adβ-gal, conforme indicado. Estas células foram então colocadas em cocultura com células respondedoras T CD8⁺ isoladas de baço de camundongos infectados com *T. cruzi* 15 dias antes. O número de células produtoras de IFN-γ foi determinado por ELISPOT. Resultados representativos de pelo menos dois experimentos independentes. SFC: células formadoras de spots. Barras representam média ± erro padrão de amostras em triplicata. Entre os grupos indicados não houve diferença significativa (ANOVA de dois fatores e teste Tukey *post hoc*).

BMDC diferenciadas *in vitro* em presença de GM-CSF recombinante são largamente utilizadas como modelo para o estudo de mecanismos de apresentação de antígenos para linfócitos T, ainda que estas células diferenciadas em cultura não correspondam exatamente às DC encontradas *in vivo*. Para confirmar a capacidade de DC apresentarem antígenos de *T. cruzi* para linfócitos T CD8⁺, observada com BMDC, foram também realizados experimentos com DC *bona fide*, isoladas do linfonodo drenante de camundongos 5 dias após infecção via s.c. com 1×10^4 formas sanguíneas de *T. cruzi* cepa Y. Como as DC constituem uma população heterogênea, estas foram separadas em três subpopulações: CD8⁺, CD8⁻ e plasmacitóides (pDC), como mostrado na Figura 8a. Em comparação às DC isoladas de animais *naïve*, as três subpopulações exibiram níveis mais altos de expressão da molécula coestimuladora CD86 após infecção com *T. cruzi* (Figura 8b). Ainda, apesar das diferenças quantitativas, os três subtipos de DC foram capazes de apresentar antígenos de *T. cruzi* restritos a H-2^b *ex vivo*, como avaliado por

ELISPOT para IFN- γ após cocultura com linfócitos T CD8⁺ isolados do baço de animais previamente infectados com o parasito (Figura 8c).

Figura 8 – Apresentação *ex vivo* de antígenos de *T. cruzi* para linfócitos T CD8⁺ por DC isoladas do linfonodo.

Camundongos C57BL/6 *naïve* ou infectados via s.c. 5 dias antes com 1×10^4 formas sanguíneas de *T. cruzi* tiveram os linfonodos drenantes retirados e as subpopulações de DC foram separadas por *sorting* como mostrado em a. A marcação de CD86 nas células de animais *naïve* (sombreado) e infectados (linha cheia) é mostrada em b. A capacidade destas células apresentarem antígenos para linfócitos CD8⁺ retirados do baço de animais infectados com *T. cruzi* 15 dias antes é mostrada em c, num ensaio de ELISPOT para IFN- γ . SFC: células formadoras de *spots*. Resultados representativos de pelo menos dois experimentos independentes. Barras representam média $\pm$ erro padrão de amostras em triplicata com *pool* de DC retiradas de 10 animais. Asteriscos indicam diferença significativa entre as subpopulações indicadas (**P<0,01 ***P<0,001, ANOVA de dois fatores e teste Tukey *post hoc*).

Portanto, células dendríticas *bona fide*, especialmente as que expressam CD8, também são capazes de apresentar antígenos de *T. cruzi* quando colocadas em cocultura com linfócitos T CD8⁺ previamente induzidos *in vivo*. Estes dados corroboraram os achados iniciais utilizando BMDC e validaram seu emprego como modelo para o estudo da apresentação de antígenos para linfócitos T.

Em sequência, foram investigadas as vias de processamento de antígenos expressos por *T. cruzi* e adenovírus. Pela via canônica de apresentação de antígenos restritos a MHC classe I, peptídeos são gerados no citosol através do processamento pelo proteassomo de proteínas maduras ou DRiPs recém-sintetizados e os peptídeos gerados são então translocados para o retículo endoplasmático pela molécula transportadora TAP. No entanto, a apresentação de antígenos de parasitos intracelulares, os quais muitas vezes residem em vacúolos parasitóforos, ainda que transientemente, é mais complexa e pode se dar de modo TAP-independente, como ocorre em *Leishmania spp*, por exemplo (BERTHOLET et al., 2006). Neste caso, em vez de acessar o citoplasma, os peptídeos podem ser processados e ligados a moléculas de MHC classe I pela via lisossomal após a fusão de vesículas. Para entender o papel de TAP no processamento de antígenos de *T. cruzi* restritos a H-2^b foram geradas BMDC de animais C57BL/6 ou TAP-1^{-/-} e estas células foram incubadas com tripomastigotas de *T. cruzi* ou com AdASP-2. As BMDC foram então colocadas em cocultura com linfócitos T CD8⁺ ou com linfócitos T CD4⁺ isolados do baço de animais infectados com *T. cruzi* 15 dias antes e a apresentação de antígenos para estes linfócitos foi quantificada por um ensaio de ELISPOT para IFN- γ . A ausência de TAP causa deficiência de linfócitos T CD8⁺, o que pode servir para confirmar o fenótipo dos animais TAP^{-/-} (Figura 9a). Em comparação às células normais, BMDC derivadas de animais deficientes de TAP exibiram níveis mais baixos de MHC classe I, como esperado para esta deficiência, enquanto modularam igualmente a expressão de MHC classe II, CD40, CD80 e CD86 em resposta a *T. cruzi*, AdASP-2 ou LPS (Figura 9b). Ainda, quando comparadas BMDC normais com deficientes em TAP, ambas foram capazes de apresentar antígenos para linfócitos T CD8⁺ *in vitro* se carregadas com o peptídeo VNHRFTLV que corresponde ao epítipo imunodominante da ASP-2 restrito a H-2K^b (Figura 9c). No entanto, quando o peptídeo processado não foi incubado com as BMDC, mas estas foram expostas ao *T. cruzi* ou AdASP-2, exigindo o processamento dos antígenos protéicos, a deficiência de TAP impediu

completamente a apresentação antigênica para linfócitos T CD8⁺ (Figura 9d). Coerentemente, este requerimento de TAP para o processamento de antígenos expressos pelo parasito e pelo vírus mostrou-se específico para a via de MHC classe I, uma vez que as BMDC deficientes em TAP foram comparáveis às BMDC normais quanto à capacidade de apresentar antígenos expressos pelo *T. cruzi* ou AdASP-2 para linfócitos T CD4⁺ (Figura 9e).

Figura 9 – Papel de TAP na apresentação *in vitro* de antígenos expressos por *T. cruzi* e adenovírus para linfócitos T CD8⁺.

Animais C57BL/6 (WT) ou deficientes em TAP-1 (TAP^{-/-}) tiveram o fenótipo confirmado pela marcação de CD8 nas células do baço, como mostrado em a. BMDC foram derivadas destes animais e deixadas sem tratamento ou incubadas com LPS, *T. cruzi*, ou com AdASP-2 e a marcação das moléculas H-2K^b, I-A^b, CD40, CD80 e CD86 foi avaliada nas células CD11c⁺ por citometria de fluxo (b). BMDC destes grupos ou ainda carregadas com o peptídeo VNHRFTLV foram colocadas, conforme indicado, em cocultura com linfócitos T CD8⁺ (c e d) ou T CD4⁺ (e) isolados do baço de animais previamente infectados com *T. cruzi* e o número de células secretoras de IFN- γ foi determinado por ELISPOT. Resultados representativos de pelo menos três experimentos independentes. SFC: células formadoras de spots. Barras representam média $\pm$ erro padrão de amostras em triplicata. Asteriscos indicam diferença significativa entre WT e TAP^{-/-} (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*).

A necessidade de TAP para a apresentação de antígenos de *T. cruzi* para linfócitos T CD8⁺ indica que os epítomos do parasito devem ser translocados do citosol para o retículo endoplasmático, como classicamente descrito para o processamento de antígenos restritos a MHC classe I. Isto sugere que proteínas de *T. cruzi* devem ser degradadas no citosol, possivelmente pelo proteassomo. Para testar esta hipótese, BMDC foram derivadas de animais C57BL/6 e incubadas com *T. cruzi* ou AdASP-2 na presença ou ausência de epoxomicina, um inibidor químico do proteassomo originalmente isolado de uma cepa de *Actinomyces* sp. Como controles, BMDC foram incubadas com *T. cruzi* ou AdASP-2 em presença de DMSO (utilizado como diluente da epoxomicina). A presença de epoxomicina não impediu a modulação da expressão de MHC classe I, MHC classe II ou CD86 nas células CD11c⁺, tampouco induziu morte das BMDC que pudesse ser detectada pela incorporação de 7-AAD (Figura 10a). Alternativamente, BMDC tratadas ou não com epoxomicina foram carregadas com o peptídeo VNHRFTLV. Ou ainda, BMDC foram deixadas sem nenhum tipo de tratamento ou recebimento de antígenos. Estas BMDC foram cocultivadas com linfócitos T CD8⁺ isolados do baço de animais previamente infectados com *T. cruzi* e, novamente, a capacidade de apresentação de antígenos foi avaliada por ELISPOT para IFN- γ . Como mostrado na Figura 10b, o tratamento com epoxomicina reduziu significativamente a capacidade de apresentação de antígenos das BMDC para linfócitos T CD8⁺, tanto quando da incubação com *T. cruzi* como AdASP-2, mas não quando carregadas com o peptídeo VNHRFTLV, indicando que esta redução de fato se deveu a um problema no processamento dos epítomos. Portanto, o processamento de antígenos de *T. cruzi* restritos a MHC classe I se dá pela via citosólica, dependente do proteassomo.

Figura 10 – Papel do proteassomo na apresentação *in vitro* de antígenos expressos por *T. cruzi* e adenovírus para linfócitos T CD8⁺.

BMDC foram derivadas de animais C57BL/6 e deixadas sem infecção, peptídeo, nem tratamento algum (não tratado) ou foram incubadas com o peptídeo VNHRFTLV, ou com *T. cruzi*, ou com AdASP-2 em presença do diluente DMSO ou do inibidor de proteassomo epoxomicina (EPO). A marcação de H-2K^b, I-A^b e CD86 ou com 7-AAD nas BMDC CD11c⁺ é mostrada em a. Estas BMDC foram colocadas em cocultura com linfócitos T CD8⁺ isolados do baço de animais previamente infectados com *T. cruzi* e o número de células secretoras de IFN- γ foi determinado por ELISPOT (b). Resultados representativos de pelo menos três experimentos independentes. SFC: células formadoras de *spots*. Barras representam média $\pm$ erro padrão de amostras em triplicata. Asteriscos indicam diferença significativa entre DMSO e EPO (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*).

As subunidades catalíticas β do proteassomo 20S podem se apresentar na configuração padrão ou podem ser trocadas pelas formas alternativas $\beta 1i$, $\beta 2i$ e $\beta 5i$ que caracterizam o imunoproteassomo, cujo papel específico na geração de epítopos restritos a MHC I vem sendo investigado ultimamente. Para determinar o papel específico do imunoproteassomo na geração de epítopos expressos por *T. cruzi* ou AdASP-2 reconhecidos por linfócitos T CD8⁺, inicialmente foram geradas BMDC de animais C57BL/6 normais ou TKO, os quais são geneticamente deficientes das três subunidades que definem o imunoproteassomo (KINCAID et al., 2012). Como nas DC a expressão do imunoproteassomo é constitutiva, espera-se que as BMDC normais expressem as subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ mesmo se não

expostas a IFN- γ . As BMDC TKO deficientes de $\beta 1i$, $\beta 2i$ e $\beta 5i$ foram comparáveis às BMDC derivadas de animais C57BL/6 em sua capacidade de modular a expressão de CD86 em resposta a *T. cruzi* ou AdASP-2, porém a marcação de H-2K^b foi reduzida, o que seria esperado no caso de uma menor provisão de peptídeos restritos a MHC classe I pelas BMDC de fundo TKO (Figura 11a). Ainda, as BMDC normais ou deficientes de imunoproteassomo foram igualmente capazes de secretar IL-12 quando estimuladas com o parasito ou o vírus (Figura 11b). Quando BMDC normais ou TKO previamente expostas a *T. cruzi* ou AdASP-2 foram colocadas em cocultura com linfócitos T CD8⁺ isolados do baço de animais previamente infectados com *T. cruzi*, pôde-se observar uma menor capacidade de apresentação de antígenos *in vitro* na ausência do imunoproteassomo (Figura 11c). No entanto, como esperado, esta deficiência foi restrita à apresentação de epítopos restritos a MHC classe I, já que linfócitos T CD4⁺ de camundongos previamente infectados com *T. cruzi* responderam igualmente secretando IFN- γ quando estimulados com BMDC normais ou TKO que haviam sido previamente expostas ao *T. cruzi* ou AdASP-2 (Figura 11d).

Em conjunto, os resultados obtidos *in vitro* apresentados até aqui demonstram que DC expostas a *T. cruzi* ou AdASP-2 são capazes de prover os três sinais necessários para ativar linfócitos T CD8⁺ i) antígeno ligado a MHC classe I; ii) coestímulo e iii) citocinas. Ainda, o processamento dos epítopos de *T. cruzi* e adenovírus que se ligam a moléculas de MHC classe I e são reconhecidos por linfócitos T CD8⁺ *in vitro* ocorre pela via citosólica: dependente de proteassomo e TAP. A expressão das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ que formam o imunoproteassomo teve também um papel considerável no incremento do processamento de epítopos de *T. cruzi* e AdASP-2 que são apresentados no contexto de MHC classe I.

Figura 11 – Papel do imunoproteassomo na apresentação *in vitro* de antígenos expressos por *T. cruzi* e adenovírus para linfócitos T CD8⁺.

BMDC foram diferenciadas de animais C57BL/6 (WT) ou de animais deficientes das três subunidades do imunoproteassomo (TKO) e incubadas com *T. cruzi*, AdASP-2 ou deixadas sem nenhum tratamento. A marcação de H-2K^b e CD86 nas BMDC CD11c⁺ foi avaliada por citometria de fluxo (a). A quantificação de IL-12 p70 no sobrenadante das culturas de BMDC foi realizada por ELISA (b). As BMDC de cada animal foram colocadas em cocultura com linfócitos T CD8⁺ (c) ou T CD4⁺ (d) isolados do baço de animais previamente infectados com *T. cruzi* e o número de células secretoras de IFN-γ foi determinado por ELISPOT. Resultados representativos de pelo menos dois experimentos independentes. SFC: células formadoras de spots. Barras representam média ± erro padrão de amostras em triplicata. Asteriscos indicam diferença significativa entre WT e TKO (**P<0,001 ANOVA de dois fatores e teste Tukey post hoc).

4.2 Papel do imunoproteassomo na resposta de linfócitos T CD8⁺ induzidos *in vivo* pela infecção com *T. cruzi* e vacinação genética

Uma vez que os resultados iniciais de cocultura de DC e linfócitos T *in vitro* apontaram para a importância do imunoproteassomo na apresentação de antígenos de *T. cruzi* para linfócitos T CD8⁺, foram realizados experimentos subsequentes para avaliar seu papel *in vivo*. Animais C57BL/6 foram deixados sem infectar ou infectados por via subcutânea com 1×10^4 formas sanguíneas de *T. cruzi* cepa Y e após 12 dias foram coletados o baço, fígado e coração de cada animal. Em comparação aos animais não infectados controle, a infecção por *T. cruzi* induziu maior transcrição de mRNA das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ que caracterizam o imunoproteassomo, enquanto a transcrição das subunidades convencionais $\beta 1$, $\beta 2$ e $\beta 5$ do proteassomo 20S foi pouco alterada, conforme análise por PCR semi-quantitativo (Figura 12).

Figura 12 – Indução da transcrição de mRNA do imunoproteassomo em animais infectados com *T. cruzi*.

Animais C57BL/6 (n=5) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar. Após 12 dias, os níveis de transcrição de mRNA das subunidades catalíticas do proteassomo ($\beta 1$, $\beta 2$ e $\beta 5$) e das subunidades correspondentes do imunoproteassomo ($\beta 1i$, $\beta 2i$ e $\beta 5i$) foram quantificados no baço, coração e fígado através de PCR semi-quantitativo. Barras indicam mediana $\pm$ erro padrão de cada grupo para o cálculo de expressão relativa pelo método $\Delta \Delta Ct$ com amostras em duplicata. *Hprt* foi utilizado como controle endógeno. Asteriscos indicam diferença estatística entre as subunidades do proteassomo convencional e imunoproteassomo (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*).

Este resultado demonstrando a indução do imunoproteassomo após a infecção com *T. cruzi* sustentou a hipótese de que as subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ são importantes para a resposta imune em resposta à infecção e poderiam contribuir para o *priming* de linfócitos T CD8⁺ específicos que reconhecem epítomos do parasito *in vivo*. Por isso, estudamos a resposta dos linfócitos T CD8⁺ em animais normais (C57BL/6) ou totalmente deficientes de imunoproteassomo (TKO).

Após uma primeira análise do perfil desses animais ficou notório que, independentemente de estarem ou não infectados com *T. cruzi*, os animais deficientes de imunoproteassomo apresentaram níveis mais baixos de expressão de MHC classe I nas DC em comparação aos seus pares normais (Figura 13a), o que poderia ser esperado no caso da deficiência do imunoproteassomo diminuir a quantidade de epítomos processados que se ligam e estabilizam as moléculas de MHC classe I. Esta quantidade reduzida de moléculas H-2 também se refletiu em menor porcentagem e número total de linfócitos T CD8⁺, evidenciado principalmente nos animais infectados, como mostrado na Figura 14 (a-b). No entanto, os níveis basais de MHC classe II (Figura 13b) e a porcentagem e número total de linfócitos T CD4⁺ é similar quando se compararam os animais normais e deficientes de imunoproteassomo (Figura 14e-f).

Além da frequência e número de linfócitos T CD8⁺ totais serem reduzidos na ausência do imunoproteassomo, também a frequência e número de linfócitos T CD8⁺ com fenótipo de células efetoras que reconheceram o epítomo cognato (CD44^{alto} CD62L^{baixo}) foi menor nos animais TKO infectados há 20 dias com *T. cruzi* quando comparados aos animais normais C57BL/6 (Figura 14c-d), enquanto a ativação dos linfócitos T CD4⁺ não sofreu alteração quando da ausência completa de imunoproteassomo (Figura 14g-h).

Em conjunto, estes dados podem sugerir que a deficiência do imunoproteassomo *in vivo* afeta exclusivamente o compartimento de linfócitos T CD8⁺, tanto no estado basal como em resposta à infecção por *T. cruzi*, mas não afeta os linfócitos T CD4⁺, o que é coerente com a participação do imunoproteassomo na via de processamento dos epítomos restritos a MHC classe I.

Figura 13 – Expressão de moléculas de MHC em DC do baço de animais normais ou totalmente deficientes de imunoproteassomo.

Animais C57BL/6 (WT) ou deficientes das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ do imunoproteassomo (TKO) ($n=3$) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (*naïve*). Após 20 dias, a marcação de H-2K^b (a) e I-A^b (b) foi avaliada por citometria de fluxo nas células CD19⁻ CD3⁻ I-A^{b+} CD11c⁺ retiradas do baço destes animais. Resultados representados como valores individuais juntamente a barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (** $P<0,01$ *** $P<0,001$ ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes. gMFI: mediana geométrica da intensidade de fluorescência.

Figura 14 – Efeito do imunoproteassomo na frequência e número de linfócitos T totais e efetores em animais infectados com *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ do imunoproteassomo (TKO) (n=3) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (naïve). Após 20 dias, a frequência (a) e o número (b) de linfócitos T CD8⁺ totais no baço foi avaliada, bem como a frequência (c) e número (d) de linfócitos com fenótipo CD44^{alto} CD62L^{baixo} dentre os linfócitos T CD8⁺ totais. Análises correspondentes foram realizadas para os linfócitos T CD4⁺ (e-h). Resultados representados como valores individuais juntamente a barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (* $P < 0,05$ ** $P < 0,01$ *** $P < 0,001$ **** $P < 0,0001$ ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Como forma de estimar o repertório dos linfócitos T ativados 20 dias após a infecção de animais normais ou deficientes de imunoproteassomo com *T. cruzi*, foram marcadas com anticorpos as diferentes formas da região variável da cadeia β do TCR ($V\beta$). Esta análise foi realizada nos linfócitos T $CD8^+$ e T $CD4^+$ que exibiram fenótipo $CD44^{\text{alto}}$ $CD62L^{\text{baixo}}$. Como mostra a Figura 15, a distribuição de frequências dos diferentes tipos de $V\beta$ dos linfócitos T $CD8^+$ $CD44^{\text{alto}}$ $CD62L^{\text{baixo}}$ nos animais infectados com *T. cruzi* foi diferente quando comparados os animais C57BL/6 e TKO, enquanto nos animais não infectados esta distribuição foi similar. Já para os linfócitos T $CD4^+$ $CD44^{\text{alto}}$ $CD62L^{\text{baixo}}$ não houve diferença na distribuição de frequências dos diferentes tipos de $V\beta$, tanto nos animais *naïve* como nos infectados com *T. cruzi*.

Figura 15 – Efeito do imunoproteassomo na frequência de clones de linfócitos T em animais infectados com *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ do imunoproteassomo (TKO) ($n=4$) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (*naïve*). Após 20 dias, a clonalidade dos linfócitos T $CD8^+$ $CD44^{\text{alto}}$ $CD62L^{\text{baixo}}$ (a-b) e T $CD4^+$ $CD44^{\text{alto}}$ $CD62L^{\text{baixo}}$ (c-d) foi estimada por marcação do TCR $V\beta$. Resultados obtidos com o *pool* das amostras de cada grupo.

A sugestão de que diferentes clones de linfócitos T CD8⁺ são induzidos pela infecção por *T. cruzi* na ausência completa do imunoproteassomo, apontada na Figura 15, levou-nos a investigar a resposta das células específicas para diferentes epítomos conhecidos do parasito restritos a H-2K^b. Dentre os epítomos descritos, o de sequência VNHRFTLV representa o mais imunodominante. Por isso, os linfócitos T CD8⁺ específicos que reconhecem este epítomo foram marcados com pentâmeros de moléculas H-2K^b ligadas a VNHRFTLV. Como mostrado na Figura 16, a indução de linfócitos T CD8⁺ específicos para VNHRFTLV 20 dias após a infecção com *T. cruzi* aconteceu apenas na presença do imunoproteassomo, sugerindo a necessidade das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ para o processamento e apresentação deste epítomo durante o *priming* dos linfócitos T CD8⁺.

Figura 16 – Dependência do imunoproteassomo para a resposta primária de linfócitos T CD8⁺ específicos para o epítomo VNHRFTLV induzidos pela infecção por *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=3) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (*naïve*). Após 20 dias, os linfócitos T CD8⁺ específicos para o epítomo VNHRFTLV foram marcados, como mostrado em a. A frequência (b) e número total (c) de células específicas no baço também são mostrados. São apresentados valores individuais e barras que indicam a mediana $\pm$ erro

padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (**P<0,01 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Para confirmar este resultado referente à resposta imune específica para o epítipo imunodominante de *T. cruzi* restrito a H-2K^b e estudar a resposta específica para outros epítopos em camundongos normais e deficientes do imunoproteassomo infectados com *T. cruzi*, foram realizados ainda ensaios de marcação intracelular de citocinas (ICS) e ELISPOT de esplenócitos reestimulados *ex vivo* com os peptídeos VNHRFTLV, bem como ANYKFTLV e ANYDFTLV (os quais correspondem aos epítopos subdominantes de *T. cruzi* restritos a H-2K^b conhecidos), além de VNDYTLV e ANYNFTLV (os quais possuem sequências similares aos epítopos descritos e que eventualmente poderiam ser reconhecidos). Os resultados destes ensaios demonstraram que, quando comparados os animais C57BL/6 e TKO 20 dias após a infecção com *T. cruzi*, a resposta imune dos linfócitos T CD8⁺ específicos foi sempre reduzida na ausência de imunoproteassomo, em termos de frequência e número absoluto de células com marcação intracelular para TNF e/ou IFN-γ (Figura 17), bem como em termos de número de células secretoras de IFN-γ que formaram spots (Figura 18). Ainda, quando a qualidade da resposta de citocinas para o epítipo VNHRFTLV foi comparada, viu-se que as células respondedoras dos animais TKO produziam majoritariamente TNF, enquanto nos animais normais foi preponderante a produção de IFN-γ, que é fundamental para o controle da infecção por *T. cruzi* (Figura 17d). Por outro lado, a resposta de citocinas de linfócitos T CD4⁺ dos animais C57BL/6 e TKO infectados com *T. cruzi* pôde ser detectada por ICS mesmo sem reestímulo específico dos esplenócitos *ex vivo* e para este grupo de células a deficiência de imunoproteassomo não teve impacto nenhum na produção de TNF e/ou IFN-γ (Figura 19).

Portanto, os animais completamente desprovidos de imunoproteassomo infectados com *T. cruzi* exibiram uma resposta de linfócitos T CD8⁺ deficitária, em comparação a animais normais, no que diz respeito à quantidade de células T CD8⁺ com fenótipo de células efetoras, à expansão do clone imunodominante e à produção de citocinas por clones de diferentes especificidades. Ainda, a clonalidade estimada dos linfócitos T CD8⁺ ativados pela infecção com *T. cruzi* foi ligeiramente distinta entre animais normais e sem imunoproteassomo.

Figura 17 - Impacto do imunoproteassomo na produção de citocinas por linfócitos T CD8⁺ específicos induzidos pela infecção por *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=3) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (*naïve*). Após 20 dias, os esplenócitos foram reestimulados com meio ou com os peptídeos indicados e a marcação intracelular de TNF e IFN- γ foi avaliada nas células T CD8⁺, como ilustrado em a. A frequência (b) e número total (c) de células respondedoras são mostrados. A análise da combinação de citocinas produzidas após reestímulo com VNHRFTLV é colocada em d. São apresentados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (* $P < 0,05$ **** $P < 0,0001$ ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Figura 18 - Efeito do imunoproteassomo no número de células específicas produtoras de IFN- γ induzidas pela infecção por *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=3) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (*naïve*). Após 20 dias, os esplenócitos foram reestimulados com meio ou com os peptídeos indicados e o número de células produtoras de IFN- γ foi determinado por ELISPOT. São apresentados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes. SFC: células formadoras de *spots*.

Figura 19 - Impacto do imunoproteassomo na produção de citocinas por linfócitos T CD4⁺ induzidos pela infecção por *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=3) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (*naïve*). Após 20 dias, os esplenócitos foram coletados e a marcação intracelular de TNF e IFN-γ foi avaliada nas células T CD4⁺. A frequência (a) e número total (b) de células respondedoras são mostrados. São apresentados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (*P<0,05 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Em seguida, foi investigado o papel do imunoproteassomo na indução de linfócitos T CD8⁺ pela vacinação genética experimental contra *T. cruzi*. Para isto, foi utilizado um protocolo de dose-reforço heterólogo, empregando o plasmídeo plgSPclone9 (inserido de *Asp2*) na primeira imunização, e reforço com AdASP-2 após 21 dias. Como controle negativo, animais foram imunizados com o plasmídeo vazio pcDNA3 na primeira imunização e com Adβ-gal no reforço. Este protocolo foi previamente desenvolvido em nosso laboratório e mostrou-se altamente eficiente na geração de linfócitos T CD8⁺ efetores e de memória de longa duração, os quais conferem proteção contra o desafio letal com *T. cruzi* em uma linhagem de camundongos altamente susceptíveis (DE ALENCAR et al., 2009; RIGATO et al., 2011). A indução de resposta dos linfócitos T CD8⁺ induzidos por esta estratégia de

vacinação foi então avaliada 15 dias após a administração do reforço em animais normais ou totalmente desprovidos de imunoproteassomo.

Novamente, o número de linfócitos T CD8⁺ totais ou com fenótipo CD44^{alto} CD62L^{baixo} foram reduzidos nos animais TKO imunizados, em comparação aos animais C57BL/6, enquanto no compartimento de linfócitos T CD4⁺ não houve reduções nestes parâmetros (Figura 20). A clonalidade dos linfócitos T CD8⁺ ativados pela vacinação também mostrou-se diferente entre os animais que expressam ou não as subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$, como estimado com o emprego do painel de anticorpos que marcam a região variável da cadeia β do TCR. Ainda, esta diferença de clonalidade não foi pronunciada nos animais que receberam apenas os vetores controle, tampouco no compartimento de linfócitos T CD4⁺ (Figura 21).

No que diz respeito à expansão do clone de linfócitos T CD8⁺ que reconhece o epítipo imunodominante VNHRFTLV encontrado na proteína ASP-2, avaliada pela marcação com pentâmeros, foi observada uma drástica redução na frequência e número destas células no animais desprovidos de imunoproteassomo vacinados, em comparação aos seus pares selvagens (Figura 22). Além disto, a frequência e número de linfócitos T CD8⁺ identificados pela marcação intracelular de TNF e/ou IFN- γ após reestimulação *ex vivo* de esplenócitos com o peptídeo VNHRFTLV foram significativamente reduzidos nos animais TKO em relação aos controles C57BL/6 (Figura 23). No entanto, a qualidade desta resposta, no que se refere à combinação de citocinas produzidas, não foi particularmente alterada (Figura 23). Mais uma vez, através do ensaio de ELISPOT para quantificação do número de células produtoras de IFN- γ em resposta à reestimulação com VNHRFTLV foi confirmada a redução na resposta dos animais totalmente desprovidos de imunoproteassomo em comparação aos normais (Figura 24).

Figura 20 - Efeito do imunoproteassomo na frequência e número de linfócitos T totais e efetores em animais geneticamente vacinados contra *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ do imunoproteassomo (TKO) (n=4) foram geneticamente imunizados com DNA plasmidial e adenovírus recombinantes inseridos dos genes *Asp2* (ASP-2) ou *Glb1* (β -gal), como descrito na metodologia. Após 15 dias da última imunização, a frequência (a) e o número (b) de linfócitos T CD8⁺ totais no baço foi avaliada, bem como a frequência (c) e número (d) de linfócitos com fenótipo CD44^{alto} CD62L^{baixo} dentre os linfócitos T CD8⁺ totais. Análises correspondentes foram realizadas para os linfócitos T CD4⁺ (e-h). São mostrados valores individuais juntamente a barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (* P<0,05 **P<0,01 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Figura 21 - Efeito do imunoproteassomo na frequência de clones de linfócitos T em animais geneticamente vacinados contra *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes das subunidades $\beta 1i$, $\beta 2i$ e $\beta 5i$ do imunoproteassomo (TKO) (n=4) foram geneticamente imunizados com DNA plasmidial e adenovírus recombinantes inseridos de *Asp2* (ASP-2) ou *Glb1* (β -gal), como descrito na metodologia. Após 15 dias da última imunização, a clonalidade dos linfócitos T $CD8^+ CD44^{alto} CD62L^{baixo}$ (a-b) e T $CD4^+ CD44^{alto} CD62L^{baixo}$ (c-d) foi estimada por marcação do TCR V β . Resultados obtidos com o pool das amostras de cada grupo.

Figura 22 - Dependência do imunoproteassomo para a indução de resposta de linfócitos T CD8⁺ específicos para o epítipo VNHRFTLV induzidos por vacinação genética contra *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=4) foram geneticamente imunizados com DNA plasmidial e adenovírus recombinantes que codificam *Asp2* (ASP-2) ou *Glb1* (β-gal), como descrito na metodologia. Após 15 dias da última imunização, os linfócitos T CD8⁺ específicos para o epítipo VNHRFTLV foram marcados com pentâmeros, como mostrado em a. A frequência (b) e número total (c) de células específicas no baço também são mostrados. São apresentados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Figura 23 - Impacto do imunoproteassomo na produção de citocinas por linfócitos T CD8⁺ específicos induzidos pela vacinação genética contra *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=4) foram geneticamente imunizados com DNA plasmidial e adenovírus recombinantes que codificam *Asp2* (ASP-2) ou *Glb1* (β-gal), como descrito na metodologia. Após 15 dias da última imunização, os esplenócitos foram reestimulados com meio ou com o peptídeo VNHRFTLV e a marcação intracelular de TNF e IFN-γ foi avaliada nas células T CD8⁺, como ilustrado em a. A frequência (b) e número total (c) de células respondedoras são mostrados. A análise da combinação de citocinas produzidas após reestímulo com VNHRFTLV é colocada em d. São apresentados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (***P<0,001 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes.

Figura 24 - Efeito do imunoproteassomo no número de células específicas produtoras de IFN- γ induzidas pela vacinação genética contra *T. cruzi*.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=4) foram geneticamente imunizados com DNA plasmidial e adenovírus recombinantes que codificam *Asp2* (ASP-2) ou *Glb1* (β -gal), como descrito na metodologia. Após 15 dias da última imunização, os esplenócitos foram reestimulados com meio ou com o peptídeo VNHRFTLV e o número de células produtoras de IFN- γ foi determinado por ELISPOT. São apresentados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultado representativo de dois experimentos independentes. SFC: células foradoras de spots.

Em conjunto, os dados apresentados até aqui indicam que a expressão das subunidades β 1i, β 2i e β 5i que definem o imunoproteassomo é relevante para a indução de imunidade mediada pelos linfócitos T CD8⁺ em resposta à infecção com *T. cruzi* e à vacinação genética contra o parasito.

Considerando a importância dos linfócitos T CD8⁺ para o controle da infecção pelo *T. cruzi*, a próxima etapa a ser investigada consistiu em analisar o efeito do imunoproteassomo na susceptibilidade ao protozoário. Inicialmente, animais

C57BL/6 normais ou animais simultaneamente desprovidos de $\beta 1i$, $\beta 2i$ e $\beta 5i$ foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* por via s.c. e tiveram o coração e baço coletados após 20 dias. Os órgãos então tiveram o DNA extraído e submetido a uma reação de PCR quantitativo para identificar a presença de *T. cruzi*. Como apresentado na Figura 25, a quantidade de parasitos nos tecidos de animais TKO foi significativamente superior à encontrada nos animais normais, sugerindo a importância do imunoproteassomo para a resistência ao *T. cruzi*.

Figura 25 – Efeito do imunoproteassomo no parasitismo de *T. cruzi* no coração e baço.

Animais C57BL/6 (WT) ou deficientes em imunoproteassomo (TKO) (n=3) foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. ou deixados sem infectar (naïve). Após 20 dias, o coração e baço foram coletados de cada animal e estes órgãos foram submetidos a PCR quantitativo para detecção de *T. cruzi*. São apresentados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT e TKO (****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.)

Em seguida, animais C57BL/6 ou TKO foram geneticamente vacinados com plgSPclone9 e AdASP-2 ou pcDNA3 e Ad β -gal, como descrito na metodologia. Após 15 dias da última imunização, estes animais foram desafiados com 1×10^4 formas sanguíneas de *T. cruzi* por via s.c. e a parasitemia no sangue e a mortalidade foram acompanhadas. Como se pode observar na Figura 26, os animais normais vacinados geneticamente com *Asp2* controlaram melhor a parasitemia do que o grupo controle, enquanto em ambos os casos os animais sobrevivem ao

desafio, repetindo os resultados prévios do nosso grupo. Em contraste, os animais totalmente desprovidos de imunoproteassomo são mais susceptíveis à infecção com *T. cruzi* e sucumbem ao desafio. Notavelmente, ainda, a vacinação genética com *Asp2* não teve nenhum efeito na proteção dos animais TKO.

Figura 26 – Importância do imunoproteassomo para a resistência à infecção com *T. cruzi* e proteção induzida por vacinação genética.

Animais C57BL/6 (WT) ou deficientes das três subunidades do imunoproteassomo (TKO) (n=5) foram geneticamente vacinados com DNA plasmidial e adenovírus recombinantes que codificam *Asp2* (ASP-2) ou *Glb1* (β -gal), como descrito na metodologia. Após 15 dias da última imunização, os animais foram desafiados com 1×10^4 formas sanguíneas de *T. cruzi* via s.c. e a parasitemia no sangue (a) e mortalidade (b) foram acompanhadas. Os valores da parasitemia foram transformados para logaritmos de base 10 e comparados no dia do pico (8) por ANOVA de um fator seguido de teste Tukey *post hoc*, mostrando que: i) os animais WT vacinados com *Asp2* exibiram níveis mais baixos de parasitemia em comparação com os outros grupos ($P < 0,01$), ii) os valores de parasitemia dos animais TKO (vacinados com *Asp2* ou controle) foram mais altos do que nos animais WT ($P < 0,01$) e iii) entre os animais TKO não houve diferença de parasitemia quando comparados os animais vacinados com *Asp2* e com os vetores controle. As curvas de sobrevivência foram comparadas pelo teste Mantel-Cox log-rank e demonstraram que: i) todos os grupos de animais WT sobreviveram mais do que os animais TKO ($P < 0,01$) e ii) entre os animais TKO vacinados com *Asp2* e controle não houve diferença estatística na sobrevivência. Resultados representativos de dois experimentos independentes.

Com estes resultados, pode-se propor que o imunoproteassomo tem um papel elementar na indução de resposta de linfócitos T CD8⁺ induzidos pela infecção com *T. cruzi* e vacinação genética com adenovírus recombinante, contribuindo assim para o controle da infecção pelo parasito.

4.3 Interferência causada por DC expostas a *T. cruzi* no *priming* de linfócitos T CD8⁺ *in vivo*

Outro aspecto pouco compreendido da imunidade mediada por linfócitos T CD8⁺ durante a infecção pelo *T. cruzi* consiste nos mecanismos reponsáveis por esta resposta ser subótima. A denominação de subótima, neste caso, foi associada à indução tardia destes linfócitos (TZELEPIS et al., 2006) e a um fenótipo de baixa viabilidade, correlacionado com alta expressão do marcador pró-apoptótico CD95 (VASCONCELOS; BRUNA-ROMERO; et al., 2012). Fisiologicamente, esta condição subótima pode ser corrigida pela vacinação genética com vetor adenoviral recombinante, que acelera a indução dos linfócitos T CD8⁺ e melhora seu *fitness*, de modo a proteger animais de uma linhagem altamente susceptível mesmo quando a vacinação se dá no mesmo dia do desafio (VASCONCELOS; BRUNA-ROMERO; et al., 2012).

Para entender melhor como o *T. cruzi* interfere na indução de linfócitos T CD8⁺ *in vivo*, nós consideramos ideal desenvolver um modelo controlado para a indução ótima destes linfócitos onde então o impacto específico do parasito pudesse ser evidenciado. O racional para o desenvolvimento deste modelo consistiu na hipótese de que a interação entre DC e linfócitos T CD8⁺ durante o *priming in vivo* poderia ser modificada pela presença do protozoário *T. cruzi*. Para testar esta possibilidade, o modelo desenvolvido consistiu na geração *in vitro* de BMDC, as quais foram estimuladas com LPS, carregadas com o peptídeo da ovalbumina restrito a H-2K^b SIINFEKL e subsequentemente transferidas para camundongos C57BL/6 que haviam recebido previamente linfócitos T CD8⁺ OTI transgênicos com alta afinidade para o epítipo SIINFEKL, como esquematizado na Figura 27a e descrito em detalhe na metodologia. Estes linfócitos T CD8⁺ transgênicos proliferaram quando estimulados *in vivo* por BMDC carregadas com o peptídeo cognato, como pôde ser avaliado pela frequência de células marcadas com o tetrâmero H-2K^b-SIINFEKL (Figura 27b-c), incorporação de BrdU pelas células tetrâmero-positivas (Figura 27c-d), bem como pela frequência de células marcadas com anticorpos que reconhecem as regiões variáveis do TCR dos linfócitos T CD8⁺ OTI (Figura 27e-f). No entanto, quando as BMDC utilizadas para indução da resposta destes linfócitos foram previamente incubadas *in vitro* por 24 h com *T. cruzi*, a proliferação das células OTI foi significativamente reduzida (Figura 27). Além disto, a diferenciação dos linfócitos OTI em células efetoras com fenótipo CD44^{alto}

CD62L^{baixo} foi reduzida quando as BMDC foram anteriormente expostas ao *T. cruzi* (Figura 27g-h).

A marcação intracelular das citocinas IL-2, TNF e IFN- γ nos linfócitos T CD8⁺ também foi avaliada nos animais que sofreram indução *in vivo* de células OTI seguindo o protocolo esquematizado na Figura 27a. Os esplenócitos destes animais foram reestimulados *ex vivo* com diferentes concentrações do peptídeo SIINFEKL. Em todas as concentrações utilizadas para reestimulação, a produção de IL-2, TNF e IFN- γ pelas células OTI foi menor no grupo que recebeu BMDC carregadas com o peptídeo cognato e que foram pré-incubadas com *T. cruzi* (Gr.3) em comparação ao grupo onde as BMDC não foram expostas ao parasito (Gr.2) (Figura 28a-c). Além disso, enquanto no Gr.2 as células que responderam produzindo citocinas foram majoritariamente polifuncionais, produzindo duas ou três citocinas simultaneamente, no Gr.3 as células OTI respondedoras foram preponderantemente monofuncionais, produzindo apenas uma das três citocinas avaliadas (Figura 28d). Quando o marcador genérico de degranulação CD107a foi adicionado a esta análise, o mesmo padrão foi observado, com maior frequência de células polifuncionais sendo induzido por BMDC que não foram previamente expostas ao *T. cruzi* (Figura 28e). A produção de IL-10, IL-17A ou IL-4 por esplenócitos CD4⁺ ou CD8⁺ reestimulados com SIINFEKL não foi observada em nenhum dos grupos, como avaliado por ICS (não mostrado).

Ainda, foi realizado um ensaio de ELISPOT para detectar o número de esplenócitos que secretaram IFN- γ de modo específico, após reestimulação *ex vivo* com diferentes concentrações do peptídeo SIINFEKL reconhecido pelos linfócitos OTI. Novamente, este ensaio demonstrou a função prejudicada das células OTI em produzirem IFN- γ quando o *priming* foi realizado por BMDC que foram pré-expostas ao *T. cruzi* (Figura 28f-g).

Portanto, além da expansão e ativação prejudicadas, a função efetora dos linfócitos T CD8⁺ OTI foi significativamente comprometida quando do *priming* por BMDC pré-expostas a *T. cruzi*.

Figura 27 – Impacto das BMDC expostas a *T. cruzi* na proliferação e ativação de linfócitos T CD8⁺ OTI *in vivo*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2) podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3), como esquematizado em a. Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células marcadas com o tetrâmero H-2K^b-SIINFEKL (b-c), incorporação de BrdU nas células tetrâmero-positivas (d-e), e marcação das cadeias Vα2 e Vβ5 do TCR dos linfócitos OTI (f-g). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efetoras de fenótipo CD44^{alto} CD62L^{baixo} (h-i). São apresentados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre Gr.2 e Gr.3 (**P<0,01 ***P<0,001 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos três experimentos independentes. #Para permitir a comparação, as células do Gr.1 são tetrâmero-negativas.

Figura 28 - Impacto das BMDC expostas a *T. cruzi* na produção de citocinas por linfócitos T CD8⁺ OTI *in vivo*.

Camundongos C57BL/6 ($n=3$) foram injetados com células OTI e imunizados no dia seguinte com BMDC previamente estimuladas *in vitro* com LPS (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2) podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3). Após 5 dias da transferência de BMDC, a produção de citocinas pelos linfócitos OTI foi estimada por ICS para IL-2, TNF e IFN- γ após reestimulação dos esplenócitos com diferentes concentrações (de 1×10^{-6} M a 3×10^{-11} M) do peptídeo SIINFEKL (a). Um exemplo da marcação obtida em cada grupo com a maior concentração de SIINFEKL utilizada é mostrado em b, e a frequência total das células respondedoras é apresentada em c. A análise da frequência de células polifuncionais entre as células respondedoras considerando cada citocina uma função (d) ou incluindo a marcação com CD107a (e) também é mostrada. O número de esplenócitos

que secretou IFN- γ após reestimulação com SIINFEKL também foi avaliado por ELISPOT (f-g). São apresentados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre Gr.2 e Gr.3 (** $P < 0,01$ ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos três experimentos independentes.

Esta interferência causada pelo *T. cruzi* no *priming in vivo* de linfócitos T CD8⁺ demonstra que o protozoário é capaz de transformar uma resposta ótima em subótima e isto de fato só foi observado quando o parasito foi incubado com as BMDC utilizadas para indução da resposta, mas não quando BMDC foram incubadas com AdASP-2, como avaliado pela frequência de células marcadas com anticorpos que reconhecem o TCR V β 5 (Figura 29b), a marcação de CD44 e CD62L das células CD8⁺ V β 5⁺ (Figura 29c) e a produção de TNF e IFN- γ por esplenócitos reestimulados com SIINFEKL (Figura 29d).

Figura 29 – Indução *in vivo* de linfócitos T CD8⁺ OTI por BMDC previamente expostas a adenovírus.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2) podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3) ou com AdASP-2 (Gr.4), como esquematizado em a. Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR V β 5 dos linfócitos OTI (b). Também é mostrada a frequência das células CD8⁺ V β 5⁺ diferenciadas em células efectoras de fenótipo CD44^{alto} CD62L^{baixo} (c). A frequência das células CD8⁺ produtoras de TNF e/ou IFN- γ após reestímulo com SIINFEKL é apresentada em d. São mostrados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos indicam diferença estatística entre Gr.2 e Gr.3 (** $P < 0,01$ *** $P < 0,001$

ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos três experimentos independentes.

Uma possível explicação para o prejuízo da resposta dos linfócitos T CD8⁺ OTI induzidos por BMDC previamente expostas ao *T. cruzi* poderia ser a competição pela mesma célula apresentadora de antígeno das células OTI com os clones de linfócitos T CD8⁺ específicos para antígenos do parasito. Para testar esta possibilidade, avaliamos a resposta de linfócitos T CD8⁺ específicos para SIINFEKL e para os epítomos VNHRFTLV e ANYKFTLV de *T. cruzi* restritos a H-2K^b 5 dias após a imunização dos animais dos Gr.2 (que receberam BMDC carregadas com SIINFEKL) e Gr.3 (que receberam BMDC expostas a *T. cruzi* antes de serem carregadas com SIINFEKL), seguindo o mesmo protocolo esquematizado na Figura 27a. Por ICS para identificação das células específicas produtoras de TNF e IFN- γ , foi observado, no entanto, que neste intervalo de 5 dias não ocorreu indução de linfócitos T CD8⁺ específicos para *T. cruzi* no Gr.3 (Figura 30a), excluindo a possibilidade de que a falta de resposta dos linfócitos específicos para SIINFEKL deveu-se à competição de vários clones pela mesma BMDC. De fato, isto repete dados já descritos por nosso grupo acerca da cinética atrasada da resposta de linfócitos T CD8⁺ que reconhecem antígenos de *T. cruzi* (TZELEPIS et al., 2006).

Alternativamente, seria razoável propor a possibilidade de que, assim como há um atraso na resposta dos clones de linfócitos T CD8⁺ que reconhecem os epítomos do *T. cruzi*, o protozoário poderia causar um atraso na resposta dos linfócitos T CD8⁺ OTI estimulados pelas BMDC carregadas com SIINFEKL. Esta alternativa foi investigada pela avaliação da resposta de citocinas dos linfócitos T CD8⁺ mais tardiamente, 12 dias depois da transferência de BMDC para os Gr.2 e Gr.3. Aqui, também, os resultados indicaram que esta hipótese não é correta, já que neste dia os linfócitos T CD8⁺ OTI continuaram deficitariamente induzidos no Gr.3 em comparação ao Gr.2, enquanto a resposta específica para epítomos de *T. cruzi* pôde ser observada no Gr.3 (Figura 30b).

Figura 30 – Resposta de citocinas de linfócitos T CD8⁺ OTI e específicos para epítomos de *T. cruzi* após imunização com BMDC.

Camundongos C57BL/6 (n=3) foram injetados com células OTI e imunizados no dia seguinte com BMDC previamente estimuladas *in vitro* com LPS e carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3). Após 5 (a) ou 12 (b) dias da transferência de BMDC, a produção de citocinas pelos linfócitos T CD8⁺ foi estimada por ICS para TNF e IFN- γ após reestimulação dos esplenócitos com os peptídeos SIINFEKL, VNHRFTLV ou ANYKFTLV. Um exemplo representativo da marcação obtida em cada grupo é mostrado. Resultados representativos de dois experimentos independentes.

Outra possível explicação para a resposta subótima dos linfócitos T CD8⁺ OTI seria a interferência causada pelo *T. cruzi* na funcionalidade das BMDC quanto à capacidade de apresentar antígenos. Para testar esta possibilidade, inicialmente foi avaliada a viabilidade das BMDC expostas ao *T. cruzi* em comparação a BMDC não tratadas, como controle negativo, e BMDC tratadas com 5 µg/mL de actinomicina D por 5 h como controle positivo para indução de morte celular, a qual foi avaliada por citometria de fluxo após marcação das células CD11c⁺ com 7-AAD, anexina V (Figura 31a) e sondas fluorescentes que detectam as formas ativas das caspases 3 e 7 (Figura 31b). Os resultados indicaram que a incubação com *T. cruzi* por 24 h não induziu morte das BMDC. Ainda, foram comparadas BMDC estimuladas com LPS (como utilizado no Gr.2 dos ensaios *in vivo*) e BMDC expostas ao *T. cruzi* e estimuladas com LPS (como utilizado no Gr.3), quanto à expressão de H-2K^b, I-A^b, CD40, CD80 e CD86 (Figura 31c). Verificou-se, assim, que o *T. cruzi* não inibiu a coestimulação induzida por LPS. Estas BMDC também tiveram níveis elevados de transcrição de mRNA das citocinas IL-6, IL-12 p35, TNF, IL-23 p19, IL-27 p28 e IL-10 em comparação a BMDC não tratadas (Figura 31d), ainda que os níveis induzidos por LPS sozinho tenham sido superiores aos induzidos por *T. cruzi* + LPS. Quando as BMDC incubadas ou não com *T. cruzi* foram estimuladas com LPS e carregadas com o peptídeo SIINFEKL, de modo idêntico ao utilizado nos Gr.2 e Gr.3 dos ensaios *in vivo*, pôde-se marcar o complexo H-2K^b-SIINFEKL na superfície das células CD11c⁺ com o anticorpo 25-D1.16, observando-se que na presença ou ausência de *T. cruzi* a quantidade de SIINFEKL carregada pelas BMDC foi comparável (Figura 31e). Por fim, foi realizado um ensaio funcional de apresentação de antígenos por estas BMDC para linfócitos OTI *naïve*, através da cocultura destas células por 72 h e detecção do número de células que responderam produzindo IFN-γ, o qual demonstrou que as BMDC pré-expostas ou não a *T. cruzi* e carregadas com SIINFEKL foram igualmente capazes de estimular linfócitos T CD8⁺ OTI *naïve in vitro* (Figura 31f). Em conjunto, estes resultados indicam que as BMDC utilizadas para induzir resposta dos linfócitos T CD8⁺ cognatos *in vivo* estavam viáveis e foram capazes de prover coestímulo, citocinas e antígenos para o *priming* das células OTI, mesmo quando previamente expostas ao *T. cruzi*. Ainda, o *priming* deficiente dos linfócitos T CD8⁺ OTI por BMDC expostas a *T. cruzi* foi observado *in vivo*, mas não *in vitro*, sugerindo o possível envolvimento de outras células na supressão desta resposta.

Figura 31 – Funcionalidade *in vitro* das BMDC expostas a *T. cruzi*, estimuladas com LPS e carregadas com SIINFEKL.

BMDC foram derivadas de camundongos C57BL/6 e incubadas por 24 h com meio, *T. cruzi* ou actinomicina D como controle positivo para indução de morte e a viabilidade celular foi avaliada por marcação com 7-AAD e anexina V (a) ou com sondas fluorescentes que detectam as formas ativas das caspases 3 e 7 (b). Alternativamente, BMDC foram expostas ao *T. cruzi* na presença ou ausência de LPS e a marcação de MHC e moléculas coestimuladoras foi avaliada nas células CD11c⁺ (c). Os níveis de transcrição de mRNA de citocinas em BMDC estimuladas com *T. cruzi* ou *T. cruzi* + LPS foram avaliados por PCR semi-quantitativo utilizando *Gapdh* como controle endógeno e BMDC não tratadas como referência (d). BMDC incubadas ou não com *T. cruzi* foram estimuladas com LPS, carregadas com o peptídeo SIINFEKL, e a marcação do complexo H-2K^b-SIINFEKL nas células CD11c⁺ foi realizada com o anticorpo 25-D1.16 (e). Estas BMDC foram também colocadas em cocultura por 72 h com linfócitos T CD8⁺ isolados de camundongos OTI *naïve* e o número de células estimuladas secretoras de IFN- γ foi detectado por ELISPOT (f). As barras indicam a mediana $\pm$ erro padrão de cada grupo. Não foi observada diferença entre os grupos BMDC LPS SIINFEKL e BMDC *T. cruzi* LPS SIINFEKL (ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes.

A despeito destes resultados obtidos *in vitro*, foram também realizados experimentos *in vivo* para investigar a possibilidade do *T. cruzi* atuar diretamente na BMDC, eventualmente comprometendo sua capacidade de apresentar o peptídeo SIINFEKL. Para isto, animais C57BL/6 receberam transferência adotiva de células OTI e foram imunizados no dia seguinte com BMDC estimuladas com LPS e carregadas com SIINFEKL por via *i.v.* no plexo retro-orbital direito, juntamente com BMDC incubadas apenas com *T. cruzi* injetadas no plexo retro-orbital esquerdo. Neste protocolo, esperava-se que, no caso do *T. cruzi* prejudicar a capacidade intrínseca da BMDC apresentar o peptídeo SIINFEKL para o linfócito T CD8⁺ OTI cognato, esta capacidade seria restaurada pela injeção da BMDC que carrega o peptídeo separada da BMDC que foi exposta ao parasito. No entanto, esta estratégia falhou em induzir o *priming* otimizado dos linfócitos OTI, avaliado pela expansão das células TCR-específicas (Figura 32b), sua diferenciação em fenótipo CD44^{alto} CD62L^{baixo} (Figura 32c), e a produção de citocinas em resposta ao reestímulo com SIINFEKL (Figura 32d).

Estes dados corroboram a sugestão de que a resposta subótima dos linfócitos OTI induzidos *in vivo* por BMDC na presença de *T. cruzi* não se deve à capacidade do parasito comprometer a habilidade da BMDC apresentar antígenos, o que pode sugerir a indução ativa pelo parasito de mecanismos de supressão do *priming* dos linfócitos T CD8⁺, que poderia ainda envolver outra(s) célula(s).

Figura 32 – Indução de linfócitos T CD8⁺ OTI *in vivo* após a transferência concomitante de BMDC carregadas com SIINFEKL e BMDC expostas a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda concomitantemente injetados com BMDC previamente expostas ao *T. cruzi* (Gr.3), como esquematizado em a. Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR Vα2 e Vβ5 dos linfócitos OTI (b). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efectoras de fenótipo CD44^{alto} CD62L^{baixo} (b). A frequência das células CD8⁺ produtoras de TNF e/ou IFN-γ após reestímulo com SIINFEKL é apresentada em c. São mostrados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre Gr.2 e Gr.3 (**P<0,01 ***P<0,001 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos três experimentos independentes.

Para continuar investigando possíveis mecanismos responsáveis pela resposta deficiente dos linfócitos OTI quando induzidos por BMDC que haviam sido expostas ao *T. cruzi*, decidimos caracterizar em detalhe o fenótipo destes linfócitos. Isto foi realizado repetindo-se o protocolo de *priming in vivo* das células OTI (esquematizado na Figura 27a e detalhadamente descrito na metodologia) e avaliando-se a expressão de vários marcadores de superfície nas células H-2K^b-SIINFEKL tetrâmero-positivas, incluindo marcadores de ativação, regulação, exaustão e migração de linfócitos T CD8⁺, além de fatores de transcrição e de marcadores relacionados à morte celular. Dentre mais de 40 parâmetros avaliados, as diferenças encontradas nos linfócitos OTI induzidos na presença de *T. cruzi*

(Gr.3) em comparação aos linfócitos induzidos na sua ausência (Gr.2) foram: i) a marcação de antígenos relacionados à ativação de linfócitos T como CD44, CD11a, CD49d, CD38 e CD27 ligeiramente mais baixa; ii) a marcação de CD95 e PDL-1 mais alta; iii) a marcação mais alta de caspases 3 e 7 ativas e a maior frequência de células com dupla marcação com anexina V e 7-AAD e iv) os marcadores de migração CCR6 e CXR4 mais altos e CXCR3 mais baixo (Figura 33).

Figura 33 – Alterações no fenótipo dos linfócitos T CD8⁺ OTI induzidos *in vivo* por BMDC expostas a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2) podendo ter sido ainda previamente expostas ao *T. cruzi* (Gr.3). Após 5 dias da transferência de BMDC, os linfócitos T CD8⁺ foram marcados com o tetrâmero H-2K^b-SIINFEKL juntamente com outros marcadores de superfície (a). A marcação intranuclear de fatores de transcrição nas células tetrâmero-positivas também foi avaliada (b), bem como a expressão da molécula antiapoptótica bcl-2 e as formas ativas das caspases 3 e 7 (c). Foi também avaliada nas células tetrâmero-positivas a marcação com 7-AAD e anexina V (d). São apresentados valores representativos de cada grupo. Resultados de pelo menos dois experimentos independentes. Para permitir a comparação, as células do Gr.1 são CD8⁺ H-2K^b-SIINFEKL⁺.

O fenótipo dos linfócitos T CD8⁺ OTI induzidos *in vivo* por BMDC carregadas com SIINFEKL e previamente expostas ao *T. cruzi* reflete em muitos aspectos o fenótipo dos linfócitos T CD8⁺ específicos para o epítipo VNHRFTLV induzidos pela infecção experimental com *T. cruzi*, especialmente quanto ao aumento da expressão de CD95 e perfil pró-apoptótico (VASCONCELOS; BRUNA-ROMERO; et al., 2012). Como as células que expressam CD95 são sabidamente propensas à morte, o mecanismo pelo qual o *T. cruzi* torna a resposta dos linfócitos T CD8⁺ subótima poderia ser via indução de CD95. Para testar esta possibilidade, cruzamos animais OTI com animais Fas^{lpr}, os quais exibem uma mutação na molécula CD95 que a torna não-funcional. Após alguns retrocruzamentos, foram obtidos animais OTI^{lpr/lpr}, onde todos os linfócitos T CD8⁺ são transgênicos, específicos para SIINFEKL e funcionalmente deficientes em CD95. Células OTI^{lpr/lpr} destes animais foram transferidas para animais C57BL/6 e o protocolo de indução de resposta destes linfócitos por BMDC carregadas com SIINFEKL e expostas ou não a *T. cruzi* foi executado, conforme esquematizado na Figura 34a e detalhadamente descrito na metodologia. Em comparação a células OTI^{wt/wt}, as células dos animais OTI^{lpr/lpr} não marcaram positivamente para CD95, confirmando o fenótipo esperado (Figura 34b). No entanto, os linfócitos T CD8⁺ OTI^{lpr/lpr} induzidos *in vivo* por BMDC expostas ao *T. cruzi* e carregadas com SIINFEKL continuaram respondendo de modo subótimo, em comparação à resposta induzida por BMDC que não foram expostas ao parasito, como avaliado pela frequência de células TCR-específicas (Figura 34c), fenótipo CD44^{alto} CD62^{baixo} das células CD8⁺ Vα2⁺ Vβ5⁺ (Figura 34d) e produção de TNF e IFN-γ por células CD8⁺ re-estimuladas com SIINFEKL *ex vivo* (Figura 34e).

Portanto, a indução de CD95 pelo *T. cruzi* não é exclusivamente responsável pelo *priming* subótimo dos linfócitos T CD8⁺. Se a expressão de CD95 é uma causa ou consequência da resposta deficiente destes linfócitos ainda não está claro, mas vale dizer que os dados apresentados aqui não descartam a possibilidade da ocorrência de outras vias redundantes independentes de CD95 que associariam a indução de morte dos linfócitos T CD8⁺ aos motivos pelos quais sua indução é prejudicada na presença do *T. cruzi*.

Figura 34 – Indução *in vivo* de linfócitos T CD8⁺ OTI deficientes de CD95 por BMDC expostas a *T. cruzi* e carregadas com SIINFEKL.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI^{lpr/lpr} e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3), como esquematizado em a. A deficiência de CD95 nas células OTI^{lpr/lpr} em comparação a células OTI^{wt/wt} foi confirmada pela marcação de CD95 nos linfócitos T CD8⁺ (b). Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR Vα2 e Vβ5 dos linfócitos OTI (b). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efetoras de fenótipo CD44^{alto} CD62L^{baixo} (b). A frequência das células CD8⁺ produtoras de TNF e/ou IFN-γ após reestímulo com SIINFEKL é apresentada em c. São mostrados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre Gr.2 e Gr.3 (***P<0,001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes.

Como todas as hipóteses testadas até aqui mostraram-se negativas, resolvemos investigar também o perfil dos linfócitos T CD4⁺ neste modelo, já que estas células são amplamente descritas como mediadoras de regulação da resposta imune e poderiam também interferir no *priming* dos linfócitos T CD8⁺ OTI. Inicialmente, fizemos a indução *in vivo* das células OTI por BMDC carregando o peptídeo cognato na presença ou ausência de *T. cruzi*, como esquematizado na Figura 27a, e avaliamos nas células T CD4⁺ totais os mesmos parâmetros anteriormente apresentados para os linfócitos OTI. Além destes parâmetros, também foram avaliadas a proliferação dos linfócitos T CD4⁺, por incorporação de

BrdU *in vivo*, e a marcação na superfície de Nrp1 e GITR, os quais são marcadores de células T reguladoras (Treg). Como mostrado na Figura 35, as diferenças encontradas nos linfócitos T CD4⁺ dos animais que receberam BMDC previamente expostas ao *T. cruzi* (Gr.3) em comparação aos animais que receberam BMDC livres do parasito (Gr.2) foram: i) maior incorporação de BrdU *in vivo*, ii) a maior frequência de linfócitos T CD4⁺ CD62L^{baixo}, iii) níveis consideravelmente mais altos de expressão de PDL-1 e CXCR4; iv) níveis ligeiramente mais altos de CTLA-4 e GITR; v) menor frequência de células Foxp3⁺ e Nrp1⁺ e vi) níveis ligeiramente mais altos de CD95, mais baixos de Bcl2, mais altos de caspases 3 e 7 ativas e maior frequência de células duplamente marcadas para anexina V e 7-AAD.

Figura 35 - Alterações no fenótipo dos linfócitos T CD4⁺ em camundongos injetados com BMDC expostas a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2) podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3). Após 5 dias da transferência de BMDC, os linfócitos T CD4⁺ foram marcados juntamente com outros marcadores de superfície (a). A marcação intranuclear de fatores de transcrição nas células T CD4⁺ também foi avaliada (b), bem como a expressão da molécula antiapoptótica Bcl-2 e as formas ativas das caspases 3 e 7 (c). Também foi avaliada a marcação com 7-AAD e anexina V

(d), além da incorporação de BrdU *in vivo* pelos linfócitos T CD4⁺ (e). São apresentados valores representativos de cada grupo. Resultados de pelo menos dois experimentos independentes.

Portanto, foi possível observar a maior indução de linfócitos T CD4⁺ nos animais que receberam BMDC previamente expostas ao *T. cruzi*. Estes linfócitos também exibiram um perfil pró-apoptótico. Quanto à presença de Tregs, os marcadores utilizados não permitiram identificar a indução pronunciada de algum subtipo específico.

Para investigar o papel dos linfócitos T CD4⁺ no *priming* dos linfócitos T CD8⁺ OTI induzidos *in vivo* por BMDC que foram pré-expostas ao *T. cruzi*, este protocolo foi repetido utilizando-se animais deficientes de CD4 como receptores das células transferidas (Figura 36a). Estes animais eram, de fato, desprovidos de linfócitos T CD4⁺, enquanto o compartimento T CD8⁺ estava presente (Figura 36b). Neste sistema, as BMDC pré-expostas ao *T. cruzi* e carregadas com SIINFEKL foram tão eficientes quanto as BMDC carregadas com o peptídeo na ausência do parasito em induzir a proliferação dos linfócitos OTI, como avaliado pela frequência de células CD8⁺ TCR-específicas (Figura 36c), frequência de células positivas para o tetrâmero H-2K^b-SIINFEKL (Figura 36d) e incorporação de BrdU *in vivo* pelas células tetrâmero-positivas (Figura 36e). Ainda, a diferenciação das células CD8⁺ Vα2⁺ Vβ5⁺ no fenótipo CD44^{alto} CD62L^{baixo} (Figura 36f) e a produção de TNF e IFN-γ após reestimulação com SIINFEKL (Figura 36g) foi comparável entre os Gr.2 e Gr.3.

Portanto, estes dados sugerem que linfócitos T CD4⁺ contribuem para a resposta subótima dos linfócitos T CD8⁺ induzidos na presença de *T. cruzi*.

Figura 36 – Indução *in vivo* de linfócitos T CD8⁺ OTI na ausência de linfócitos T CD4⁺ por BMDC expostas a *T. cruzi*.

Camundongos CD4^{-/-} (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3), como esquematizado em a. A deficiência de CD4 foi confirmada pela marcação de CD4 e CD8 nos esplenócitos (b). Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR Vα2 e Vβ5 dos linfócitos OTI (c), além da marcação com o tetrâmero H-2Kb-SIINFEKL (d) e incorporação *in vivo* de BrdU pelas células tetrâmero-positivas (e[#]). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efetoras de fenótipo CD44^{alto} CD62L^{baixo} (f) e a frequência das células CD8⁺ produtoras de TNF e/ou IFN-γ após reestímulo com SIINFEKL (g). São mostrados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Não houve diferença entre Gr.2 e Gr.3 (ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes. [#]Para permitir a comparação, as células do Gr.1 são tetrâmero-negativas.

Para confirmar o papel dos linfócitos T CD4⁺ induzidos por DC expostas ao *T. cruzi* na supressão do *priming* das células OTI *in vivo*, foram realizados experimentos de transferência adotiva de células. Como esquematizado na Figura 37a, animais C57BL/6 foram injetados com BMDC previamente incubadas com *T. cruzi* e após 5 dias os linfócitos T CD4⁺ destes animais foram isolados do baço e transferidos para novos animais C57BL/6 que haviam previamente recebido células OTI. Juntamente com a transferência de 15×10^6 linfócitos T CD4⁺, estes animais receberam a injeção de BMDC carregando o peptídeo SIINFEKL. Como controle, o *priming* dos linfócitos OTI foi induzido em animais C57BL/6 sem a transferência de linfócitos T CD4⁺. Neste protocolo, a supressão da resposta dos linfócitos T CD8⁺ específicos para SIINFEKL pôde ser induzida pela transferência de linfócitos T CD4⁺ de animais que haviam sido expostos a *T. cruzi*, como avaliado pela marcação com o tetramero H-2K^b-SIINFEKL (Figura 37b), marcação do TCR dos linfócitos OTI (Figura 37c), diferenciação das células TCR-específicas no fenótipo CD44^{alto} CD62L^{baixo} (Figura 37d) e produção de TNF e IFN- γ após reestimulação com SIINFEKL (Figura 37e). Estes dados, portanto, confirmaram o envolvimento de linfócitos T CD4⁺ induzidos por *T. cruzi* na supressão do *priming* de linfócitos T CD8⁺ OTI *in vivo*.

De fato, é amplamente descrita na literatura a capacidade reguladora de linfócitos T CD4⁺ sobre a resposta de outros linfócitos T, o que pode se dar por inúmeros mecanismos distintos, incluindo entre os principais a produção da citocina imunoreguladora IL-10. Portanto, a etapa seguinte deste estudo consistiu em avaliar o papel da IL-10 na supressão do *priming* de linfócitos OTI. Para isto, foi realizada a indução destes linfócitos em animais deficientes de IL-10, como descrito na Figura 38a. A deficiência de IL-10 foi confirmada por genotipagem e pela quantificação da citocina no sobrenadante de BMDC derivadas de camundongos IL-10^{-/-} e estimuladas com LPS *in vitro* (Figura 38b). Os resultados do experimento, no entanto, indicaram que a expansão (Figura 38c-d), diferenciação (Figura 38e) e função efetora (Figura 38f) das células OTI estimuladas *in vivo* por BMDC carregando o peptídeo cognato foi subótima quando da exposição da BMDC ao *T. cruzi*, mesmo na ausência de IL-10.

Figura 37 - Indução *in vivo* de linfócitos T CD8⁺ OTI após transferência adotiva de linfócitos T CD4⁺ gerados em presença de BMDC expostas a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2 e Gr.3), podendo ter sido ainda transferidos com linfócitos T CD4⁺ isolados de animais que receberam BMDC incubadas com *T. cruzi* 5 dias antes (Gr.3), como esquematizado em a. Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR Vα2 e Vβ5 dos linfócitos OTI (b), além da marcação com o tetrâmero H-2K^b-SIINFEKL (c). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efectoras de fenótipo CD44^{alto} CD62L^{baixo} (d) e a frequência das células CD8⁺ produtoras de TNF e/ou IFN-γ após reestímulo com SIINFEKL (e). São mostrados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença entre Gr.2 e Gr.3 (*P<0,05 **P<0,01 ***P<0,001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes.

Figura 38 - Indução *in vivo* de linfócitos T CD8⁺ OTI em animais deficientes de IL-10 por BMDC previamente expostas a *T. cruzi*.

Camundongos IL-10^{-/-} (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3), como esquematizado em a. A deficiência de IL-10 foi confirmada pela quantificação de citocinas por CBA no sobrenadante de BMDC normais ou IL-10^{-/-} estimuladas com LPS (b). Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR Vα2 e Vβ5 dos linfócitos OTI (c), além da marcação com o tetrâmero H-2K^b-SIINFEKL (d). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efetoras de fenótipo CD44^{alto} CD62L^{baixo} (e) e a frequência das células CD8⁺ produtoras de TNF e/ou IFN-γ após reestímulo com SIINFEKL (f). São mostrados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos representam diferença entre Gr.2 e Gr.3 (*P<0,05 **P<0,01 ***P<0,001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes.

Os linfócitos T CD4⁺ reguladores foram classicamente descritos como CD25⁺, ainda que este marcador não seja absolutamente específico. No entanto, tal marcador ainda não existe, e CD25 e Foxp3 são os marcadores do *estado da arte* para distinguir células T CD4⁺ reguladoras. Para observar se Tregs medeiam a supressão dos linfócitos OTI *in vivo*, novamente foi executado o protocolo de *priming* por BMDC pré-incubadas com *T. cruzi* em animais tratados com o anticorpo PC61, que depleta as células CD25⁺ (Figura 39a). Como observado pela marcação dos esplenócitos com o anticorpo 7D4, que marca CD25, o tratamento com PC61 eliminou as células CD4⁺ CD25⁺ do baço (Figura 39b). Mesmo assim, a supressão do priming dos linfócitos T CD8⁺ OTI continuou ocorrendo após a transferência de BMDC carregando SIINFEKL que haviam sido anteriormente incubadas com *T. cruzi*, como avaliado pela marcação do TCR (Figura 38c), tetrâmero (Figura 38d), CD44 e CD62L (Figura 38e) e ICS (Figura 38f). Então, estes resultados indicam que células T CD4⁺ CD25⁺ não estão envolvidas na geração de resposta subótima dos linfócitos T CD8⁺ induzidos na presença do *T. cruzi*.

Figura 39 - Efeito da depleção de células CD25⁺ na indução *in vivo* de linfócitos T CD8⁺ OTI por BMDC expostas a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram injetados com o anticorpo PC61 e transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3), como esquematizado em a. A depleção das células CD25⁺ por PC61 foi confirmada pela marcação de esplenócitos com o anticorpo 7D4 (anti-CD25) (b). Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células positivamente marcadas para o TCR Vα2 e Vβ5 dos linfócitos OTI (c), além da marcação com o tetrâmero H-2K^b-SIINFEKL (d). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efetoras de fenótipo CD44^{alto} CD62L^{baixo} (e) e a frequência das células CD8⁺ produtoras de TNF e/ou IFN-γ após reestímulo com SIINFEKL (f).

São mostrados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos representam diferença entre Gr.2 e Gr.3 (* $P < 0,05$ ** $P < 0,01$ *** $P < 0,001$ **** $P < 0,0001$ ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes.

Por outro lado, para investigar o envolvimento de linfócitos T CD4⁺ Foxp3⁺ na supressão do *priming* das células OTI *in vivo*, foram realizados novos experimentos de transferência adotiva de células. Como esboçado na Figura 40a, animais congênicos CD45.1 que expressam GFP sob controle do promotor de *FoxP3* foram injetados com BMDC previamente incubadas com *T. cruzi* ou LPS e após 5 dias os linfócitos T CD4⁺ destes animais foram isolados do baço e a fração GFP⁺ (Foxp3⁺) foi separada por *sorting* e transferida para novos animais C57BL/6 que haviam previamente recebido células OTI. No mesmo dia da transferência de linfócitos T CD4⁺ Foxp3⁺, estes animais receberam a injeção de BMDC estimuladas com LPS e carregadas com o peptídeo SIINFEKL. Como controle, o *priming* dos linfócitos OTI foi induzido em animais sem a transferência de linfócitos T CD4⁺ Foxp3⁺. A transferência das células para o baço dos animais receptores foi confirmada pela marcação de CD45.1 e expressão de GFP (Figura 40b). Neste protocolo, a supressão da resposta dos linfócitos T CD8⁺ específicos para SIINFEKL pôde ser induzida pela transferência específica de 1×10^6 linfócitos T CD4⁺ Foxp3⁺ de animais que haviam sido injetados com BMDC previamente expostas ao *T. cruzi*, como avaliado pela marcação com o tetrâmero H-2K^b-SIINFEKL (Figura 40c), marcação do TCR (Figura 40d), CD44 e CD62L nas células TCR específicas (Figura 40e) e ICS após reestímulo com SIINFEKL (Figura 40f). Esta supressão ocorreu de modo significativamente mais intenso do que após a transferência de um número equivalente de linfócitos T CD4⁺ Foxp3⁺ induzidos na ausência de *T. cruzi* (Gr.4). Notavelmente, ainda, as células OTI induzidas na presença de linfócitos T CD4⁺ Foxp3⁺ isolados de animais que haviam sido injetados com BMDC previamente expostas ao *T. cruzi* foram menos polifuncionais em comparação à resposta após transferência de linfócitos T CD4⁺ Foxp3⁺ isolados de animais que não foram expostos a *T. cruzi* (Figura 40g-h). Ainda, quando foram transferidos números equivalentes de células T CD4⁺ GFP⁻ dos animais induzidos com BMDC *T. cruzi* não houve supressão da resposta dos linfócitos OTI (não mostrado). Estes dados, portanto, sugerem o envolvimento específico de linfócitos T CD4⁺ Foxp3⁺ induzidos por *T. cruzi* na supressão do *priming* de linfócitos T CD8⁺ OTI *in vivo*.

Figura 40 - Indução *in vivo* de linfócitos T CD8⁺ OTI após transferência adotiva de linfócitos T CD4⁺ Foxp3⁺ gerados em presença de BMDC expostas ou não a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2, Gr.3 e Gr.4), podendo ter sido ainda transferidos com linfócitos T CD4⁺ Foxp3⁺ isolados de animais CD45.1⁺ Foxp3-GFP que receberam BMDC incubadas com *T. cruzi* (Gr.3) ou LPS (Gr.4) 5 dias antes, como esquematizado em a. Após 5 dias da transferência de BMDC e linfócitos T CD4⁺, a presença dos linfócitos transferidos foi avaliada pela marcação de CD45.1 e GFP (b). A proliferação dos linfócitos T CD8⁺ OTI foi estimada pela frequência de células positivamente marcadas com o tetrâmero H-2K^b-SIINFEKL (c) e TCR Vα2 e Vβ5 dos linfócitos OTI (d). Também é mostrada a frequência das células CD8⁺ Vα2⁺ Vβ5⁺ diferenciadas em células efectoras de fenótipo CD44^{alto} CD62L^{baixo} (e) e a frequência das células CD8⁺ produtoras de IL-2 e/ou TNF e/ou IFN-γ após reestímulo com SIINFEKL (f), bem como as análises da frequência de células polifuncionais dentre as que responderam produzindo citocinas (g-h). São mostrados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença entre os grupos apontados (*P<0,05 **P<0,01 ***P<0,001 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.) Resultados representativos de pelo menos dois experimentos independentes.

Mecanismos descritos em camundongos e humanos pelos quais Treg silenciam a resposta de linfócitos T CD8⁺ podem ser mediados por TGF- β e/ou CTLA-4 (BHADRA et al., 2014; MAEDA et al., 2014). Além disto, o aumento da marcação de CTLA-4 nos linfócitos T CD4⁺ dos animais que foram injetados com BMDC pré-expostas ao *T. cruzi* (Figura 35) pode sugerir sua participação na regulação do *priming* dos linfócitos OTI *in vivo*. Por isso, foram também investigadas a participação de CTLA-4 e TGF- β na supressão da resposta dos linfócitos T CD8⁺ induzida por *T. cruzi*. A estratégia utilizada consistiu no tratamento de animais C57BL/6 com os anticorpos que bloqueiam CTLA-4 ou TGF- β (9D9 e 1D11, respectivamente) durante o *priming in vivo* dos linfócitos OTI por BMDC previamente expostas ao *T. cruzi*, como esquematizado na Figura 41a. Após 5 dias da transferência das BMDC, foi observado que o tratamento com o anti-TGF- β recuperou parcialmente a capacidade de expansão dos linfócitos T CD8⁺ OTI induzidos por BMDC carregadas com SIINFEKL que foram previamente expostas a *T. cruzi*, como avaliado pela frequência de células CD8⁺ marcadas positivamente com o tetrâmero H-2K^b-SIINFEKL (Figura 41b), enquanto o tratamento com anti-CTLA-4 recuperou a capacidade destas células T CD8⁺ OTI produzirem citocinas efectoras (Figura 41c) e reverteu, em parte, sua capacidade polifuncional (Figura 41d-e). Portanto, TGF- β e CTLA-4 estão envolvidos na supressão induzida por *T. cruzi* do *priming in vivo* de linfócitos T CD8⁺.

Figura 41 - Efeito dos anticorpos que bloqueiam CTLA-4 ou TGF- β na indução *in vivo* de linfócitos T CD8⁺ OTI por BMDC expostas a *T. cruzi*.

Camundongos C57BL/6 (n=3) foram transferidos com células OTI e injetados com IgG policlinal, com o anticorpo 9D9 (anti-CTLA-4) ou com o anticorpo 1D11 (anti-TGF- β) e imunizados com BMDC previamente estimuladas *in vitro* com LPS apenas (Gr.1) ou também carregadas com o peptídeo SIINFEKL (Gr.2), podendo ter sido ainda previamente incubadas com *T. cruzi* (Gr.3-5), como esquematizado em a. Após 5 dias da transferência de BMDC, a proliferação dos linfócitos T CD8⁺ foi estimada pela frequência de células CD8⁺ positivamente marcadas com o tetrâmero H-2K^b-SIINFEKL (b). Também é mostrada a frequência das células CD8⁺ produtoras de TNF e/ou IFN- γ após reestímulo com SIINFEKL (c), bem como as análises da frequência de células polifuncionais dentre as que responderam produzindo citocinas (d-e). São mostrados valores individuais e barras que indicam a mediana $\pm$ erro padrão de cada grupo. Asteriscos representam diferença entre os grupos indicados (*P<0,05 **P<0,01 ANOVA de dois fatores e teste Tukey *post hoc*).

Os resultados apresentados até aqui utilizando o modelo controlado de indução *in vivo* de linfócitos T CD8⁺ transgênicos OTI com alta afinidade pelo epítopo cognato sugerem que a resposta subótima de linfócitos T CD8⁺ induzidos na presença de *T. cruzi* se deve à indução pelo parasito de células T CD4⁺ Foxp3⁺ que suprimem o *priming*. Portanto, resolvemos desafiar esta hipótese num contexto de indução de clones naturais de linfócitos T CD8⁺ durante a infecção experimental pelo *T. cruzi*. Para isto, foram utilizados camundongos DREG, os quais expressam o receptor símio da toxina diftérica juntamente com a proteína eGFP (DTR-eGFP) sob controle do promotor de *FoxP3*. Portanto, a injeção de toxina diftérica nestes animais causa a depleção momentânea específica das células que expressam Foxp3. Animais DREG ou animais normais da mesma ninhada foram infectados com 1×10^4 formas sanguíneas de *T. cruzi* por via *s.c.* na base da cauda e tratados com toxina diftérica nos dois dias subsequentes. A parasitemia no sangue foi acompanhada e a resposta de linfócitos T CD8⁺ específicos para os epítopos de *T. cruzi* restritos a H-2K^b VNHRFTLV, ANYDFTLV e ANYKFTLV foi avaliada 22 dias após a infecção. Como mostrado na Figura 42a, a depleção de células Foxp3⁺ aumentou a resistência dos animais à infecção com *T. cruzi*, o que foi correlacionado com uma resposta mais pronunciada de linfócitos T CD8⁺ específicos para epítopos do parasito, como avaliado por marcação com o pentâmero H-2K^b VNHRFTLV (Figura 42c) e marcação intracelular de IL-2, TNF e IFN- γ em esplenócitos reestimulados com os peptídeos VNHRFTLV, ANYDFTLV e ANYKFTLV *ex vivo* (Figura 42d). Ainda, na ausência de células Foxp3⁺ foi observada a maior frequência de esplenócitos que produziram simultaneamente mais de uma citocina em resposta ao reestímulo com VNHRFTLV (Figura 42e). Portanto, a depleção de células Foxp3⁺ logo após o desafio é capaz de melhorar a resposta de linfócitos T CD8⁺ e contribuir para o controle da infecção por *T. cruzi*.

Figura 42 - Parasitemia e resposta de linfócitos T CD8⁺ após infecção por *T. cruzi* em animais depletados de células Foxp3⁺.

Camundongos DREG (Foxp3 DTR) ou animais normais da mesma ninhada (WT) foram infectados com *T. cruzi* e tratados com toxina diftérica (DT) nos dois dias seguintes. A parasitemia no sangue foi acompanhada (a) e a resposta imune foi avaliada pela marcação dos linfócitos T CD8⁺ do baço com o pentâmero H-2K^b-VNHRFTLV (b). Também é mostrada a frequência das células CD8⁺ produtoras de TNF e/ou IFN- γ após reestímulo com os peptídeos VNHRFTLV, ANYDFTLV e ANYKFTLV (c), bem como a análise da combinação de citocinas produzidas pelas células CD8⁺ que responderam positivamente ao reestímulo com VNHRFTLV (d).

5 DISCUSSÃO

O estudo da resposta de linfócitos T CD8⁺ utilizando o modelo murino experimental de infecção com *T. cruzi* e vacinação genética com *Asp2* inserido em um vetor adenoviral permitiu a identificação de bases celulares e moleculares que controlam a expansão, diferenciação e função destes linfócitos. Especificamente, os dados apresentados nesta tese demonstram, pela primeira vez, a participação marcante do imunoproteassomo e de Treg CD4⁺ CD25⁻ durante a resposta de linfócitos T CD8⁺ induzidos *in vivo* na infecção por *T. cruzi*. Além disto, a parte inicial deste estudo caracterizou a interação do parasito com as células que apresentam antígenos e permitiu, também pela primeira vez, a descrição da via de processamento dos epítomos de *T. cruzi* restritos a MHC classe I.

Células dendríticas derivadas de medula óssea expostas ao *T. cruzi* cepa Y ou vetor adenoviral recombinante aumentam a expressão de moléculas coestimuladoras e MHC de modo dependente de MyD88, sugerindo a importância de TLR para sua maturação (Figura 3). De fato, a importância de TLR, particularmente TLR2 (ALMEIDA et al., 2000; CAMPOS et al., 2001; ROBERT et al., 2002), TLR4 (OLIVEIRA et al., 2004), TLR7 (CAETANO et al., 2011) e TLR9 (BAFICA et al., 2006) no reconhecimento de *T. cruzi* já foi bem caracterizada, além da descrição de ligantes de TLR expressos em formas tripomastigotas e epimastigotas, como recentemente revisado (KAYAMA; TAKEDA, 2010).

Curiosamente, *T. cruzi* inativado por congelamento/descongelamento não foi capaz de induzir maturação de DC, repetindo resultados de outro grupo (KAYAMA et al., 2009). Estes resultados podem indicar, entre outras possibilidades, que ligantes de TLR expressos em formas amastigotas do parasito no citoplasma sejam críticos para a ativação de DC, uma vez que apenas as formas vivas do parasito são capazes de escapar do vacúolo parasitóforo, acessar o citosol e diferenciar-se em amastigotas. Além disso, foi sugerido que em presença de IFN- γ , DC podem ser induzidas à maturação por *T. cruzi* (cepa Tulahuen) de modo independente de TLR (KAYAMA et al., 2009). Isto sugere a participação de outros receptores citoplasmáticos no reconhecimento do parasito. Neste sentido, mais recentemente foi descrita a importância de NOD1 (SILVA et al., 2010), bem como ASC (SILVA et al., 2013) e NLRP3 (GONCALVES et al., 2013) no reconhecimento de *T. cruzi*.

Além de expressar moléculas coestimuladoras, DC são importantes fontes de citocinas que polarizam a resposta de linfócitos T. Citocinas relevantes para o controle da infecção por *T. cruzi* incluem a IL-12 (ALIBERTI et al., 1996; GRAEFE et

al., 2003; MICHAILEWSKY et al., 2001), IL-6 (GAO; PEREIRA, 2002; PONCE et al., 2012; SAVINO et al., 2007), IL-23 (BORGES et al., 2013; ERDMANN et al., 2013), IL-27 (HAMANO et al., 2003) e IL-1 β (GONCALVES et al., 2013; SILVA et al., 2013), mas raros são os trabalhos que mostram a célula provedora destas citocinas. Neste trabalho, é sugerida a contribuição de DC como fontes de citocinas descritas como críticas para o controle da infecção por *T. cruzi*. Notavelmente, a produção *in vitro* de IL-1 β por DC expostas a *T. cruzi* foi acentuada e contrastou com o observado para DC expostas a AdASP-2 (Figura 4). O papel da IL-1 β na indução de células T CD8⁺ tem sido sugerido em alguns modelos de infecções virais (PANG; ICHINOHE; IWASAKI, 2013), mas não está claro para a infecção por *T. cruzi*. Experimentos preliminares do nosso grupo utilizando animais deficientes para o receptor de IL-1 imunizados com AdASP-2 e desafiados ou apenas infectados com *T. cruzi* revelaram um perfil de parasitemia, mortalidade e produção de TNF e IFN- γ por células T CD8⁺ específicas comparáveis ao observado para animais normais (dados não mostrados). Não obstante, o papel de moléculas como ASC, caspase-1 e NLRP3, cuja importância para a produção de IL-1 β na infecção por *T. cruzi* já foi descrita (GONCALVES et al., 2013; SILVA et al., 2013), merece ainda ser investigado em mais detalhe quanto ao impacto na resposta de linfócitos T CD8⁺ específicos induzidos pela infecção e imunização com AdASP-2.

Além de expressar MHC, moléculas coestimuladoras e citocinas, DC expostas a *T. cruzi* ou AdASP-2 foram capazes de apresentar antígenos para linfócitos T CD8⁺ efetores *in vitro* (Figura 5). Ainda, em experimentos de coinfeção de BMDC foi demonstrado que *T. cruzi* não inibe a apresentação de epítomos de adenovírus restritos a MHC classe I (Figura 6), e também adenovírus não interfere na apresentação dos epítomos de *T. cruzi* (Figura 7).

Pela via canônica de apresentação de antígenos restritos a MHC classe I, peptídeos são gerados no citosol pelo processamento de proteínas via proteassomo e translocados para o retículo endoplasmático pela molécula transportadora TAP, como revisado (ROCK; SHEN, 2005). No entanto, o processamento de antígenos de parasitos intracelulares, os quais muitas vezes residem em vacúolos parasitóforos, ainda que transientemente, pode se dar por outras vias. Já foi sugerido, por exemplo, que antígenos de *Leishmania major* são ligados em moléculas de MHC classe I ainda no fagolisossomo, sendo apresentados de modo independente de TAP, sem necessidade de alcançar o citosol (BERTHOLET et al., 2006). Já no caso

de *Plasmodium berghei*, a apresentação de antígenos para linfócitos T CD8⁺ ocorre pela via citosólica, dependente de TAP (COCKBURN et al., 2011). Aqui, pela primeira vez foi formalmente demonstrado que os antígenos de *T. cruzi* restritos a MHC classe I são processados pela via citosólica, assim como classicamente descrito para os vírus, de modo dependente de TAP e proteassomo (Figuras 9 e 10). Ainda, o envolvimento do imunoproteassomo no processamento e apresentação *in vitro* de epítomos reconhecidos por linfócitos T CD8⁺ foi demonstrado neste estudo, tanto para a infecção com *T. cruzi* como AdASP-2 (Figura 11).

Estendendo os resultados iniciais dos experimentos *in vitro*, foi demonstrada a indução *in vivo* da transcrição das subunidades que constituem o imunoproteassomo em animais infectados com *T. cruzi* (Figura 12). Estes resultados aparentemente contradizem estudos *in vitro* de outro grupo que relatam a não indução, ou mesmo a inibição da síntese do imunoproteassomo por células HeLa infectadas com *T. cruzi* (CAMARGO et al., 2014; FARIA; LIMA; DE SA, 2008). Possíveis explicações para estas diferenças podem residir na dualidade entre o modelo *in vitro* e *in vivo*, ou ainda na quantidade de parasitos utilizados em cada caso: enquanto os animais foram infectados com 10.000 formas sanguíneas de *T. cruzi*, os experimentos com células HeLa utilizaram 40 parasitos/célula, atingindo níveis de infecção muito acima das condições fisiológicas. Outra possível explicação para os resultados divergentes pode estar na diferença entre o modelo murino e a resposta de células humanas. Para resolver esta questão, no futuro próximo serão quantificados os níveis de transcrição de mRNA de $\beta 1i$, $\beta 2i$ e $\beta 5i$ em amostras de pacientes cardiopatas chagásicos, pacientes com cardiopatia dilatada idiopática e controles saudáveis, em colaboração com o Prof. Dr. Edécio Cunha-Neto, da Faculdade de Medicina da USP.

O modelo murino de infecção com *T. cruzi* apresentado aqui indicou ainda o expressivo envolvimento do imunoproteassomo na expansão, ativação e função de linfócitos T CD8⁺ específicos para diferentes epítomos do parasito *in vivo* (Figuras 13-18). Paralelamente, não foi observada nenhuma participação do imunoproteassomo na resposta de linfócitos T CD4⁺ (Figuras 14, 15 e 19). Mais uma vez, a resposta de linfócitos T CD8⁺ específicos induzidos pela vacinação genética com *Asp2* foi fortemente dependente da expressão do imunoproteassomo (Figuras 20-24).

O envolvimento da subunidade $\beta 5i$ do imunoproteassomo já havia sido documentado na ativação de linfócitos T CD8⁺ e resistência à infecção por

Toxoplasma gondii (TU et al., 2009). No entanto, como nunca reportado antes para um patógeno, neste trabalho é demonstrado que a falta completa do imunoproteassomo teve um impacto drástico no aumento da susceptibilidade à infecção pelo *T. cruzi* e na anulação total da proteção induzida por uma estratégia de vacinação genética que normalmente seria protetora (Figuras 25 e 26).

Possivelmente, o modelo de infecção experimental de animais TKO pelo *T. cruzi* realçou a importância do imunoproteassomo porque: i) os animais utilizados aqui eram completamente deficientes de todas as subunidades do imunoproteassomo, ii) neste modelo o controle da infecção é fortemente dependente de linfócitos T CD8⁺ (TARLETON et al., 1992; TZELEPIS et al., 2006), que são as células que reconhecem os epítomos gerados pelo imunoproteassomo, e iii) a infecção pelo *T. cruzi* induz níveis altos de produção de IFN- γ detectável no soro (CAETANO et al., 2011), um estímulo sabidamente indutor do imunoproteassomo.

Ainda, é plausível especular que a relevância do IFN- γ para o controle da infecção pelo *T. cruzi* (MICHAILOWSKY et al., 2001) esteja associada também à sua capacidade de induzir a expressão do imunoproteassomo nas células infectadas (Figura 12), durante a efetuação da resposta dos linfócitos T CD8⁺ citotóxicos. Em outras palavras, além da expressão do imunoproteassomo pelas células apresentadoras de antígenos durante o *priming* dos linfócitos T CD8⁺, a efetuação da resposta pelos linfócitos citotóxicos seria uma segunda fonte de resistência à infecção onde há participação do imunoproteassomo expresso nas células-alvo, induzida por IFN- γ .

Outra possível explicação para a susceptibilidade maior dos animais TKO pode estar associada ao descontrole da inflamação do miocárdio. Corroborando essa hipótese, já foi relatado o envolvimento do imunoproteassomo na proteção contra miocardite em um modelo de infecção por coxsackievirus B3 (OPITZ et al., 2011). Apesar da cepa Y, utilizada neste trabalho, não possuir um tropismo específico por tecidos cardíacos (MELO; BRENER, 1978), outras cepas, como CL e Colombiana causam pronunciada inflamação do miocárdio e poderiam representar bons modelos para esta investigação (MICHAILOWSKY et al., 2001).

A deficiência de todas as subunidades que definem o imunoproteassomo em um sistema *in vivo* só foi possível com a geração dos animais TKO, utilizados neste trabalho. Como apresentado aqui e por outros, esta deficiência afeta profundamente a provisão de epítomos reconhecidos por linfócitos T CD8⁺. A provisão comprometida

e alterada destes epítomos observada quando o processamento pelo proteassomo é realizado unicamente pelas subunidades catalíticas convencionais resulta na expressão de níveis mais baixos de MHC classe I, e por conseguinte em um número de linfócitos T CD8⁺ reduzido, como mostrado nas Figuras 13 e 20 e reportado na literatura (KINCAID et al., 2012). A expressão reduzida de MHC classe I e a falta do imunoproteassomo são inextricáveis, assim como é o número dos linfócitos T CD8⁺. Mesmo assim, é reportado na literatura que quando animais TKO são comparados a animais TAP^{+/-}, os quais apresentam redução similar aos animais TKO quanto à expressão de MHC classe I e número de linfócitos T CD8⁺, a resposta destes linfócitos permanece mais baixa nos animais TKO, indicando que, de fato, o processamento dos epítomos pelo imunoproteassomo tem uma contribuição majoritária para o fenótipo observado (KINCAID et al., 2012). Além disso, o experimento apresentado na Figura 11 demonstra que mesmo quando DC TKO interagem com linfócitos T CD8⁺ WT *in vitro*, a apresentação de antígenos é reduzida em comparação às DC WT, o que sugere que de fato existe um efeito que independe do compartimento de linfócitos T CD8⁺, mas depende do processamento dos epítomos.

Ainda, como controle para verificar se a relevância do imunoproteassomo observada nos experimentos de infecção com *T. cruzi* e vacinação *in vivo* deste trabalho pode ser atribuída ao papel direto das moléculas $\beta 1i$, $\beta 2i$ e $\beta 5i$ no processamento dos epítomos, e não às alterações no compartimento dos linfócitos T CD8⁺ causadas pela falta do imunoproteassomo, foram utilizados animais C57BL/6 irradiados e reconstituídos com medula óssea de animais TKO ou C57BL/6. Neste modelo, o repertório dos linfócitos T CD8⁺ deve ser igual nos dois grupos, pois a seleção dos linfócitos T é realizada por células normais do epitélio tímico, enquanto as células apresentadoras de antígeno de origem mielóide são deficientes ou dotadas de imunoproteassomo, respectivamente. Os animais quiméricos reconstituídos com medula TKO e infectados com *T. cruzi* (Figura Suplementar 1) ou vacinados com AdASP-2 (Figura Suplementar 2) exibiram resposta reduzida de linfócitos T CD8⁺ específicos em comparação aos animais reconstituídos com medula normal, portanto corroborando a proposição de que os resultados observados nesta tese de fato decorrem do papel do imunoproteassomo no processamento de antígenos expressos por *T. cruzi* ou adenovírus.

Outra questão que há muito vem sendo investigada refere-se às causas da indução de resposta de linfócitos T CD8⁺ ser subótima durante a infecção pelo *T. cruzi*. Alguns autores sugerem que isto se deve à falta de ligantes de TLR no parasito (PADILLA; SIMPSON; TARLETON, 2009), enquanto outros observam a indução dos linfócitos T CD8⁺ indistinta entre animais WT e Myd88^{-/-} infectados com *T. cruzi* (OLIVEIRA et al., 2010).

Mais um aspecto associado à resposta subótima de linfócitos T CD8⁺ na infecção por *T. cruzi* refere-se à viabilidade destas células. É documentado que o bloqueio da via apoptótica Fas/FasL pela administração de anticorpos anti-FasL em animais infectados com *T. cruzi* resulta em menor parasitemia, maior número de linfócitos T ativados, aumento na produção de óxido nítrico e bloqueio da morte por ativação de linfócitos T CD8⁺ (GUILLERMO et al., 2007). Mais recentemente, nós demonstramos que linfócitos T CD8⁺ específicos para epítomos de *T. cruzi* expressam altos níveis de CD95 em resposta à infecção e são induzidos à morte quando incubados com anticorpos anti-CD95 (VASCONCELOS; BRUNA-ROMERO; et al., 2012). Surpreendentemente, no entanto, durante a infecção concomitante à imunização com AdASP-2 as células T CD8⁺ específicas não aumentaram a expressão de CD95 e permaneceram viáveis. Os mecanismos responsáveis pela indução de CD95 neste caso, contudo, permanecem desconhecidos e também não foi possível responder se a indução de morte celular era causa ou consequência da resposta subótima dos linfócitos T CD8⁺.

Para melhor entender como *T. cruzi* interfere no *priming in vivo* de linfócitos T CD8⁺, no presente estudo nós induzimos uma resposta artificial e ótima contra um antígeno inerte sem o parasito e então avaliamos o impacto específico do *T. cruzi* nesta indução. Num modelo onde o fenótipo e número de células que apresentam o epítomo cognato, a quantidade de antígeno e coestímulo, e a afinidade do clone do linfócito T CD8⁺ respondedor eram os mesmos, a presença do *T. cruzi* foi capaz de tornar a proliferação, diferenciação e função dos linfócitos T CD8⁺ subótima (Figuras 27 e 28), o que não foi observado quando da adição de AdASP-2 (Figura 29). Avaliando a funcionalidade das DC *in vitro* (Figura 31) e separando a célula dendrítica exposta ao *T. cruzi* da célula que apresenta o epítomo cognato *in vivo* (Figura 32) nós ainda excluimos a possibilidade de que o impacto do *T. cruzi* no *priming* dos linfócitos T CD8⁺ OTI pudesse ser devido à disfunção da célula dendrítica infectada, como ocorre no modelo de infecção por *Plasmodium sp*, por

exemplo (WILSON et al., 2006). Pelo contrário, os nossos resultados sugeriram a indução ativa de mecanismos supressores da resposta dos linfócitos T CD8⁺ *in trans* (Figura 32). Além disso, o *T. cruzi* foi capaz de causar alterações no fenótipo dos linfócitos T CD8⁺ OTI, que reproduziram o fenótipo dos linfócitos T CD8⁺ específicos para o epítipo imunodominante do parasito (Figura 33). Assim como os clones naturalmente responsivos a *T. cruzi*, os linfócitos OTI exibiram um perfil pró-apoptótico, com elevada expressão de CD95. No entanto, utilizando abordagens genéticas não foi possível atribuir a expressão exclusiva de CD95 às causas da resposta subótima das células OTI (Figura 34). Ainda assim, os resultados aqui apresentados indicam que outras vias de morte celular, como as induzidas pela sinalização via PD-1, merecem ser investigadas quanto ao impacto na resposta dos linfócitos T CD8⁺.

A resposta dos linfócitos T CD4⁺, por outro lado, foi induzida por *T. cruzi*, como observado pela proliferação *in vivo* e frequência de células CD62L^{baixo} (Figura 35). Estes linfócitos induzidos por *T. cruzi* foram os responsáveis pela supressão do *priming* dos linfócitos T CD8⁺ OTI (Figuras 36 e 37). No entanto, o mecanismo dessa supressão foi independente de IL-10 (Figura 38). Este resultado é compatível com nossos experimentos nos quais observamos que animais deficientes em IL-10 infectados com *T. cruzi* apresentam resposta imune mediada por linfócitos T CD8⁺ idêntica à de animais normais C57BL/6 (dados não publicados).

Ao depletar as células CD25⁺ com anticorpos, nós também demonstramos que os linfócitos T CD4⁺ CD25⁺ não participam da supressão dos linfócitos T CD8⁺ OTI (Figura 39). No modelo de infecção por *T. cruzi*, essa observação também foi obtida por dois outros grupos de modo independente (KOTNER; TARLETON, 2007; SALES et al., 2008). Os autores desses estudos concluíram, portanto, que Treg CD4⁺ CD25⁺ não atuam na supressão do *priming* de linfócitos T CD8⁺ que respondem contra o parasito. No entanto, utilizando abordagens mais sofisticadas para identificação de Treg pela expressão de Foxp3, nós demonstramos que a transferência das células T CD4⁺ Foxp3⁺ induzidas na presença de *T. cruzi* é capaz de suprimir o *priming in vivo* dos linfócitos T CD8⁺ OTI de modo significativamente maior do que a transferência de células T CD4⁺ Foxp3⁺ isoladas de animais não infectados (Figura 40). Em conjunto, portanto, estes resultados sugerem que linfócitos T CD4⁺ CD25⁻ Foxp3⁺ induzidos por *T. cruzi* são responsáveis pela resposta subótima dos linfócitos T CD8⁺. Corroborando esta afirmação, a depleção

in vivo especificamente das células Foxp3⁺ no camundongo DEREK logo após o desafio tornou a resposta dos linfócitos T CD8⁺ que reconhecem epítomos de *T. cruzi* mais forte e aumentou a resistência à infecção (Figura 42).

Quanto à origem das Treg que suprimem a resposta dos linfócitos T CD8⁺ no modelo deste estudo, é possível que sejam realmente iTreg induzidas direta ou indiretamente pelo *T. cruzi*, já que foram funcionalmente distintas das Treg isoladas de animais não infectados (Figura 40). Além disto, a expressão de Nrp1, um marcador de nTreg, foi reduzida nos linfócitos T CD4⁺ dos animais que receberam BMDC expostas ao *T. cruzi* (Figura 35). No entanto, no corrente *estado da arte* não existe uma maneira precisa e inequívoca de diferenciar fenotipicamente nTreg de iTreg, o que dificulta responder a esta questão de modo conclusivo.

A frequência de células CD4⁺ Foxp3⁺ induzidas em animais que receberam *T. cruzi* foi menor do que a encontrada em animais dos grupos-controle (Figura 35). Uma hipótese alternativa e especulativa que contemple esta observação seria a capacidade do *T. cruzi* tornar os linfócitos T CD8⁺ mais susceptíveis à regulação pelas Treg, sem necessariamente induzir sua expansão. Já foi descrito, por exemplo, que a sinalização via Myd88 em linfócitos T CD4⁺ pode romper a capacidade destes linfócitos serem regulados (SCHENTEN et al., 2014). Talvez fosse possível que algum mecanismo similar ocorresse nos linfócitos T CD8⁺ e fosse especificamente inibido, direta ou indiretamente, pelo *T. cruzi*.

Após algumas semanas da infecção pelo *T. cruzi* a resposta dos linfócitos T CD8⁺ é intensa. Isto pode indicar que os resultados apresentados aqui são restritos ao *priming* durante os primeiros dias da exposição *in vivo* ao parasito. Uma possibilidade é que os linfócitos T CD4⁺ supressores do priming dos linfócitos T CD8⁺ morram e então liberem a indução dos linfócitos T CD8⁺, o que seria condizente com seu perfil proapoptótico (Figura 35). Além disso, em animais infectados com *T. cruzi* por 20 dias, o número e frequência de linfócitos T CD4⁺ totais diminuiu em comparação aos animais *naïve* (Figura 14), o que poderia resultar da sua morte induzida pelo parasito.

Os experimentos utilizando anticorpos para bloqueio *in vivo* sugerem ainda que TGF-β e CTLA-4 restringem respectivamente a proliferação e função dos linfócitos T CD8⁺ induzidos na presença de *T. cruzi* (Figura 41). No caso do CTLA-4, existem dados na literatura que corroboram nossos resultados, indicando que o bloqueio da molécula com anticorpos melhora a indução de linfócitos T CD8⁺ e o

controle da infecção pelo *T. cruzi* (DOS SANTOS et al., 2015; MARTINS et al., 2004). Também um trabalho utilizando animais que não respondem a TGF- β infectados com *T. cruzi* relatou maior proliferação dos linfócitos T CD8⁺, mas não a sua restauração funcional. O mesmo trabalho sugeriu, ainda, que TGF- β é importante para proteger os animais da imunopatologia na fase crônica da infecção (MARTIN et al., 2007).

Como em toda investigação científica, os resultados encontrados aqui levam a novas questões, por exemplo: Quais são as propriedades das DC expostas ao *T. cruzi* que desengatilham a indução de Treg supressoras do *priming* dos linfócitos T CD8⁺? Quais são as citocinas envolvidas na polarização das Treg neste caso? Qual é a especificidade do TCR dos linfócitos T CD4⁺ CD25⁻ Foxp3⁺ induzidos? A supressão observada depende de contato? Para suprimir a resposta dos linfócitos, expressão de CTLA-4 ocorreria exclusivamente nas células Foxp3⁺? Quanto tempo dura esta supressão? Apesar de abrir todas estas perguntas, os modelos utilizados neste trabalho contribuem para o entendimento mais detalhado e claro de como o *T. cruzi* induz o *priming* dos linfócitos T CD8⁺ *in vivo* e sugerem o processamento pela via citosólica, a participação elementar do imunoproteassomo, além de um novo mecanismo de regulação destes linfócitos por células CD4⁺ CD25⁻ Foxp3⁺.

6 CONCLUSÕES

Utilizando o modelo de infecção experimental pelo *T. cruzi* e vacinação com vetor adenoviral recombinante contendo um gene do parasito, foi possível identificar novas bases celulares e moleculares que governam a resposta de linfócitos T CD8⁺. Estas bases incluem o envolvimento do imunoproteassomo e de Tregs CD4⁺ CD25⁻ Foxp3⁺ respectivamente na indução e na supressão do *priming* induzido pelo *T. cruzi*.

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ANEXOS

Figura Suplementar 1 - Resposta de linfócitos T CD8⁺ específicos em camundongos C57BL/6 reconstituídos com medula óssea TKO e infectados com *T. cruzi*.

Camundongos C57BL/6 (n=3) foram irradiados, reconstituídos com medula óssea normal ou TKO e após 8 semanas infectados ou não com *T. cruzi*, como esquematizado em a. Duas semanas depois da infecção, o fenótipo das quimeras TKO-WT foi avaliado pela marcação de H-2K^b nas células CD11c⁺ I-A^b⁺ do baço (b). A resposta de linfócitos T CD8⁺ específicos foi avaliada pela marcação destas células com pentâmeros H-2K^b-VNHRFTLV (c-d) e ICS após reestímulo com VNHRFTLV, ANYKFTLV e ANYDFTLV (e-f). A clonalidade dos linfócitos T CD8⁺ CD44^{alto} CD62L^{baixo} foi avaliada pela marcação das cadeias Vβ do TCR. São apresentados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT-WT e TKO-WT (*P<0,05 ***P<0,001 ****P<0,0001 ANOVA de dois fatores e teste Tukey *post hoc*.)

Figura Suplementar 2 - Resposta de linfócitos T CD8⁺ específicos em camundongos C57BL/6 reconstituídos com medula óssea TKO e imunizados com AdASP-2.

Camundongos C57BL/6 (n=3) foram irradiados, reconstituídos com medula óssea normal ou TKO e após 8 semanas imunizados com Adβ-gal ou AdASP-2, como esquematizado em a. Duas semanas depois da imunização, o fenótipo das quimeras TKO-WT foi avaliado pela marcação de H-2K^b nas células CD11c⁺ I-A^b do baço (b). A resposta de linfócitos T CD8⁺ específicos foi avaliada pela marcação destas células com pentâmeros H-2K^b-VNHRFTLV (c-d) e ICS após reestímulo com VNHRFTLV (e-f). A clonalidade dos linfócitos T CD8⁺ CD44^{alto} CD62L^{baixo} foi avaliada pela marcação das cadeias Vβ do TCR (g-h). São apresentados valores individuais e barras que indicam a mediana ± erro padrão de cada grupo. Asteriscos indicam diferença estatística entre os animais WT-WT e TKO-WT (***P<0,001 ANOVA de dois fatores e teste Tukey *post hoc*.)

Pathogen-Induced Proapoptotic Phenotype and High CD95 (Fas) Expression Accompany a Suboptimal CD8⁺ T-Cell Response: Reversal by Adenoviral Vaccine

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Abstract

MHC class Ia-restricted CD8⁺ T cells are important mediators of the adaptive immune response against infections caused by intracellular microorganisms. Whereas antigen-specific effector CD8⁺ T cells can clear infection caused by intracellular pathogens, in some circumstances, the immune response is suboptimal and the microorganisms survive, causing host death or chronic infection. Here, we explored the cellular and molecular mechanisms that could explain why CD8⁺ T cell-mediated immunity during infection with the human protozoan parasite *Trypanosoma cruzi* is not optimal. For that purpose, we compared the CD8⁺ T-cell mediated immune responses in mice infected with *T. cruzi* or vaccinated with a recombinant adenovirus expressing an immunodominant parasite antigen. Several functional and phenotypic characteristics of specific CD8⁺ T cells overlapped. Among few exceptions was an accelerated expansion of the immune response in adenoviral vaccinated mice when compared to infected ones. Also, there was an upregulated expression of the apoptotic-signaling receptor CD95 on the surface of specific T cells from infected mice, which was not observed in the case of adenoviral-vaccinated mice. Most importantly, adenoviral vaccine provided at the time of infection significantly reduced the upregulation of CD95 expression and the proapoptotic phenotype of pathogen-specific CD8⁺ cells expanded during infection. In parallel, infected adenovirus-vaccinated mice had a stronger CD8 T-cell mediated immune response and survived an otherwise lethal infection. We concluded that a suboptimal CD8⁺ T-cell response is associated with an upregulation of CD95 expression and a proapoptotic phenotype. Both can be blocked by adenoviral vaccination.

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Competing Interests: I have read the journal's policy and have the following conflicts: R.T.G., M.M.R., A.V.M. and O.B.-R. are named inventors on patent applications covering *Trypanosoma cruzi* vectored vaccines and immunization regimens.

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Introduction

The digenetic intracellular protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) is the etiologic agent of Chagas' disease, an acute and chronic illness affecting millions of individual in the Americas [1]. After contact with *T. cruzi*, humans and mice develop MHC class Ia-restricted CD8⁺ T cells specific for immunodominant parasite epitopes [2–7]. In highly susceptible hosts, such as A/Sn mice, the immune response is unable to control acute-phase pathology and prevent death. In most hosts, however, these specific CD8⁺ T cells are critical for survival following infection, even when limited numbers of parasites initiate infection [2,8]. Despite the CD8⁺ T

cell-mediated immune response, *T. cruzi* usually survives and establishes a life-long chronic infection. Parasite persistence is an important element of chronic-phase pathologies that occur many years or even decades after initial infection [9–15]. Therefore, in any circumstance, the CD8⁺ T cell-mediated immune response completely eliminate the parasite. The reason for this ineffective or suboptimal immune response is not fully understood. Several potentially significant mechanisms have been described for parasite evasion of the immune response and may account for short- and long-term parasite survival [16].

In contrast to the observations made during experimental mouse infection, immunization with adenoviral vaccines express-

Author Summary

Killer lymphocytes are important mediators of the immunological resistance against infections caused by virus, bacteria and parasites. In some circumstances, however, these lymphocytes are unable to properly eliminate the microorganisms which survive, causing death or establishing chronic infections. The purpose of our study was to understand why these killer cells do not succeed during infection with a human protozoan parasite. For that purpose, we compared the immune responses in animals infected or vaccinated. Many characteristics of these killer cells were similar. Among few exceptions was an accelerated immune response in vaccinated animals when compared to infected ones. Also, we observed on the surface of the killer lymphocytes from infected, but not from vaccinated animals, an increased expression of a protein involved in signaling cell death. Most importantly, vaccine significantly reduced the higher expression of this cell-death receptor. In parallel, these animals had a stronger immune response and cured infection. We concluded that a deficient killer cell response observed during infection was associated with an upregulation of this cell-death receptor and it was changed by vaccination.

ing a parasite immunodominant antigen can elicit a strong CD8⁺ T cell-mediated immunity against acute and late pathologies after an infectious challenge with *T. cruzi* [17–21]. Although it is well established that these specific CD8⁺ T cells induced by vaccination eliminate parasites, it is not clear which properties of these T cells are responsible for sustaining highly effective immunity against *T. cruzi*-induced pathology. It is paradoxical that specific CD8⁺ T cells elicited by infection or vaccination are cytotoxic *in vivo* and secrete IFN- γ , which are mechanisms that mediate protective immunity against *T. cruzi* [5,17].

To understand this problem, we hypothesized that CD8⁺ T cells induced during infection had different functional or phenotypic properties from specific T cells generated by adenoviral vaccines. Accordingly, our approach consisted of a detailed comparison of the kinetics, function, and phenotype of the CD8⁺ T cells induced by either experimental infection or immunization with recombinant adenoviral vaccine expressing the immunodominant antigen, amastigote surface protein 2, of *T. cruzi* (AdASP-2). Essentially, our results showed that parasite infection and AdASP-2 vaccination elicited effector CD8⁺ T cells that mostly overlapped in phenotype and function. An important and recurrent exception was the overexpression of the apoptotic receptor CD95 on the surface of the specific CD8⁺ T cells observed during *T. cruzi* infection. In contrast, AdASP-2 vaccine induced T cells that failed to upregulate CD95 expression. Moreover, immunization with the AdASP-2 vaccine performed simultaneously with the infectious challenge pre-programmed specific CD8⁺ T cells to mediate protective immunity. In adenoviral-vaccinated animals, protective CD8⁺ T cells that expand after an infectious challenge had reduced CD95 expression and a reduced proapoptotic phenotype. Our observation indicates that CD95 expression by suboptimal CD8⁺ T cells during infection may be one of the key factors leading to an early increase in apoptosis and a reduced immune response. Furthermore, adenoviral vaccines may pre-program pathogen-specific T cells by reducing the levels of CD95 expression and apoptosis and, therefore, promote T cell accumulation and efficacy.

Results

Protective CD8⁺ T cells are elicited by a single dose of AdASP-2 vaccine provided shortly before or at the same time as an infectious challenge

Recently, the AdASP-2 vaccine was described [17–21]. This vaccine expresses the immunodominant antigen ASP-2, a member of the *trans*-sialidase superfamily of surface proteins of *T. cruzi*. This antigen is abundantly expressed by intracellular amastigotes and contains 2 immunodominant epitopes (one for H-2K^k [TEWETGQI] and one for H-2K^b [VNHRFTLV]) recognized by protective CD8⁺ T cells during mouse infection with the Y strain of *T. cruzi* (Figure 1A and B, [ref. 3,5]).

To establish a simple method to study the induction of protective CD8⁺ T cells against *T. cruzi* infection, we immunized

Figure 1. Schematic representation of the ASP-2 antigen and its expression in intra-cellular amastigotes of *T. cruzi*. **A)** The protein is a prototypical member of the *trans*-sialidase family of *T. cruzi* surface antigens containing a putative signal peptide at the N-terminal region, 2 ASP-box sequences, and a VTV-box at the C-terminal domain. The protein is attached to the membrane through a glycosphosphatidylinositol anchor. **B)** ASP-2 expression in intra-cellular amastigotes was determined by immunofluorescence with the specific MAb K22. HeLa cells were infected for 48 h with trypomastigotes of the Y strain. After fixation, indirect immunofluorescence and DAPI staining were performed and imaged under fluorescence microscopy. Bar, 14 μ m. Photomicrography kindly provided by Dr. Clara Claser (Singapore Immunology Network -SIgN, Singapore). doi:10.1371/journal.ppat.1002699.g001

susceptible A/Sn mice with a single dose of AdASP-2 vaccine. As shown in Figure 2A, i.m. immunization with increasing doses of AdASP-2 (ranging from 10^6 to 10^8 pfu) 7 days prior to infection led to a dose-dependent reduction in peak parasitemia. Vaccination also significantly retarded or reduced mouse mortality (Figure 2B). As a control, mice injected with the higher dose of adenovirus vector expressing the β -galactosidase protein (Ad β -gal) developed higher parasitemia (Figure 2A) and died sooner than the other mouse groups following experimental challenge (Figure 2B).

From the experiment described above, we considered a single dose of $\geq 10^8$ pfu sufficient to protect against a challenge with *T. cruzi*. The timing of administration of the adenoviral vaccine was

determined by immunizations performed from 7 days before up to 7 days after infection. As shown in Figure 2C and 2D, even when administered on the same day as the infectious challenge, immunization with AdASP-2 vaccine elicited significant protective immunity, impairing the development of parasitemia and improving mouse survival. In contrast, administration of AdASP-2 vaccine after the challenge had limited or no impact on infection.

To determine whether the route of immunization affected protective immunity, we injected AdASP-2 vaccine on the same day as infection by the i.m., s.c., or i.p. routes. While mice immunized i.m. or s.c. controlled parasitemia and survived

Figure 2. Parasitemia and mortality in A/Sn mice immunized with AdASP-2 vaccine and challenged with trypomastigotes. **A)** A/Sn mice were immunized i.m. with the indicated doses (pfu) of AdASP-2 vaccine or control Ad β -gal. Seven days later, mice were challenged s.c. with 150 bloodstream trypomastigotes of the Y strain of *T. cruzi*. Parasitemia was followed daily from days 9 to 13 after challenge. The results represent the mean $\pm$ SD values for 6 mice. At the peak parasitemia (day 11), values of mice immunized with each different dose were compared by one-way ANOVA and Tukey HSD tests and the results were as follows: **i)** Groups of mice that received doses of 10^8 or 10^7 pfu of AdASP-2 vaccine had values lower than the group of mice injected with Ad β -gal ($P < 0.01$). **B)** Kaplan–Meier curves for survival of the different groups were compared using the logrank test. The results of the comparisons were as follows: **i)** Groups of mice injected with 10^8 or 10^7 pfu of AdASP-2 vaccine survived longer than the group injected with Ad β -gal ($P < 0.01$). **C)** A/Sn mice were immunized i.m. on the indicated days before or after infection with AdASP-2 vaccine or control Ad β -gal (2×10^8 pfu/mouse). All mice were challenged s.c. as described above. Although the day of the peak parasitemia was different for each group, we used the maximal values for statistical comparison. The results of the comparisons were as follows: **i)** Mice vaccinated on day -7 (red) with AdASP-2 vaccine had values lower than those of any other group. **ii)** Mice vaccinated on days 0 (green) or +4 (yellow) with AdASP-2 vaccine had values lower than the group of mice injected with Ad β -gal (black) or AdASP-2 on day +7 (blue, $P < 0.01$). **D)** Kaplan–Meier curves for survival of the different groups were compared and the results were as follows: **i)** Groups of mice vaccinated with AdASP-2 vaccine on days -7 (red) or 0 (green) survived longer than the groups of mice injected with Ad β -gal (black) or AdASP-2 vaccine on days +4 (yellow) or +7 (blue) ($P < 0.01$ in all cases). **E)** A/Sn mice were immunized with AdASP-2 vaccine or Ad β -gal (2×10^8 pfu/mouse) by the indicated routes (i.m., s.c., or i.p.). All mice were challenged s.c. on the same day as the immunization, as described above. The parasitemia data for each mouse group are represented in terms of mean $\pm$ SD ($n = 6$). Although the day of the peak parasitemia was different for each group, we used the maximal values for statistical comparison. The results of the comparisons were that mice vaccinated i.m. or s.c. with the AdASP-2 vaccine had values lower than those of any other groups ($P < 0.01$). **F)** Kaplan–Meier curves for survival of the different groups were compared and the results were as follows: **i)** Mice vaccinated i.m. or s.c. with the AdASP-2 vaccine survived longer than any other groups ($P < 0.01$). **ii)** Mice vaccinated i.p. with AdASP-2 vaccine survived longer than mice that received Ad β -gal ($P < 0.05$). **G)** A/Sn mice were immunized i.m. with AdASP-2 vaccine or control Ad β -gal (2×10^8 pfu/mouse). Seven days later, mice were challenged s.c. as described above. Before and after challenge, mice were treated as described in the Methods section with rat IgG (control) or anti-CD8 MAb. The parasitemia for each mouse group is represented as mean $\pm$ SD ($n = 6$). Although the day of the peak parasitemia was different for each group, we used the maximal values for statistical comparison. AdASP-2-immunized mice treated with rat IgG had a significantly lower parasitemia ($P < 0.01$) when compared to those of non-immune animals or AdASP-2-vaccinated mice treated with anti-CD8 MAb. **H)** Kaplan–Meier curves for survival of the different groups were compared and the results showed that AdASP-2-immunized mice treated with rat IgG survived significantly longer ($P < 0.01$) than non-immune animals or AdASP-2-immunized mice treated with anti-CD8 MAb. The results described above are representative of 2 or more independent experiments. doi:10.1371/journal.ppat.1002699.g002

infection, animals injected by the i.p. route showed limited control of parasite development and mouse survival (Figure 2E and 2F). Finally, to evaluate the role of CD8⁺ T cells for protection, AdASP-2-immunized mice were treated with MAb to CD8 prior to infection. The depletion of CD8⁺ T cells led to a complete reversal of immunity as evaluated by parasitemia (Figure 2G) and mouse survival (Figure 2H). We concluded that a single dose of 2×10^8 pfu of AdASP-2 vaccine administered 7 days prior to or even on the same day as the infectious challenge can be used as a simple model to elicit protective CD8⁺ T cells against an otherwise lethal infection with *T. cruzi*.

To determine whether AdASP-2 vaccination cured *T. cruzi* infection, we collected 0.5 mL of the blood 24 months after challenge and transferred i.p. to naïve A/Sn mice. Four of the 15 mice (26.66%) developed patent parasitemia and died. In contrast, 100% of control mice injected with 150 parasites developed patent parasitemia and died (data not shown). Based on that, we considered that AdASP-2 vaccination in fact cured *T. cruzi* infection in 73.33% of the vaccinated animals. The absence of infection can not be attributed to passively transferred immunity because injection of 0.5 mL of blood of vaccinated mice admixed with 100 viable bloodstream trypomastigotes also does not confer protection to naïve A/Sn (data not shown).

Higher numbers of multifunctional specific CD8⁺ T lymphocytes are present as a result of the adenovirus vaccination

The fact that AdASP-2 vaccine administered simultaneously with the infectious challenge could provide a significant degree of protective immunity raised the possibility that vaccine- or *T. cruzi*-induced CD8⁺ T cells had different traits. To understand the cellular basis for the biological properties of the CD8⁺ T cells elicited by infection or vaccination, we compared certain functional and phenotypic profiles.

Groups of A/Sn mice were immunized according to the protocol described in Figure 3A. In these experiments, vaccination and challenge were performed simultaneously to avoid any obvious boosting effect that might interfere with interpretation of the results. Our analysis of the immune response was done on day 19 post infection just prior to the Adβ-gal-immunized control mice started to die. We observed consistently similar frequencies of peptide-specific cells in mice that were infected with *T. cruzi* or vaccinated with AdASP2 (Figure 3B). Most important was the fact that significantly more peptide-specific cells were detected in infected AdASP-2-vaccinated mice. The frequency of specific CD8⁺ T cells, as determined by staining with the multimer H2K^b-TEWETGQI, was approximately 4 times higher in infected AdASP-2-immunized mice than in mice that had only been immunized or infected (Figure 3B).

To compare important functional aspects of antigen-specific CD8⁺ T cells, we performed staining of CD8⁺ T cells following *in vitro* peptide stimulation for surface mobilization of CD107a [22], a marker for T-cell degranulation, and intracellular effector cytokines (IFN-γ and TNF-α, intra-cellular cytokine staining-ICS). Estimates of frequencies of splenic functional peptide-specific CD8⁺ cells that mobilize CD107a to their surfaces, express IFN-γ or TNF-α were 6- to 9-fold higher in infected AdASP-2-immunized mice than in the spleen of mice that had been only immunized or infected (Figure 3C). Spleen cells stimulated with the peptide TEWETGQI revealed a large fraction of multifunctional CD8⁺ cells in both, infected or immunized mice (Gr.2 and Gr. 3, Figure 3D and 3E). The analysis of the frequency of CD8⁺ T cells expressing different combinations of the effector molecules in mice infected with *T. cruzi* or immunized with AdASP-2 vaccine

revealed that multifunctional triple-positive cells was the largest population in both cases, accounting for 57.36% or 47.25 % of the peptide-specific cells in infected or immunized mice, respectively. Among the double-positive cells, we found 14.15 % or 12.90 % of IFN-γ⁺/TNF-α⁺ cells in infected or vaccinated mice, respectively. The difference in terms of frequency was the higher proportion of CD107a⁺/TNF-α⁺ or CD107a⁺/IFN-γ⁺ double-positive CD8⁺ T cells in splenic cells from infected mice or immunized mice, respectively. We considered that the quality of the specific CD8⁺ T cells elicited by *T. cruzi* infection or AdASP-2 immunization generally overlapped.

The pattern of effector molecules expressed by specific CD8⁺ cells in infected AdASP-2-immunized mice retained a high frequency of triple-positive (28.28 %) and double-positive IFN-γ⁺/TNF-α⁺ cells (47.25 %) The difference we observed in this case was significantly higher frequencies of single-positive TNF-α⁺ cells in splenic cells of infected AdASP-2-immunized mice when compared to those of vaccinated or infected mice (Figure 3D and 3E).

To confirm our analyses of the immune response, we used also the C57BL/6 mouse model for experiments. Infected C57BL/6 mice do not succumb to acute infection and display a strong CD8⁺ T-cell immune response to the immunodominant epitope VNHRFTLV [2–4,17,19,20]. After s.c. infection with 10^4 parasites of *T. cruzi*, peak parasitemia occurred on day 8 or 9 post infection (Figure S1 A). We then followed the kinetics of the CD8 epitope-specific cells by 2 distinct assays, i.e., *in vivo* cytotoxicity and *ex vivo* surface mobilization of CD107a and ICS for IFN-γ and TNF-α, after s.c. parasite infection or i.m. immunization with AdASP-2 vaccine. We observed a more rapid *in vivo* cytotoxic response in mice immunized with the AdASP-2 vaccine when compared to the response of the infected ones (day 8, Figure S1 B). Nevertheless, by day 12 both groups had reached maximal levels. In infected mice, high levels of *in vivo* cytotoxicity were still detected by day 30. This high *in vivo* cytotoxicity is maintained for a long period of >90 days due to unknown reasons (3). Likewise, the number of CD8⁺ splenocytes that mobilize CD107a to their surfaces and express IFN-γ and/or TNF-α also appeared faster in mice immunized with the AdASP-2 vaccine than in infected mice (day 7, Figure S1 C). However, the immune response in vaccinated mice declined significantly at day 28 when compared to infected mice.

Splenic cells were collected from animals that had been infected or immunized and re-stimulated *in vitro* with the peptide VNHRFTLV. At day 7, multi-functional double positive CD107a⁺/IFN-γ⁺ and triple-positive peptide-specific CD8⁺ cells accounted for the largest populations of peptide-specific cells from immunized mice (Figure S1 D). At day 15, the multifunctional triple-positive cells accounted for the largest population in infected or AdASP-2 immunized mice (Figure S1 E). Among the double-positive cells, we found higher frequencies of IFN-γ⁺/TNF-α or CD107a⁺/IFN-γ⁺ cells in infected or vaccinated mice, respectively. A difference in terms of frequency was also observed by the higher proportion of CD107a single-positive CD8⁺ T cells in splenic cells from immunized mice (Figure S1 E).

At day 28, the frequencies of the distinct sub-populations changed little in infected mice compared to day 15. In the case of AdASP-2 immunized mice CD107a⁺/IFN-γ⁺ cells accounted for the largest population with the decline of multifunctional triple-positive cells (Figure S1 F). An increase was also seen by the higher proportion of CD107a single-positive CD8⁺ T cells in splenic cells from immunized mice.

In addition, we compared the frequencies of specific T cells in groups of mice that have been immunized and challenged

Figure 3. Specific CD8⁺ T cell-mediated immune responses of infected, immunized or infected AdASP-2 immunized A/Sn mice. **A)** A/Sn mice were immunized i.m. with Adβ-gal or AdASP-2 vaccine (2×10^8 pfu/mouse). On the same day, half of the mice were challenged s.c. with trypomastigotes of the Y strain of *T. cruzi* (150 bloodstream parasites/mouse). **B)** Nineteen days after the immunization/challenge, we estimated the frequency of splenic H2K^k-TEWETGQI⁺ CD8⁺ cells. The results are presented in terms of each mouse (dots) and medians (bars). **C)** Splenic cells were cultured in the presence of anti-CD107a and anti-CD28, with or without the peptide TEWETGQI. After 12 h, cells were stained for CD8, IFN-γ, and TNF-α. Frequencies were initially estimated for any CD8⁺ that expressed surface CD107a, IFN-γ or TNF-α after stimulation *in vitro* with peptide TEWETGQI. **D)** Subsequently, we estimated the subpopulations of CD8⁺ cells expressing each individual molecule or combination (surface CD107a, IFN-γ or TNF-α). The results are presented as the mean $\pm$ SD frequencies of CD8⁺ cells for 4 mice. The asterisks and crosses denote significantly higher numbers of peptide-specific cells than in the group of mice immunized with Adβ-gal or all other groups, respectively ($P < 0.05$). **E)** Pie charts show the fraction of peptide-specific cells expressing the indicated molecules. The results are expressed as the mean values for 4 mice per group. The results are representative of 2 independent experiments.
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according to the protocol described in Figure S2 A. On day 21, there was an increase in the frequency of peptide-specific CD8⁺ T cells from infected AdASP-2-immunized mice (Gr.4) according to the expression of effector molecules (Figure S2 B). Despite these numerical differences, the composition of the distinct T-cell subpopulation expressing one or more of these molecules was not dramatically different between infected (Gr. 2) and infected AdASP-2-immunized mice (Gr. 4, Figure S2 C and S2D). The multifunctional T cells that expressed 2 or more effector molecules accounted for more than 66% of the cells. However, differences between Gr. 2 and Gr. 4 can be seen when single-positive T cells

are compared (Figure S2 C and S2 D). As we observed earlier in Figure S1 F, there was a tendency for the accumulation of more differentiated T cells (triple-positive) in infected mice (Gr. 2). Further, the distribution was more heterogeneous in the cells from AdASP-2-vaccinated mice (Gr. 3 and Gr. 4) than in the cells from infected mice (Gr. 2).

Together with the results obtained from A/Sn mice, we considered that the size and quality of the specific CD8⁺ T cells elicited by *T. cruzi* infection and AdASP-2 immunization were not dramatically different. The magnitude was similar and in terms of effector molecules, they generally overlapped at the peak of the

immune response. Noteworthy were the observations that in AdASP-2 immunized mice the immune response was faster and in infected AdASP-2-immunized mice there was a significant increase in the frequency of specific CD8⁺ T cells.

A relevant question raised from these experiments was the possibility that the increase in the frequency of specific CD8⁺ T cells was due to a bystander activation caused by the AdASP-2 vaccine. To evaluate this possibility, we determined whether the frequency of CD8⁺ T cells specific for a sub-dominant epitope of *T. cruzi* was also increased in infected AdASP-2-immunized mice. For that purpose, we estimated the immune response of H-2K^b-restricted CD8⁺ cells specific for the epitope TsKb-20. We found that AdASP-2 immunization prior to infection (Gr. 4) generated a significant increase in the frequencies of VNHRFTLV specific CD8⁺ cells as estimated by the multimer staining or by expression of IFN- γ and/or TNF- α (Figure S3 B and C, respectively). In contrast, AdASP-2 immunization prior to infection did not cause an increase in the frequency of TsKb-20 specific CD8⁺ cells when compared to mice that had been immunized with control Ad β -gal and infected with *T. cruzi* (Gr.2, Figure S3 C). In fact, the frequencies of TsKb-20 specific CD8⁺ cells were lower. This observation provided a strong argument against any bystander effect of the AdASP-2 immunization.

Prevention of up-regulation of CD95 expression on specific CD8⁺ T cells from mice primed with the adenovirus based vaccine and simultaneously challenged with blood stream trypomastigotes

Based on the fact that the magnitude and quality of the immune response was similar in infected or immunized mice, we pursued a further characterization of the phenotype of these specific cells that could account for a higher frequency of specific CD8⁺ T cells in infected AdASP-2-immunized mice. Splenic cells were stained for CD8, the H2K^k-TEWETGQI multimer, and various T-cell surface markers. Cells were collected from animals infected, immunized, or infected-AdASP-2 immunized as described in Figure 3A. We found a similar pattern of expression for many molecules analyzed when we compared infected (Gr. 2) or AdASP-2-immunized (Gr. 3) mice. Specific CD8⁺ T cells upregulated the expression of CD11a, CD44, PD-1 and KLRG-1. On the other hand, they downregulated the expression of CD62L and CD127. Several molecules, such as CD95L, CCR7, and CTLA-4, had unchanged expression patterns when compared to those of naive CD8⁺ T cells (Figure 4).

Some molecules were expressed differently by antigen-specific CD8⁺ T cells from infected or immunized mice. CD11c, CD25, CD43 (1B11), CD69 and CD122 were expressed only on a subset of specific CD8⁺ T cells of infected mice but not on CD8⁺ T cells from immunized mice. CD27, BTLA and β 7 expression were down regulated only on a subset of specific CD8⁺ T cells of immunized mice but not on CD8⁺ T cells from infected mice. To our surprise, we consistently found that the apoptotic receptor CD95 (Fas) was upregulated only on the surface of specific CD8⁺ T cells from infected but not immunized mice.

When we analyzed the phenotype of the specific CD8⁺ T cells of infected AdASP-2-immunized mice (Gr. 4), we found that they matched the ones observed on the specific CD8⁺ T cells from AdASP-2 immunized mice (Gr. 3). This observation suggests that vaccination has a dramatic influence on the phenotype of the cells expanded after the infectious challenge.

To confirm these results, we analyzed the phenotype of splenic CD8⁺ cells from immunized or infected C57BL/6 mice. Splenic cells were collected from animals that had been infected or

immunized 28 or 14 days earlier, respectively, and stained for CD8, the H2K^b-VNHRFTLV multimer and various T-cell surface markers. These days were selected because they represented the peak of their immune response (Figure S1). Overall, we found a very similar pattern of expression for most molecules analyzed. Specific CD8⁺ T cells upregulated the expression of CD11a, CD43, CD44, CD49d, CD95L, and KLRG-1. On the other hand, they downregulated the expression of CD27, CD62L, CD127, and BTLA. Several molecules, such as CD25, CD69, CCR7, and CTLA-4, had unchanged expression patterns when compared to those of naive CD8⁺ T cells (Figure S4 A).

Only a few molecules were expressed differently by antigen-specific CD8⁺ T cells from infected and immunized mice. CD11c was expressed only on a subset of specific CD8⁺ T cells of infected mice but in all CD8⁺ T cells from immunized mice. CD122 was expressed more strongly in specific CD8⁺ T cells from mice immunized with the AdASP-2 vaccine; nevertheless, the expression was still low. On the other hand, PD1, CD43 (1B11), and CD183 were upregulated only on the surface of specific CD8⁺ T cells from immunized but not infected mice (Figure S4 A).

Consistent with the results described in A/Sn infected mice, we found that the apoptotic receptor CD95 was upregulated only on the surface of specific CD8⁺ T cells from infected but not immunized mice. The kinetics of CD95 expression on the surface of specific CD8⁺ T cells of infected mice revealed that the maximum expression occurred at 14 to 28 days after infection. Thereafter, a significant decline was observed. In contrast, the expression of CD95 on specific CD8⁺ T cells from AdASP-2-immunized mice did not change during the evaluation period in comparison to that of naive CD8⁺ T cells (Figure S4 B).

MyD88 activation, IL-12 and IFN-type 1 are described as important inflammatory mediators during *T. cruzi* infection in mice [23]. Accordingly, we tested whether the upregulation of CD95 on the surface of specific CD8⁺ T cells was dependent on their expression. For that purpose, we used genetically deficient (KO) mice that failed to express MyD88, p40 (IL-12/IL-23) or IFN-1 receptor. As shown in Figure S5, specific CD8⁺ T cells from infected MyD88, IL-12/IL-23 or IFN-1 receptor KO mice upregulated CD95 on their surface at the same level as WT mice. These results suggested that upregulation of CD95 was independent of the expression of any of these molecules.

The fact that specific H2K^k-TEWETGQI⁺ CD8⁺ cells expressed higher amounts of the apoptotic receptor CD95 led us to determine whether they could be indeed in a proapoptotic state. Splenic H2K^k-TEWETGQI⁺ CD8⁺ cells were analyzed in infected, vaccinated or infected AdASP-2-immunized mice 19 days after challenge/infection. We estimated their frequencies, expression of CD95 and annexin V ligand and their *in vivo* proliferative capacity. As earlier described, a significantly higher frequency of H2K^k-TEWETGQI⁺ CD8⁺ cells was detected in the spleen of infected-AdASP-2 immunized mice (Gr. 4, Figure 5A). Also, the expression of CD95 was higher on the surface of specific CD8⁺ from infected mice (Gr. 2, Figure 5B, C and D). Most relevant was the fact that we found that a higher frequency of H2K^k-TEWETGQI⁺ CD95⁺ CD8⁺ cells from Gr. 2 (infected) mice stained positive for annexin V (Gr. 2, Figure 5C). This frequency was significantly higher when compared to that of cells from Gr. 4 (mean values, 58.05% $\pm$ 6.38% and 17.29% $\pm$ 6.42%, $n = 3$, respectively; $P < 0.05$).

To confirm that the lower frequency of specific CD8⁺ T cells was not due to a diminished proliferative capacity of cells from infected mice, we evaluated their *in vivo* proliferative capacity. We found that a similar proportion of the H2K^k-TEWETGQI⁺ CD8⁺ cells incorporated BrdU *in vivo* in infected or infected-

Gr. 1 Adβ-gal
 Gr. 2 Adβ-gal + *T. cruzi*
 Gr. 3 AdASP-2
 Gr. 4 AdASP-2 + *T. cruzi*

Figure 4. Phenotypic characterization of specific CD8⁺ T cells of infected and/or AdASP-2 immunized A/Sn mice. A/Sn mice were infected or immunized as described in the legend of Figure 3A. Splenic cells were collected nineteen days after infection/immunization. The histograms show FACS analysis on CD8⁺ cells (Gr. 1) and H-2K^b-TEWETGQI⁺ CD8⁺ cells (Gr. 2, 3, and 4) and the indicated marker (blue). Control cells

were from naive mice (red lines). Results of CD44, KLRG1 and CD183 staining are presented as MFI and frequencies of the CD44^{High}, KLRG1^{High} or CD183^{High} cells, respectively. On the other hand, results of CD27 and CD62L staining are presented as MFI and frequencies of the CD27^{Low} or CD62L^{Low} cells. Representative analyses are shown from pools of cells from 3 mice. Stainings were performed 2 or more times with identical results. doi:10.1371/journal.ppat.1002699.g004

AdASP-2 vaccinated mice, indicating that the proliferative capacity of these cells was not significantly different (Gr. 2 and Gr. 4, Figure 5C). From these experiments, we concluded that in the highly susceptible A/Sn mice, H2K^K-TEWETGQI⁺ CD8⁺ cells were proliferating at a similar rate in infected or infected-AdASP-2 immunized mice. Nevertheless, H2K^K-TEWETGQI⁺ CD8⁺ cells from infected mice expressed higher levels of the apoptotic receptor CD95 and the earlier apoptotic marker ligand for annexin V.

We subsequently confirmed these observations using C57BL/6 mice. These mice were immunized and/or infected as described in Figure S2 A. As shown in Figure S6 B, although a clear pattern of upregulation of CD95 can be observed in cells from infected

animals (Gr. 2), no or limited regulation were observed in cells from animals that were only immunized with AdASP-2 vaccine (Gr. 3) or from infected-AdASP-2 immunized mice (Gr. 4), respectively. Most important, we found that the frequency of H2K^b-VNHRFTLV⁺ CD8⁺ cells from Gr. 2 (infected) mice that stained for annexin V was higher than those of the other mouse groups (Figure S6 B).

The observations that the specific CD8⁺ T cells showed a higher CD95 expression and a pro-apoptotic phenotype *in vivo* strongly suggest that these cells have a lower survival rate. Initially, we tested their behavior upon CD95 engagement. Upon *in vitro* exposure to α CD95, specific-CD8⁺ cells from infected mice (Gr. 2) survived poorly. We estimated that 88.2% of them died

Figure 5. Frequency, phenotypic characterization and proliferative capacity of specific CD8⁺ T cells. The protocol of the experiment was as described in the legend of Figure 3A. **A)** Nineteen days after the immunization/challenge, we estimated the frequency of splenic H2K^K-TEWETGQI⁺ CD8⁺ cells. FACS charts are for a representative mouse (median) from 3 mice. Numbers represent frequencies of splenic cells. **B)** Splenic cells were stained for CD8, H2K^K-TEWETGQI and CD95 prior to analysis by FACS (blue lines). Control cells were from naive mice (red lines). **C)** The histograms show FACS analysis on CD8⁺ cells (Gr. 1) and H2K^K-TEWETGQI⁺ CD8⁺ cells (Gr. 2, 3, and 4) stained for CD95 and annexin V ligand. Numbers represent frequencies of CD8⁺ cells (Gr.1) or H2K^K-TEWETGQI⁺ CD8⁺ cells (Gr. 2, 3, and 4). **D)** Mice were treated with BrdU for 4 days prior to euthanasia and splenic cells were stained for CD8, H2K^K-TEWETGQI, CD95 and BrdU prior to FACS analysis. The histograms show analysis on CD8⁺ cells (Gr. 1) or H2K^K-TEWETGQI⁺ CD8⁺ cells (Gr. 2, 3, and 4) stained for CD95 and incorporated BrdU. Numbers represent frequencies of CD8⁺ cells (Gr.1) or H2K^K-TEWETGQI⁺ CD8⁺ cells (Gr. 2, 3, and 4). Mean numbers represents MFI for CD95 staining. Representative analyses (medians) are shown for 3 mice per experiment. The experiment was performed two or more times with similar results. doi:10.1371/journal.ppat.1002699.g005

Figure 6. *In vitro* survival capacity of specific CD8⁺ T cells from infected and/or AdASP-2 immunized mice. A) A/Sn mice were immunized i.m. with Adβ-gal or AdASP-2 vaccine (2×10^8 pfu/mouse). On the same day, half of the mice were challenged s.c. with trypomastigotes of the Y strain of *T. cruzi* (150 bloodstream parasites/mouse). Twenty days after the immunization/challenge, splenic cells were cultured in the presence of αCD95 (10 μg/mL) or Medium. After 24 h, we estimated the frequencies of splenic H2K^d-TEWETGQI⁺ CD8⁺ cells. Results are from pools of cells from four mice. The experiment was performed twice with similar results. Mice were immunized i.m. with 2×10^8 pfu of the recombinant adenovirus and challenged s.c. with 10^4 trypomastigotes of *T. cruzi* as described in the Figure S2 A. Twenty one days after adenoviral immunization, splenic cells were immediately stained with anti-CD8 and H2K^d-VNHRFTLV prior to FACS analysis (Day 0). Alternatively, they were labeled with CFSE and cultured in the absence or presence of 10 μM VNHRFTLV (Day 6). After that period, the cells were stained with anti-CD8 and H2K^d-VNHRFTLV prior to FACS analyses. Results are from pools of cells from three mice. These mice were also analyzed individually and the results were identical. The numbers represent the frequency of CD8⁺ cells in each quadrant. The asterisks and crosses denote significantly higher numbers of peptide-specific cells than in the group of mice immunized with Adβ-gal or all other groups, respectively ($P < 0.05$). doi:10.1371/journal.ppat.1002699.g006

(Figure 6A). In contrast, under the same conditions, 73% of the cells from AdASP-2-immunized mice (Gr. 3) survived. In the case of AdASP-2-immunized and infected mice (Gr. 4), we observed that a fraction of the cells (45%) survived the exposure to αCD95. However, considering the high initial frequency of these cells, the total of 0.54% of total splenic cells was still higher than the other groups.

To test the *in vitro* proliferation capacity, we stimulated splenic cells collected from infected C57BL/6 mice with the cognate peptide VNHRFTLV. We observed that after 6 days in culture, specific CD8⁺ cells from infected mice (Gr. 2) were no longer detected (Figure 6B). In contrast, specific CD8⁺ cells from AdASP-2-immunized mice, infected (Gr. 4) or not (Gr. 3) with *T. cruzi*,

were still alive. In the presence of the peptide, they also proliferated. Based on that, we conclude that, *in vitro*, the ability to survive to CD95 engagement or to survive for long periods of time even in the presence of the antigen, was strikingly different between specific CD8⁺ T cells from *T. cruzi* infected or AdASP-2 immunized mice. The possible reason why we found proliferating cells *in vivo*, but not *in vitro*, is that *in vivo*, as the cells die, they were being replenished by new ones which proliferate for few cycles. *In vitro*, as they die, they disappear.

Because experimental *T. cruzi* infection can vary widely according to the combination of mouse and parasite strain used, we attempted to reproduce some of our results using a different experimental model. For that purpose, we immunized BALB/c mice with an adenoviral vaccine expressing the *trans*-sialidase antigen of *T. cruzi* (AdTS), according to the protocol described in Figure S7 A. AdTS contains the immunodominant H-2K^d-restricted epitope, IYNGVQVSI [2,3,5,7]. Immunization with AdTS vaccine significantly reduced the parasitemia caused by parasites of the Brazil strain (Figure S7 B). On day 28 post infection, we again observed a higher frequency of specific cells in mice simultaneously immunized with AdTS vaccine when compared to those in the other mouse groups. The frequency of specific CD8⁺ cells as determined by staining with the multimer H-2K^d-IYNGVQVSI was 3–4 times higher in infected AdTS-immunized mice than in mice that had only been infected or immunized (Figure S7 C). Estimates of frequencies of peptide-specific CD8⁺ cells by ICS assays also detected 6.89- or 18.92-times higher CD107a- and IFN-γ- and/or TNF-α-positive cells in infected AdTS-immunized mice than in infected or immunized mice, respectively (Figure S7 D).

To extend the observations for CD95 expression by specific CD8⁺ T cells, we compared the phenotypes of H2K^d-IYNGVQVSI⁺ CD8⁺ cells from the different mouse groups. A clear pattern of upregulated expression of CD95 was observed in specific CD8⁺ T cells from infected animals (Gr. 2, Figure S8 B). In contrast, cells collected from mice immunized with the AdTS vaccine (Gr. 3) did not modulate CD95 expression. H2K^d-IYNGVQVSI⁺ CD8⁺ cells from mice immunized with the AdTS vaccine before challenge expressed significantly lower levels of CD95 than those of specific CD8⁺ T cells from infected animals (Gr. 2, Figure S8 B). This different pattern was not observed when we estimated the expression of KLRG1 and CD95L on the surface of these same cells (Figure S8 C and D, respectively). The difference is also observed after different days of infection (Figure S8 E). We concluded from these experiments that *T. cruzi* infection with 2 distinct strains of the parasite leads to an upregulation of CD95 expression on parasite-specific CD8⁺ T cells. On the other hand, immunization with distinct adenoviral vaccines does not modulate CD95 expression on transgene-specific CD8⁺ T cells. Finally, priming with the adenoviral vaccines prior to or simultaneously with the challenge partially prevents aberrant CD95 expression on parasite-specific CD8⁺ T cells in these different infection models.

Discussion

In the present study, we investigated the cellular and molecular mechanisms that may account for the suboptimal immunity of specific CD8⁺ T cells generated during infection with *T. cruzi* in mice. Accordingly, our strategy was to compare the suboptimal immune response elicited by parasite infection with the response elicited by a highly effective adenoviral vaccine expressing the parasite's immunodominant epitopes. Our main hypothesis was that CD8⁺ T cells induced by *T. cruzi* infection had one or more

properties not present in cells expanded by adenoviral vaccines, and this caused a suboptimal immune response.

A detailed comparison of the specific CD8⁺ T cells elicited by infection or vaccination revealed that, in general, the cells overlapped in their functional or phenotypic characteristics. Epitope-specific CD8⁺ T cells were highly cytotoxic *in vivo* and secreted IFN- γ , a cytokine that is critical for control of *T. cruzi* multiplication [3,17]. Further, these cells mobilize CD107a to their surface after *in vitro* stimulation with the cognate peptide, indicating that a large proportion of these cells are prepared for granule exocytosis. This observation is supported by cytotoxicity experiments showing that the specific T cells are highly cytotoxic *in vivo*. Analysis of the expression of IFN- γ and TNF- α by ICS also revealed that after experimental infection or immunization with AdASP-2 vaccine, the majority of specific T cells were a population of multifunctional T cells that mobilized surface CD107a and expressed both cytokines. Other cytokines evaluated, such as IL-2 or IL-10, were not detected by ICS (data not shown, [18]).

Similarly to the functional aspects described above, the surface phenotype of the specific CD8⁺ T cells was characteristic of T_{effector} cells. The fact that effector functions of CD8⁺ T cells elicited by *T. cruzi* infection overlap with those elicited by the AdASP-2 vaccine emphasizes that these cell functions may be important for control of the pathogen multiplication and, in the case of the most mouse strains, the acute-phase pathology.

A clear difference we observed between the immune responses elicited by *T. cruzi* infection or AdASP-2 vaccine was in the kinetics of the immune response. The generation of cytotoxicity and IFN- γ -producing cells occurred faster in mice immunized with the AdASP-2 vaccine than in those infected with *T. cruzi* a fact previously described by us and others [2,3,4,24]. The precise reasons for the delay in the immune response after *T. cruzi* challenge are debatable and certainly may be important for the parasite to establish a successful infection. In earlier studies, the timing for the development of the T-cell immune response was critically controlled by the parasite load [2,4,24]. Because parasites contain antigen and, possibly, yet to be identified adjuvant molecules, the effect of the parasite load could be associated with either one individually, or both. Padilla et al. [24] proposed that the delay was not due to the absence of parasite antigen but to the absence of sufficient parasite TLR agonists considered as *T. cruzi* adjuvant molecules. We question this interpretation because, recently, we described that the development of the CD8⁺ T cell immune response during experimental *T. cruzi* infection progressed well in genetically deficient mice that do not express TLR-2, TLR-4, TLR-9, or even the adaptor molecule MyD88 [25]. Further, even when the host immune response was accelerated by the addition of TLR agonists, no changes in the acute-phase pathology were described [24]. Therefore, other intrinsic characteristics of the CD8⁺ T cells may explain the difference in the timing of specific CD8⁺ T-cell activation.

In accord with this possibility, an important observation from our study was that epitope-specific CD8⁺ T cells expanded during infection expressed higher levels of CD95 than naive CD8⁺ cells or epitope-specific cells from mice immunized with adenoviral vaccines. This phenomenon occurred in different mouse strains (A/Sn, C57BL/6 and BALB/c) infected with 2 distinct parasite isolates (Y and Brazil). This aspect is important because in many cases there may be differences when using different parasites isolates in terms of mechanism of immunity activated during infection [26]. This aberrant pattern of upregulated expression was the only one consistently observed when compared to any other surface adhesion/homing/activation receptor that we analyzed. In addition to the specific CD8⁺ T cells expressing

higher levels of CD95, CD95^{hi} cells stained positively for annexin V, a marker for apoptotic cells. Compatible with an increased apoptosis rate, we found that specific CD8⁺ T cells accumulated less *in vivo*. These smaller numbers cannot be explained by lower proliferation rates. *In vivo* incorporation of BrdU indicated similar proliferation rates of cells from infected and infected AdASP-2-vaccinated mice.

CD95 functions during the immune response are multiple and still the subject of intense studies [27–29]. The presence of low levels of CD95L on the surface of antigen-presenting cells (APCs) may serve as a co-stimulatory signal for naive CD95-expressing T cells [30]. On the other hand, higher levels of the ligand provide a negative signal for these same cells by blocking their activation through the CD3 complex [30,31]. The presence of CD95L on the surface of pathogen-infected APCs has been suggested as a possible mechanism to downmodulate the immune response [31]. We could not find evidence of this theory as we could not detect CD95L on the surface of dendritic cells infected with *T. cruzi* (J. Ersching and MMR, unpublished results).

Because CD95 is an important initiator of the intrinsic pathway of apoptosis in T lymphocytes, later it cooperates with Bim in retraction of the T-cell immune response [32–34]. The observation that specific CD8⁺ T cells undergo modulation of CD95 expression after infection was described in specific CD8⁺ T cells following mouse infection with LCMV or in individuals infected with HIV. In the case of LCMV infection, lower levels of CD95 expression on specific CD8⁺ T cells located in the peripheral organs is associated with an increased resistance to activation-induced cell death *in vitro* [35]. In the case of human HIV infection, higher expression of CD95 leads to increased susceptibility to CD95/CD95L-mediated apoptosis and may compromise the immune response of specific CD8⁺ T cells [36].

Upregulation of CD95 expression was also described in splenic T cells during acute infection with certain *T. cruzi* strains [37]. Most relevant biologically was that in this model of infection, *in vivo* injection of antibody to CD95L, which blocks the interaction with CD95, reduced apoptosis, improved type-1 immune responses, and reduced the infection severity as estimated by parasitemia [37]. Similar treatment with anti-CD95L or anti-PD1 during infection of the highly susceptible A/Sn mice with parasites of the Y strain of *T. cruzi* did not reduce the parasitemia or the mortality, indicating that these treatments alone cannot substitute for immunization with AdASP-2 vaccine (AFA and MMR, unpublished results). The reasons for the failure observed with the anti-CD95L treatment are not clear. However the fact that CD95 is expressed in a number of different cell types which play a diverse role during the immune response may account for the difficulty to block solely the interaction of CD95 expressed on the surface of specific-CD8⁺ T cells.

The precise reason(s) why CD95 is differentially modulated after infection with *T. cruzi* or adenoviral vaccination is unknown at present. Herein, we tested whether 2 important mediators of inflammation during *T. cruzi* experimental infection (MyD88, IL-12 and IFN type I) could account for this pattern of CD95 expression on specific CD8⁺ T cells. Our results demonstrated that during infection the CD95 upregulation process was independent of the expression of each one of these mediators individually. Because these molecules are involved directly or indirectly with most cytokines that are largely produced during *T. cruzi* infection, these results may indicate that the signal for CD95 up-regulation may be given by direct interaction between APC, CD8 and CD4.

To date and to our knowledge, generally the cellular and molecular mechanisms modulating CD95 expression on lymphocytes have been poorly explored. Cytokines IL-6 and type I IFN

were described as factors that either downregulate or upregulate CD95 expression, respectively [38,39]. The environment of *T. cruzi* infection is relatively poor in IL-6 but rich in type 1 IFN [40–42]. It is possible that this imbalance favors the expression of CD95. On the other hand, the *in vivo* environment following immunization with human adenovirus (including recombinant type 5) is rich in type 1 IFN and IL-6. This balance may block the upregulation of CD95 [43].

A very recent observation also correlated a defective CD8⁺ T cell-mediated anti-tumor immune response with the aberrant expression of CD95 and PD1 by these cells. This pattern of CD95 expression by CD8⁺ T cells was reproduced by stimulating transgenic CD8⁺ T cells *in vitro* with immature dendritic cells pulsed with the cognate peptide [44]. Whether immature or *T. cruzi*-infected dendritic cells also have the ability to upregulate the CD95 expression of CD8⁺ T cells *in vivo* is an interesting possibility that should be evaluated. Finally, cyclon, a newly identified cytokine-inducible protein produced by T cells upon TCR activation, has been shown to exert a key role in the process of CD95 expression [45]. Whether cyclon expression following TCR activation plays a role in our system also remains to be determined.

The fact that AdASP-2 vaccine fails to upregulate CD95 and changes the program of CD8⁺ T cells expanded after an infectious challenge is unexpected but may be desirable for an efficient immune response. The lower levels of CD95 expressed would render these cells resistant to parasite-induced apoptosis. In fact, we observed that adenoviral-induced CD8⁺ T cells of multiple specificities were not affected by ongoing *T. cruzi* infection (JRV and MMR, unpublished observations). These observations disfavor the hypothesis that cytokines generated during infection can nonspecifically trigger upregulation of the CD95 molecule on activated CD8⁺ T lymphocytes.

Based on our observations, we propose a model depicted in Figure 7. The initial contact of specific CD8⁺ T cells with APC loaded with *T. cruzi* antigen will lead to a molecular program characterized by a higher expression of CD95 and a lower viability (Gr. 2, *T. cruzi* infected mice). The low viability of specific CD8⁺ T cells will preclude optimal immune response. Host pathology is developed that may lead to death or chronic infection.

As oppose, initial contact with APC loaded with AdASP-2 antigens will lead to different molecular program of priming and expansion of specific CD8⁺ T cells (Gr. 3, AdASP-2 immunized mice). Upon a second contact, primed CD95^{Low} specific CD8⁺ T cells will proliferate intensively and display a significantly higher viability (Gr. 4, AdASP-2 immunized and infected mice). This accelerated proliferation coupled to a high viability would lean the equilibrium towards an efficient host immune response and parasite elimination.

The absence of an aberrant CD95 expression not only facilitates the development of a protective immune response by slowing the rate of apoptosis but it also reduces the formation of apoptotic bodies. Apoptotic bodies are described as powerful immune modulators during *T. cruzi* infection. Binding of apoptotic lymphocytes to $\alpha V\beta 3$ expressed by macrophages leads to PGE2 and TGF- β production by macrophages, followed by the induction of ornithine decarboxylase and the synthesis of putrescine, which function as growth factors for intracellular forms of *T. cruzi* [46–48].

The reduction of CD95 expression on the surface of specific CD8⁺ T cells expanded following pathogen challenge may be important not only in our infection model but also in vaccination against other infectious diseases. As mentioned above, during HIV/SIV infection, CD8⁺ T cells upregulate CD95 expression [35,49,50]. Blocking the interaction of CD95/CD95L *in vivo* by

treatment with anti-CD95L antibody preserved memory lymphocytes and cell-mediated immunity in SIV-infected primates [51]. Further, by down-modulating CD95 expression, adenoviral vaccines may reduce the formation of apoptotic bodies. As in the case of *T. cruzi* infection, these apoptotic bodies may fuel viral growth [52].

The possibility that vaccination may selectively alter specific CD8⁺ T cell phenotype and change the expression of surface receptors is only now beginning to be explored. Recently, it was described in humans that during immune responses to melanoma tumors, antigen-specific CD8⁺ T cells maintained high expression levels of the inhibitory receptor B and T lymphocyte attenuator (BTLA). Multiple vaccination doses with peptides representing CD8 epitopes in the presence, but not in the absence, of the TLR-9 agonist CpG ODN led to progressive downregulation of BTLA *in vivo*, increased resistance to BTLA-mediated inhibition, and improved T cell function [53]. This study provides important evidence that vaccination can modify antigen-specific CD8⁺ T cells, thereby qualitatively improving their function. Further, by using this same adjuvant (a TLR-9 agonist), Muraoka *et al.* [44] modified a defective CD8⁺ T cell-mediated anti-tumor immune response to a productive one by counteracting the aberrant expression of CD95 and PD1 on CD8⁺ T cells.

We consider that these results may facilitate understanding of the host–parasite relationship and present some new interpretations of the functions of T-cell vaccines. Recombinant replication-defective human and simian adenoviral vectors have proven successful against a variety of experimental infections, such as SIV, TB, malaria, toxoplasmosis, Marburg virus infection, and Ebola [54–69]. Nonetheless, the precise reason for their success is still unknown. By understanding the potential of these vectors to elicit an immune response and simultaneously to modulate acquired immune responses in the host, it might be possible to design simpler and more effective vaccine formulations.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (<http://www.cobea.org.br/>). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of Sao Paulo (Id # CEP 0426/09).

Mice and parasites

Female 5- to 8-week-old A/Sn, C57BL/6, BALB/c, p40-deficient (IL-12/IL-23 KO, [70]), MyD88- (MyD88 KO, [71]) and Interferon-I receptor (IFN-I rec KO, [72])-deficient mice were purchased from the University of São Paulo. Parasites of the Y or Brazil strain of *T. cruzi* were used in this study [2,3]. Bloodstream trypomastigotes were obtained from mice infected 7 to 28 days earlier with parasites of the Y or Brazil strain, respectively. After estimating the parasite concentration, the blood was diluted in PBS to the desired concentration. Each mouse was inoculated with 150 trypomastigotes (A/Sn), 10⁴ trypomastigotes (C57BL/6), or 10³ trypomastigotes (BALB/c) diluted in 0.2 mL PBS and administrated subcutaneously (s.c.) in the base of the tail.

Monitoring the presence of persisting bloodstream trypomastigotes

Parasite development was monitored by counting the number of bloodstream trypomastigotes in 5 μ L of fresh blood collected from

Figure 7. Proposed model for the differences in activation of specific CD8⁺ T cells. The initial contact of specific CD8⁺ T cells with APC loaded with *T. cruzi* antigen leads to a molecular program characterized by a higher expression of CD95 and a lower viability (Gr. 2). Due to the low viability of these specific CD8⁺ T cells, optimal immune response are not generated and host pathology is developed leading to death or chronic infection. In contrast, contact of APC loaded with AdASP-2 antigens leads to a molecular program of priming and expansion of specific CD8⁺ T cells (Gr. 3). Upon a second contact with *T. cruzi*, primed CD95^{Low} specific CD8⁺ T cells proliferate intensively and display a significantly higher viability (Gr. 4). This accelerated proliferation of highly viable CD8⁺ T cells lean the equilibrium towards an efficient host immune response (cure) and parasite elimination. doi:10.1371/journal.ppat.1002699.g007

the tail vein. Mouse survival was recorded daily. Twenty four months after challenge, the parasitemia was undetectable. To evaluate whether there was still a very small amount of viable circulating parasites, we collected 0.5 mL of the blood and transferred i.p. to naïve highly susceptible A/Sn mice.

Peptide synthesis

Synthetic peptides were purchased from Genscript (Piscataway, New Jersey). Peptide purity was higher than 90%. Peptide identities were confirmed by a Q-TOF Micro equipped with an electrospray ionization source (Micromass, UK). The immunodo-

minant epitopes of ASP-2 and TS were represented by AA VNHRFTLV and AA IYNVGQVSI, respectively. The subdominant epitope TsKb-20 was represented by AA ANYKFTLV (purity 99.7%).

The pentamer H2K^b-VNHRFTLV was purchased from ProImmune Inc. (Oxford, UK). The dextramers H2K^k-TEWETGQI and H2K^d-IYNVGQVSI were purchased from Immunodex (Copenhagen, Denmark).

Adenoviruses used for immunization

Recombinant human type 5 replication-defective adenoviruses expressing *T. cruzi* Amastigote Surface Protein-2 (AdASP-2) or TS (AdTS) were generated, characterized, grown, and purified as previously described [17]. Mice were inoculated intramuscularly (i.m.) in each *tibialis anterior* muscle with 50 µL of viral suspension containing the indicated plaque forming units (pfu). In some experiments mice were inoculated by the s.c. route at the base of the tail. Immunological assays were performed at the indicated days after viral inoculation.

Immunological interventions and readout assays

In vivo depletion of CD8⁺ T cells was performed by treating vaccinated A/Sn mice with 53.6.7 MAb. On days -6, -4, and -2 and before challenge with trypanomastigotes, mice were injected i.p. with a dose of 0.5 mg of anti-CD8 or control rat IgG. Seven days after challenge, each mouse received one more dose of 0.5 mg of anti-CD8 or rat IgG. The efficacy of depletion of CD8⁺ spleen cells before challenge was more than 96% in anti-CD8-treated mice compared to that of the rat IgG-treated ones (data not shown).

For the surface mobilization (expression) of CD107a and the intracellular expression of cytokines (IFN-γ and TNF-α, ICS), splenocytes collected from A/Sn, C57BL/6 or BALB/c mice were treated with ACK buffer (NH₄Cl, 0.15 M; KHCO₃, 10 mM; Na₂-EDTA 0.1 mM; pH = 7.4) for lysing the erythrocytes. Surface mobilization of CD107a and ICS were evaluated after *in vitro* culture of splenocytes in the presence or absence of the antigenic stimulus. Cells were washed 3 times in plain RPMI and resuspended in cell culture medium consisting of RPMI 1640 medium, pH 7.4, supplemented with 10 mM Hepes, 0.2% sodium bicarbonate, 59 mg/L of penicillin, 133 mg/L of streptomycin, and 10% Hyclone fetal bovine sera (Hyclone, Logan, Utah). The viability of the cells was evaluated using 0.2% trypan blue exclusion dye to discriminate between live and dead cells. Cell concentration was adjusted to 5 × 10⁶ cells/mL in cell culture medium containing anti-CD28 (2 µg/mL), brefeldin A (10 µg/mL), monensin (5 µg/mL), and FITC-labeled anti-CD107a (Clone 1D4B, 2 µg/mL; BD Pharmingen). In half of the cultures, a final concentration of 10 µM of the VNHRFTLV, TEWETGQI or IYNVGQVSI peptide were added. The cells were cultivated in flat-bottom 96-well plates (Corning) in a final volume of 200 µL in duplicate, at 37°C in a humid environment containing 5% CO₂. After 12-h incubation, cells were stained for surface markers with PerCP- and PE-labeled anti-CD8, on ice for 20 min. To detect IFN-γ and TNF-α by intracellular staining, cells were then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA, fixed in 4% PBS-paraformaldehyde solution for 10 minutes, and permeabilized for 15 minutes in a PBS, 0.1% BSA, and 0.1% saponin solution. After being washed twice, cells were stained for intracellular markers using APC- and PE-labeled anti-IFN-γ (Clone XMGI.2) and PE-labeled anti-TNF-α (clone MP6-XT22), for 20 minutes on ice. Finally, cells were washed twice and fixed in 1% PBS-paraformaldehyde. At least 300,000 cells

were acquired on a BD FACS Canto flow cytometer and then analyzed with FlowJo.

For the *in vivo* cytotoxicity assays, splenocytes collected from naive BALB/c or C57BL/6 mice were treated with ACK buffer (NH₄Cl, 0.15 M; KHCO₃, 10 mM; Na₂-EDTA 0.1 mM; pH = 7.4) for lysing the erythrocytes. These cells were divided into 2 populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE; Molecular Probes, Eugene, Oregon, USA) at a final concentration of 5 µM (CFSE_{high}) or 0.5 µM (CFSE_{low}). CFSE_{high} cells were coated for 40 min at 37°C with 1 µM of H-2K^b ASP-2 peptide VNHRFTLV. CFSE_{low} cells remained uncoated. Subsequently, CFSE_{high} cells were washed and mixed with equal numbers of CFSE_{low} cells, and 30 to 40 × 10⁶ total cells per mouse were injected intravenously (i.v.). Recipient animals were mice previously immunized with adenoviruses. Spleen cells of recipient mice were collected 4 h or 20 h after transfer as indicated on the legend of the figures, fixed with 1.0% paraformaldehyde and analyzed by flow cytometry by using a FACS Canto flow cytometer (BD, Mountain View, CA). The percentage of specific lysis was determined using the following formula:

$$1 - \left[\frac{(\%CFSE_{high} \text{ immunized} / \%CFSE_{low} \text{ immunized})}{(\%CFSE_{high} \text{ naive} / \%CFSE_{low} \text{ naive})} \right] \times 100\%.$$

For flow cytometry analyses, we used mouse splenocytes treated with ACK buffer. Single-cell suspensions were washed in PBS, stained for 10 min at RT with biotinylated MHC I multimer H-2K^b-VNHRFTLV, and stained for 20 min at 4°C with avidin-APC- and PerCP-labeled anti-CD8 antibodies (both from BD Pharmingen). Alternatively, splenic cells were stained for 10 min at RT with H2K^k-TEWETGQI and H2K^d-IYNVGQVSI dextramers (both APC-labeled) and PerCP-labeled anti-CD8 antibody. For the analyses of other cell-surface markers, single-cell suspensions from spleens of mice were stained with multimers and PerCP-labeled anti-CD8 antibody as described above. Then, FITC-labeled anti-CD11a (clone 2D7), anti-CD11c (clone HL3), anti-CD25 (clone 7D4), anti-CD31 (clone MEC13.3), anti-CD43 (clone S7), anti-CD44 (clone IM7), anti-CD49d (clone R-12), anti-CD62L (clone MEL-14), anti-CD69 (clone H1.2F3), anti-CD95 (clone Jo2), anti-CD122 (clone TM-β1), anti-CD127 (clone SB/199), anti-CD95L (MFL3), anti-BTLA (8F4), anti-PD1 (J43), anti-CTLA4 (UC10-4B9), and anti-CCR-7 (4B12) (all from BD Pharmingen). Anti-CD27 (clone LG.7F9) and anti-KLRG-1 (clone MAFA) were purchased from eBioscience (San Diego, CA). Anti-CD43 (1B11) and anti-CD183 (CXCR3-173) were purchased from Biologend (San Diego, CA). Annexin-V-PE staining was performed with a kit according to the manufacturer's instructions (BD Pharmingen). For detection of BrdU, mice were injected i.p. with 2 mg of BrdU (Sigma) for 4 days before euthanasia. The cells were treated according to the manufacturer's kit instructions and stained with anti-BrdU-APC (BD Pharmingen). At least 100,000 cells were acquired on a BD FACS Canto flow cytometer and then analyzed with FlowJo (Tree Star, Ashland, OR).

In vitro survival assay were performed by culturing the A/Sn splenic cells collected 20 days after inoculation of parasites or adenoviruses for 24 h in the presence or absence of anti-CD95 (clone Jo2, 10 µg/mL). Cell culture medium contained murine recombinant IL-2 (Sigma, 0.1 U/mL) and 10⁶ cells/0.2 mL. After this period, cells were stained with anti-CD8 and dextramers H-2K^k-TEWETGQI before FACS analyses.

Alternatively, C57BL/6 splenic cells were collected from mice at the indicated days post immunization and/or challenge. These

cells were labeled with 10 μ M of CFSE as described above and 10⁶ cells/0.2 mL maintained for 6 days in culture medium containing or not the peptide VNHRFTLV (10 μ M). After this period, cells were stained with anti-CD8 and pentamers H-2K^b-VNHRFTLV before FACS analyses.

Statistical analysis

The values of parasitemia of each individual mouse were log transformed before being compared by one-way ANOVA followed by Tukey Honesty Significance Difference tests, available at the following site: <http://faculty.vassar.edu/lowry/VassarStats.html>. The logrank test was used to compare mouse survival rates after challenge with *T. cruzi*. The differences were considered significant when the *P* value was <0.05.

Supporting Information

Figure S1 Parasitemia and kinetics of specific CD8⁺ T cell-mediated immune responses during infection or vaccination. **A)** Parasitemia of C57BL/6 mice infected s.c. with 10⁴ bloodstream trypomastigotes of *T. cruzi*. **B)** Mice were infected s.c. or not with *T. cruzi* trypomastigotes as described above. In parallel, mice were immunized i.m. with AdASP-2 vaccine (2×10⁸ pfu/mouse). At the indicated days, the *in vivo* cytotoxic activity against target cells coated with peptide VNHRFTLV was determined as described in the Methods Section. The results represent the mean ± SD values for 4 mice per group. The results are representative of 3 independent experiments. **C–F)** C57BL/6 mice were infected or immunized as described above. Control mice were either naive mice or mice immunized with Adβ-gal (2×10⁸ pfu/mouse). At the indicated days after infection or immunization, these mice had their splenic cells cultured in the presence of anti-CD107a and anti-CD28, with or without the peptide VNHRFTLV. After 12 h, cells were stained for CD8, IFN-γ, and TNF-α. Frequencies were estimated for CD8⁺ cells expressing the indicated molecules after stimulation *in vitro* with peptide VNHRFTLV. The results are expressed as the mean ± SD values for 4 mice per group. The values of cultures stimulated with peptide VNHRFTLV were always subtracted from those of cultures with medium alone. Pie charts show the fraction of peptide-specific cells expressing the indicated molecules. The results are expressed as the mean values for 4 mice per group. The asterisks and crosses denote significantly higher numbers of peptide-specific cells than in the group of mice immunized with Adβ-gal or all other groups, respectively (*P*<0.05). (PPT)

Figure S2 Specific CD8⁺ T cell-mediated immune responses of infected, immunized or infected AdASP-2 immunized C57BL/6 mice. **A)** C57BL/6 mice were immunized i.m. with 2×10⁸ pfu/mouse of the indicated recombinant adenovirus. Seven days later, half of the mice were challenged s.c. with 10⁴ trypomastigotes of the Y strain of *T. cruzi*. **B)** Twenty one, days after immunization with recombinant adenovirus, the splenic cells of these mice were cultured in the presence of anti-CD107a and anti-CD28, with or without the peptide VNHRFTLV. After 12 h, cells were stained with anti-CD8, anti-IFN-γ, and anti-TNF-α. The results are expressed as the total frequency of CD8⁺ cells stained for any of the indicated molecules (mean ± SD values for 4 mice per group). The values of cultures stimulated with peptide VNHRFTLV were always subtracted from those of cultures with medium alone. **C)** The same as described above except that the results are expressed as the frequencies of the indicated subpopulation of CD8⁺ cells stained for CD107a, IFN-γ, and TNF-α (mean ± SD values for 4 mice per group). **D)** Pie charts

show the fraction of peptide-specific cells expressing the indicated molecules. The results are expressed as the mean values for 4 mice per group. The asterisks and crosses denote significantly higher numbers of peptide-specific cells than in the group of mice immunized with Adβ-gal or all other groups, respectively (*P*<0.05). (PPT)

Figure S3 Epitope-specific CD8⁺ T cell-mediated immune responses of infected and/or immunized immunized C57BL/6 mice. **A)** C57BL/6 mice were immunized i.m. with 2×10⁸ pfu/mouse of the indicated recombinant adenovirus. Seven days later, half of the mice were challenged s.c. with 10⁴ trypomastigotes of the Y strain of *T. cruzi*. **B)** Twenty seven days after immunization with recombinant adenovirus, we estimated the frequency (%) of splenic H2K^b-VNHRFTLV⁺ CD8⁺ cells. The results represent a pool of cells from 4 mice per group. **C)** Twenty seven days after immunization with recombinant adenovirus, the splenic cells of these mice were cultured in the presence of anti-CD28, with or without the peptide VNHRFTLV or TsKb-20. After 12 h, cells were stained with anti-CD8, anti-IFN-γ, and anti-TNF-α. The results are expressed as the total frequency (%) of CD8⁺ cells stained for any of the indicated molecules (mean ± SD values for 4 mice per group). The asterisks denote significantly higher numbers of peptide-specific cells in Gr. 4 when compared to the same sample of Gr. 2 (*P*<0.05). (PPT)

Figure S4 Phenotypic characterization of specific CD8⁺ T cells induced by *T. cruzi* infection or AdASP-2 immunization. C57BL/6 mice were infected or immunized as described in the legend of Figure 2. Control mice were naive mice. **A)** Twenty-eight or 14 days after infection or immunization, respectively, these mice had their splenic cells stained with anti-CD8, H2K^b-VNHRFTLV, and the indicated marker-specific antibody labeled prior to analysis by FACS. The histograms show the expression of the markers on H2K^b-VNHRFTLV⁺ CD8⁺ T cells (green lines) or control naive CD8⁺ spleen cells (red lines). Representative analyses are shown from pools of cells from 3 mice. Experiments were performed 3 or more times with identical results. **B)** At the indicated days after infection or immunization, splenic cells were stained with anti-CD8, H2K^b-VNHRFTLV, and the indicated marker-specific antibody labeled prior to analysis by FACS. Numbers in red or green represent mean fluorescence intensity. (PPT)

Figure S5 Phenotypic characterization of epitope-specific CD8⁺ T cells induced by infection of genetically deficient C57BL/6 mice with Y strain of *T. cruzi*. C57BL/6 mice (WT, MyD88, IL-12/IL-23 KO or IFN-1 receptor KO) were infected s.c. with 10⁴ blood forms of *T. cruzi* Y strain. Control mice were naive mice of the different strains. Fourteen days after infection, the splenic cells of these mice were stained with anti-CD8, H2K^b-VNHRFTLV, anti-CD95 and anti-CD44 prior to analysis by FACS. The histograms show the expression of the markers: a) H2K^b-VNHRFTLV and CD8 on splenic cells from infected or control naive CD8⁺ spleen cells; b) CD95 and CD44 on H2K^b-VNHRFTLV⁺ (blue lines) or naive CD8⁺ cells (red lines). Numbers in red or blue represent mean fluorescence intensity. Analyses are shown for a representative from 3 mice. (PPT)

Figure S6 Phenotypic characterization of specific CD8⁺ T cells of infected and/or AdASP-2 immunized C57BL/6 mice. **A)** Mice were immunized i.m. with 2×10⁸ pfu of the

indicated recombinant adenovirus and challenged s.c. with 10⁴ trypomastigotes of *T. cruzi*. **B**) Control cells were CD8⁺ T cells from naive mice (red lines). Splenic cells were stained with anti-CD8, H2K^b-VNHRTLV, anti-CD95 and annexin V prior to FACS analysis (blue lines). The histograms show FACS analysis on CD8⁺ cells (Gr. 1) or H2K^b-VNHRTLV⁺ CD8⁺ cells (Gr. 2, 3, and 4) stained for CD95 or incorporated BrdU. Representative analyses are shown from pools of cells of 3 mice per experiment. Analyses of each individual mouse provided the same result. The results for the annexin V ligand are expressed as the percentage of positive cells. The experiment was performed 3 times with similar results. (PPT)

Figure S7 Specific CD8⁺ T cell-mediated immune responses of infected and/or AdTS immunized BALB/c mice. **A**) BALB/c mice were immunized i.m. with of Adβ-gal or AdTS vaccine (2×10⁸ pfu/mouse). One week later, half of the mice were challenged s.c. with trypomastigotes of the Brazil strain of *T. cruzi* (10³ bloodstream parasites/mouse). **B**) Parasitemia (per mL) was estimated at the indicated days following infection of mice that had been immunized with Adβ-gal (Gr. 2) or AdTS vaccine (Gr. 4). The values of parasitemia were significantly lower in mice from Gr. 4 ($P<0.05$, $n=4$). **C**) Twenty eight days after challenge (35 days after adenovirus immunization), we estimated the frequency of splenic H2K^d-IYNVGQVSI⁺ CD8⁺ cells. The results are presented in terms of each mouse (dots) and medians (bars). **D**) On that day, the splenic cells were cultured in the presence of anti-CD107a and anti-CD28, with or without the peptide IYNVGQVSI. After 12 h, cells were stained for CD8, IFN-γ, and TNF-α. The results are presented as mean ± SD frequencies of splenic CD8⁺ cells of 4 mice. The values of cultures stimulated with peptide IYNVGQVSI were subtracted from those of cultures with medium alone. The asterisks and crosses denote significantly higher numbers of peptide-specific cells than in the group of mice immunized with Adβ-gal or all other groups, respectively ($P<0.05$). **E**) Pie charts show the fraction of peptide-specific cells expressing the indicated molecules. The results are expressed as the mean values for 4 mice per group. The results are representative of 2 independent experiments. The asterisks and

crosses denote significantly higher numbers of peptide-specific cells than in the group of mice immunized with Adβ-gal or all other groups, respectively ($P<0.05$). (PPT)

Figure S8 Phenotypic characterization of specific CD8⁺ T cells of infected and/or AdTS immunized BALB/c mice. **A**) Thirty five days after adenovirus immunization, we estimated the frequencies of H2K^d-IYNVGQVSI⁺ CD8⁺ cells. Because some of these mice were challenged 7 days after immunization, this date represents 28 days after challenge with parasites (Gr. 2 and Gr. 4). FACS charts are from a representative mouse (median) from 3 mice. Numbers represent percentage of splenic cells. **B to D**) Splenic cells were stained for CD8, H2K^d-IYNVGQVSI, CD95, KLRG1, and CD95L prior to analysis by FACS. The histograms show the expression of the markers on H2K^d-IYNVGQVSI⁺ CD8⁺ cells (blue lines) or control naive CD8⁺ spleen cells (red lines). Numbers in red or blue represent mean fluorescence intensity. **E**) Kinetics of CD95 expression on H2K^d-IYNVGQVSI⁺ CD8⁺ cells (blue lines) at different days after inoculation of the parasites. Control cells were from naive mice (red lines). Representative samples (median) are shown from 3 mice per experiment. (PPT)

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Author Contributions

Conceived and designed the experiments: JRV AFA MRD JE BCGA KRB GPAM MFL MMR. Performed the experiments: JRV AFA MRD JE BCGA. Analyzed the data: JRV OBR AFA MRD JE BCGA KRB GPAM MFL MMR. Contributed reagents/materials/analysis tools: OBR AFA AVM RTG KRB GPAM. Wrote the paper: MMR MFL KRB JRV BCGA GPAM.

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Re-circulation of lymphocytes mediated by sphingosine-1-phosphate receptor-1 contributes to resistance against experimental infection with the protozoan parasite *Trypanosoma cruzi*

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ABSTRACT

T-cell mediated immune responses are critical for acquired immunity against infection by the intracellular protozoan parasite *Trypanosoma cruzi*. Despite its importance, it is currently unknown where protective T cells are primed and whether they need to re-circulate in order to exert their anti-parasitic effector functions. Here, we show that after subcutaneous challenge, CD11c⁺-dependent specific CD8⁺ T-cell immune response to immunodominant parasite epitopes arises almost simultaneously in the draining lymph node (LN) and the spleen. However, until day 10 after infection, we observed a clear upregulation of activation markers only on the surface of CD11c⁺PDCA1⁺ cells present in the LN and not in the spleen. Therefore, we hypothesized that CD8⁺ T cells re-circulated rapidly from the LN to the spleen. We investigated this phenomenon by administering FTY720 to *T. cruzi*-infected mice to prevent egress of T cells from the LN by interfering specifically with signalling through sphingosine-1-phosphate receptor-1. In *T. cruzi*-infected mice receiving FTY720, CD8 T-cell immune responses were higher in the draining LN and significantly reduced in their spleen. Most importantly, FTY720 increased susceptibility to infection, as indicated by elevated parasitemia and accelerated mortality. Similarly, administration of FTY720 to mice genetically vaccinated with an immunodominant parasite antigen significantly reduced their protective immunity, as observed by the parasitemia and survival of vaccinated mice.

We concluded that re-circulation of lymphocytes mediated by sphingosine-1-phosphate receptor-1 greatly contributes to acquired and vaccine-induced protective immunity against experimental infection with a human protozoan parasite.

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1. Introduction

T cells are important mediators of the adaptive immune response against infections caused by intracellular microorganisms, including the digenetic intracellular protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease (American trypanosomiasis). Genetic deficiency or specific treatments leading to the depletion of CD4⁺ or CD8⁺ T cells critically impairs the acquired immunity observed during experimental mouse infection [1–4]. Although, the anti-parasitic effect exerted by the T cells is

largely mediated by IFN- γ , other mediators may also participate in the efficient elimination of parasites from the host [1–4].

In inbred mouse strains or humans, MHC class II-restricted CD4⁺ T cells recognize multiple antigens from *T. cruzi* [5–9], whereas MHC class Ia-restricted CD8⁺ T cells are primarily specific for immunodominant epitopes that are expressed by surface antigens members of a large family of *T. cruzi* proteins named *trans*-sialidases (TS) [1,4,9–18]. T cells are not only critical for acquired immunity, but they are also important mediators of protective immunity in response to vaccination with recombinant proteins, plasmid DNA, and bacteria- and virus-based vaccine constructs against *T. cruzi* [19–25]. Additionally, as in the case of immunity acquired during infection, IFN- γ is a key mediator of protective immunity [25]. Despite the important role of T-cell mediated immune responses, it is currently unknown where protective T cells are primed and whether they need to re-circulate in order to exert their anti-parasitic effector functions during acquired immune responses.

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With this aim, we first evaluated the kinetics of CD8⁺ T-cell activation in the LN and spleen following a subcutaneous parasite challenge. Although the kinetics of activation in both locations were very similar, we detected the presence of clearly activated CD11c⁺ Plasmacytoid Dendritic Cells 1⁺ (PDCA-1) cells only in the LN. CD11c⁺ PDCA-1⁺ are known for their capacity to secrete large amounts of type I IFN upon activation. But most important for our purposes, very recently, they have been implicated in the priming of CD8⁺ T cells [26]. Based on that, we hypothesized that CD8⁺ T cells were activated at the LNs and re-circulated rapidly to the spleen.

To evaluate this possibility, we administered an immunosuppressive drug, FTY720, to interfere with T-cell signalling via the sphingosine-1-phosphate receptor-1 (S1P1). This receptor is expressed on T cells that respond to S1P1 by emigrating out of the thymus, LN, and bone marrow [27–29]. Following T-cell activation, S1P1 is transiently downmodulated, resulting in prolonged residence of T cells within lymphoid tissues and improved priming efficacy. FTY720 interferes with this process, since upon application, it becomes rapidly phosphorylated to FTY720-P, thus behaving as a strong S1P1 agonist. This results in sustained inhibition of S1P1 signalling, effectively trapping naive and recently activated T cells within the secondary lymphoid. Although FTY720 allows T-cell priming, it efficiently blocks migration of activated T cells from the LNs to the peripheral tissues and thereby precludes peripheral T-cell responses [27–29].

Essentially, we observed that administration of FTY720 after challenge with *T. cruzi* in mice that normally survive acute infection (C57BL/6) or susceptible vaccinated A/Sn mice led to a significant increase in the susceptibility to infection, as indicated by elevated parasitemia and accelerated mortality. Together, these results corroborate the hypothesis that re-circulation of T lymphocytes mediated by S1P1 plays an important role during acquired or vaccine-induced protective immune responses to *T. cruzi* infection.

2. Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (<http://www.cobea.org.br/>). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of Sao Paulo (Id # CEP 0426/09).

2.2. Mice and parasites

Female 8-week-old mice (C57BL/6 and A/Sn) were purchased from CEDEME (Federal University of São Paulo). Transgenic mice expressing the diphtheria toxin receptor (DTR) under control of the CD11c promoter (CD11c-DTR) on a C57BL/6 background were derived as described and were maintained in our colony as heterozygotes [30]. Blood-derived trypomastigotes of the Y strain of *T. cruzi* were obtained from A/Sn mice infected 7–8 days earlier. Each C57BL/6 or A/Sn mouse was challenged sub-cutaneously (s.c.) at the base of the tail with a final dose containing 10⁴–10⁵ or 150 parasites, respectively, in a final volume of 0.1 mL. Parasite development was monitored by counting the number of blood-derived trypomastigotes in 5 µL of fresh blood collected from the tail vein [10].

2.3. Administration of DT or FTY720

Wild type (WT) and CD11c-DTR mice were treated i.p. with 2 doses of 50 ng diphtheria toxin from *Corynebacterium diphtheriae*

(DT, Sigma), 48 h before and on the same day of challenge. In addition, infected WT mice were treated every other day, beginning on the same day of infection, with doses of 20 µg FTY720 (Cayman Chemical, Ann Arbor, MI) per mouse (1 mg/kg) in a final volume of 0.2 mL. The control mice were injected with the diluent only.

2.4. Peptides

Peptides were purchased from Genscript (Piscataway, NJ). Purity was as follows: VNHRFTLV, 97.2% and TsKb-20 (ANYKFTLV), 99.7%.

2.5. Recombinant plasmids and adenoviruses

Plasmid pIgSPCL9 and the human replication-defective adenovirus type 5 containing the *asp-2* gene were described previously [22,24,25,31]. Heterologous prime-boost immunization involved priming i.m. with 100 µg of plasmid DNA followed by a dose of viral suspension containing 2 × 10⁸ plaque-forming units (pfu) of adenovirus 21 days later in the same locations. Immunological assays or challenges were performed 14 days after viral inoculation (boost).

2.6. Phenotypic cell analyses by flow cytometry

The panel of conjugated antibodies used for FACS analyses were CD11c-FITC (clone HL3), CD19-PECy7 (clone 1D3), CD8α-PerCP (clone 53-6.7), CD86-APC (clone GL1), CD80-APC (clone 16-10A1), CD40-APC (clone 3/23) all from BD; PDCA-1-PE (clone JF05-1C2.4.1) from Miltenyi Biotec. Single-cell suspensions from Inguinal lymph nodes or spleen were stained for surface markers on ice for 20 min, and then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA fixed in 4% PBS-paraformaldehyde solution for 10 min. At least 300,000 events were acquired on a BD FACSCanto II flow cytometer and then analyzed with FlowJo (Tree Star, Ashland, OR).

2.7. Sorting of cells by flow cytometry

PDCA-1⁺ cells were isolated from LN collected from C57BL/6 mice infected 5 days earlier s.c. with 10⁴ *T. cruzi* parasites. As controls, we used PDCA-1⁺ cells isolated from LN of naïve C57BL/6 mice (*n* = 15). Inguinal lymph nodes were removed, collagenase-treated, and the single cell suspension was stained with the following antibodies: CD3 Pacific Blue (500A2), IAb FITC (25-9-17), CD11c APCCy7 (HL3) all from BD, and PDCA-1 PE (JF05-1C2.4.1) from Miltenyi Biotec. CD3⁺ IAb⁺ CD11c⁺ PDCA-1⁺ cells were then sorted in a BD FACSaria III cell sorter.

CD8⁺ cells were obtained from C57BL/6 mice (*n* = 2) s.c. infected with 10⁴ *T. cruzi* parasites. Spleens were removed 15 days after infection. Following red blood cell lysis, a single cell suspension was stained with CD8 PE (53-6.7) from BD and positive cells were subjected to sorting in a BD FACSaria III cell sorter. As determined by FACS analysis, the purity of the CD8⁺ was 98%.

2.8. Immunological T-cell assays

Ex vivo ELISPOT (IFN-γ) or *in vivo* cytotoxicity assays were performed exactly as described previously [13,25]. Briefly, the *in vivo* cytotoxicity assays, C57BL/6 splenocytes were divided into two populations and labeled with the fluorogenic dye carboxyfluorescein diacetate succinimidyl diester (CFSE Molecular Probes, Eugene, Oregon, USA) at a final concentration of 10 µM (CFSE^{high}) or 1 µM (CFSE^{low}). CFSE^{high} cells were pulsed for 40 min at 37 °C with 1 µM of the H-2 K^b ASP-2 peptide (VNHRFTLV) or TsKb-20. CFSE^{low} cells remained unpulsed. Subsequently, CFSE^{high} cells were washed and mixed with equal numbers of CFSE^{low} cells before injecting intravenously (i.v.) 30 × 10⁶ total cells per mouse. Recipient animals were mice that had been infected or not with *T. cruzi*. Spleen cells or

Fig. 1. Kinetics of the specific CD8⁺ T-cell-mediated immune responses in mice infected with *T. cruzi*. C57BL/6 mice were challenged s.c. with 10⁴ blood-borne trypomastigotes of the Y strain of *T. cruzi*. At the indicated days, IFN- γ -producing cells were estimated *ex vivo* by using the ELISPOT assay in the presence of peptides VNHRFTLV (Panel A) or TsKb-20 (Panel B). The *in vivo* cytotoxic activities were estimated against target cells coated with peptides VNHRFTLV (Panel C) or TsKb-20 (D). The results are presented as the mean of the values of 4 mice $\pm$ SD per group, except for the *in vivo* cytotoxic activity of LN cells. In that case, we pooled cells from 4 mice for analysis. Asterisks denote the frequency of SFCs in the spleen of infected mice were significantly higher than the LN cells ($P < 0.05$).

lymph node cells of recipient mice were collected 20 h after transfer, fixed with 3.7% paraformaldehyde and analyzed by FACS as described above. The percentage of specific lysis was determined using the formula:

$$1 - \frac{\%CFSE_{high} \text{ infected} / \%CFSE_{low} \text{ infected}}{\%CFSE_{high} \text{ naive} / \%CFSE_{low} \text{ naive}} \times 100\%$$

The surface mobilization of CD107a and the intracellular expression of cytokines (IFN- γ and TNF- α) were evaluated after *in vitro* culture of splenocytes in the presence or absence of an antigenic stimulus. Cells were washed 3 times in plain RPMI and resuspended in cell culture medium containing RPMI 1640 medium (pH 7.4), supplemented with 10 mM Hepes, 0.2% sodium bicarbonate, 59 mg/L penicillin, 133 mg/L streptomycin, and 10% Hyclone fetal bovine sera (Hyclone, Logan, Utah). The viability of cells was evaluated using 0.2% Trypan Blue exclusion dye to discriminate between live and dead cells. The cell concentration was adjusted to 5×10^6 cells/mL in a cell culture medium containing anti-CD28 (2 μ g/mL, BD Pharmingen), brefeldin A (10 μ g/mL, BD Pharmingen), monensin (5 μ g/mL, Sigma, St. Louis, MO), and FITC-labeled anti-CD107a (Clone 1D4B, 2 μ g/mL, BD Pharmingen). In half of the cultures, VNHRFTLV peptide was added at a final concentration of 10 μ M. Cells were cultivated in V-bottom 96-well plates (Corning)

in a final volume of 200 μ L in duplicates, at 37 $^{\circ}$ C in a humid environment containing 5% CO₂. After no more than 12 h incubation, cells were harvested and stained for surface markers with Per-CP or PE-labeled anti-CD8 on ice for 20 min. To detect IFN- γ , or TNF- α by intracellular staining (ICS), cells were then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA and then fixed and permeabilized for 20 min on ice with 100 μ L Cytofix/Cytoperm (BD Pharmingen). After washing twice with 250 μ L permwash buffer (BD Pharmingen), the cells were stained to detect intracellular markers using APC or PE-labeled anti-IFN- γ (clone XMG1.2) and PE-labeled anti-TNF- α (clone MP6-XT22). Finally, cells were washed twice and fixed in 1% PBS-paraformaldehyde. At least 300,000 events were acquired on a BD FACSCanto II flow cytometer and then analyzed with FlowJo (Tree Star, Ashland, OR).

2.9. Statistical analysis

Values are expressed as means $\pm$ SD. These values were compared using One-way ANOVA followed by Tukey's HSD tests (<http://faculty.vassar.edu/lowry/VassarStats.html>). The Logrank test was used to compare mouse survival rates after challenge with *T. cruzi* (<http://bioinf.wehi.edu.au/software/russell/logrank/>). The differences were considered significant when the P value was < 0.05 .

Fig. 2. CD11c⁺ cells are required for the CD8 T-cell immune responses of mice infected with *T. cruzi*.

WT or CD11c-DTR mice were infected s.c. with 10^4 *T. cruzi* blood parasites. 48 h before and on the day of challenge, WT and CD11c-DTR mice were treated i.p. with 50 ng of DT. One week later, IFN- γ -producing spleen cells were estimated *ex vivo* by ELISPOT in the presence of peptides VNHRFTLV or TsKb-20. Results are expressed as means $\pm$ SD of 4 mice per group and are representative of experiments performed at twice with similar results. Asterisks denote that the number of spots forming cells (SFC) from infected WT mice were significantly higher when compared to naïve or infected CD11c-DTR mice ($P < 0.01$, one-way ANOVA).

3. Results

During experimental infection of H-2^b inbred mouse strains with parasites of the Y strain of *T. cruzi*, epitopes VNHRFTLV and TsKb-20 (ANYKFTLV) are recognized by H-2K^b-restricted CD8⁺ cytotoxic T cells. In previous studies we have described that the first is the immunodominant epitope leading to a higher immune response and the second a sub-dominant epitope [10,12,13]. After s.c. challenge with infective trypomastigote forms of the parasite, detailed analyses of the kinetics of peptide-specific immune responses were determined *ex vivo* by ELISPOT and *in vivo* by cytotoxicity assays. At the indicated time points, spleen or LN cells were incubated *in vitro* with medium (control) or peptides (VNHRFTLV or TsKb-20). The number of peptide-specific IFN- γ secreting cells was determined by ELISPOT assay (13). Alternatively, at the indicated time points, target cells were labeled with CFSE and coated with peptides VNHRFTLV or TsKb-20 as described in Section 2. These cells were transferred to infected or naïve mice. Twenty hours later, spleen or LN cells were collected and the *in vivo* cytotoxicity estimated.

The results showed that the effector peptide-specific immune cells developed at a similar rate in both the draining LN and the spleen (Fig. 1A–D). The main transition occurred from days 4 to 12 in both organs, for both peptides.

To determine the role of CD11c⁺ cells during the expansion/maturation phase of the adaptive immune response, we used transgenic mice expressing the DTR under control of the CD11c promoter. When infected mice were subjected to diphtheria toxin (DT), the peptide-specific immune response in their spleen 12 days after infection was severely compromised, as measured using the ELISPOT assay (Fig. 2). These results strongly suggest that CD11c⁺ cells are important for priming of peptide-specific cells following *T. cruzi* infection.

To further evaluate the subpopulation of CD11c⁺ cells that could potentially be involved in the priming of the peptide-specific T

cells, we stained LN and spleen cells with antibodies to CD11c and PDCA-1 and the activation markers CD40, CD40L, CD80, and CD86 at different times after challenge. As depicted in Fig. 3A, a clear upregulated pattern of expression of CD40, CD80 and CD86, but not CD40L, can be seen on the surface of CD11c⁺PDCA-1⁺ cells obtained from the LN. In contrast, we detect only the upregulation of CD40 on CD11c⁺PDCA-1⁺ splenocytes at day 10 after infection (Fig. 3B).

In addition, we also stained LN and spleen cells for CD11c expression in conjunction with CD8 α in addition to the activation markers CD40, CD40L, and CD86 at different times after infection. A limited pattern of upregulation of expression of CD86 can be seen on the surface of CD11c⁺CD8 α ⁺ cells collected from the LN or spleen on days 3–7 following infection (Fig. 4A and B).

Similar analyses were also conducted for CD11c⁺CD8 α ⁺ cells collected from the spleen and LN, but we did not detect an upregulation of expression of the activation markers CD40, CD40L, CD80, or CD86 at any time point from 3 to 30 days in the spleen or LN (data not shown).

To determine whether indeed CD11c⁺PDCA-1⁺ cells could present antigen for specific CD8 lymphocytes, we purified CD11c⁺PDCA-1⁺. After sorting the cells from naïve or 5-day infected LN cells, we obtained cells that were 95.3 and 83% pure as determined by the PDCA-1 marker (Fig. 5A and B, respectively). For some unknown reason, during the purification process, some cells become negative for the marker for CD11c marker but still retained the PDCA-1 marker. The PDCA-1⁺ cells obtained from mice that were infected expressed significantly higher amounts of MHC-II-IA^b and CD80 (Fig. 5C and D, respectively). PDCA-1⁺ cells were used to stimulate purified CD8⁺ splenic cells obtained from *T. cruzi* infected mice. As shown in Fig. 5E, IFN- γ producing cells were detected only when CD8⁺ were incubated with PDCA-1⁺ cells obtained from infected mice.

The fact that CD11c⁺ cells from the spleen exhibit a limited activation phenotype suggested that perhaps most of the specific T cells found in the spleen might not be primed there. If this assumption is correct, the re-circulation of T cells could account for the CD8⁺ T-cell mediated functions detected in this organ. To test whether lymphocyte re-circulation was responsible for the immune response observed in the spleen, we treated infected mice with FTY720. This immunosuppressive drug inhibits S1P1 signalling, thus efficiently blocking re-circulation of naïve and activated T cells from the LNs into peripheral tissues, thereby preventing development peripheral T-cell responses [27–29].

Mice were infected with *T. cruzi* parasites and FTY720 or diluent were administered on the same day of challenge and every 2 days thereafter as described in Section 2. Two weeks later, the frequency of IFN- γ -producing cells were estimated *ex vivo* by using ELISPOT assay in the presence of peptides VNHRFTLV or TsKb-20. Alternatively, splenocytes were cultured in the presence or absence of peptides VNHRFTLV or TsKb-20 and the expression of surface CD107a, IFN- γ and TNF- α by ICS.

In infected mice, administration of FTY720 resulted in 2.52- or 3.05-fold increases in the frequency of IFN- γ -secreting cells from the LNs specific for VNHRFTLV or TsKb-20, respectively, as detected using the ELISPOT assay (Fig. 6). In contrast, this increase in the frequency IFN- γ -secreting peptide-specific cells in the LN was accompanied by a significant decrease of immune responses of splenic lymphocytes. Immune responses were initially determined by the frequency of IFN- γ -producing cells as measured by the ELISPOT assay (Fig. 7A). The frequency of IFN- γ -producing cells found in the spleen after FTY720 administration was reduced by 74.55% or 100% upon stimulation with peptides VNHRFTLV or TsKb-20, respectively (Fig. 7A).

Subsequently, we estimated the immune response by the detection of peptide-specific CD8⁺ cells that mobilized CD107a to their surface and expressed IFN- γ and TNF- α upon exposure to the

Fig. 3. Expression of activation markers on CD11c⁺ and PDCA-1⁺ DCs from the LNs or spleens from infected mice. WT C57BL/6 mice were infected s.c. with 10^4 *T. cruzi* blood parasites. At the indicated days after infection, LN (panel A) or splenic (Panel B) cells were collected and co-stained for the expression of CD11c, PDCA-1, and the indicated marker. The expression of the different activation markers were compared between cells collected from infected (blue lines) and naïve (red lines) mice.

peptides *in vitro*. The frequency of CD8⁺ cells that were CD107a⁺, IFN- γ ⁺ or TNF- α ⁺ was reduced by 74.61% or 84.15% after stimulation with VNHRFTLV or TsKb-20, respectively (Fig. 7B). The reduction substantially affected all the different subpopulations of CD8⁺ cells (Fig. 7C). The proportions of each population did not change significantly in the cells collected from infected mice that were administered or not with FTY720 (Fig. 7D).

To evaluate the influence of restricting T-cell re-circulation on the outcome of infection, we also monitored the parasitemia levels and survival of mice that were and were not subjected to FTY720 over the course of infection. We found that drug exposure resulted in increased parasitemia and accelerated mortality of infected mice (Fig. 8A and B, respectively). Therefore, we concluded that lymphocyte re-circulation is indeed important for the acquired protective immune response in this mouse model of acute infection.

We then sought to test the same hypothesis by applying a distinctly different approach. In this case, we used highly susceptible A/Sn mice that were genetically vaccinated by priming with

plasmid pIgSPCL9 followed by a booster immunization with AdASP-2. We previously showed that this heterologous prime-boost regimen reproducibly conferred protective immunity against a lethal challenge with *T. cruzi* [25]. Immunity was mediated by CD8⁺ T cells as depletion of these T cells renders these mice completely susceptible to infection. These CD8⁺ T cells are specific for the ASP-2 H-K^k restricted epitopes TEWETGQI, PETLGHEI or YEIVAGYI [31]. Prior to challenge, these mice exhibit a strong immune response to all three epitopes [31].

Following infection (s.c.), some of these vaccinated mice were subjected to FTY720. We then monitored the parasitemia levels and survival. We observed that drug exposure caused an increase in the parasitemia levels and accelerated the mortality of vaccinated mice (Fig. 9A and B, respectively). This observation indicates that vaccinated mice still require lymphocyte re-circulation to mount an effective immune response on subsequent challenge. This finding further corroborated our initial conclusions regarding the importance of re-circulation activity, even for the vaccine-supported

Fig. 4. Expression of activation markers on CD11c⁺ and CD8- α ⁺ DCs from the LNs or spleens of infected mice. WT C57BL/6 mice were infected s.c. with 10^4 *T. cruzi* blood parasites. At the indicated days after infection, LN (panel A) or splenic (Panel B) cells were collected and co-stained for the expression of CD11c, CD8- α , and the indicated marker. The expression of the different activation markers were compared on cells collected from infected (blue lines) and naïve (red lines) mice.

protective immune response, as seen in this second mouse model of acute infection.

4. Discussion

The CD8⁺ T-cell immune response elicited by *T. cruzi* infection in most inbred mouse strains can control multiplication of this intracellular pathogen and preclude acute-phase pathologies such as death [1,10–17]. The time at which acquired immunity develops is highly dependent on the parasite load [12,32]. In our model, with the Y strain of *T. cruzi*, we observed that the CD8⁺ T-cell immune response is only triggered at the time of the peak parasitemia [10,12]. Because the number of circulating parasites at this time is high, antigen presentation could occur in the draining LN or the spleen. However, the results of our experiments that involved the use of the immunosuppressive drug FTY720, in combination with the identification of activated CD11c⁺ cells, found mostly in the LN, clearly demonstrated that the LNs draining the parasite entrance are where the specific CD8⁺ T cells are primed. Then, they exit the LN and reach the spleen. Our results are similar to those of experimental vaccination studies with radiation-attenuated malaria parasites

[33]. In this case, the CD8⁺ T-cell response originates in the LN draining site at the site of parasite entrance in the skin, and then these cells migrate to other peripheral organs. Similar to our results, exposure to FTY720 led to accumulation of specific T cells in the draining LN and a ~85% reduction of the specific CD8⁺ T cells in the spleen [33]. Together, these results provide compelling evidence that the priming of CD8⁺ T cells can take place in the local lymphoid tissue during protozoan infection/vaccination and that a rapid re-circulation to the spleen is likely to occur. As in our case, the authors conclude that this rapid re-circulation during infection was critical for protective immunity mediated by malaria-specific CD8⁺ T cells [33].

Both studies used parasites that infect mice (*T. cruzi* or *Plasmodium yoelii*). Nevertheless, it is important to highlight that only *T. cruzi* infects humans. Also, the studies of malaria used radiation-attenuated parasites as vaccine because they do not cause infection. Therefore, it is unknown whether the same occurs during acquired immunity to experimental infection as in our case. These observations with *T. cruzi* and malaria parasites stand in contrast to other pathogens. Several studies describe re-circulation mediated by S1P1 is not critical for the protective immune responses to certain viral, bacterial and protozoan

Fig. 5. PDCA-1⁺ cells purified from infected mice present antigen to specific CD8⁺ cells.

PDCA-1⁺ cells were sorted from LN cells from mice infected 5 days earlier s.c. with *T. cruzi* parasites as described in Section 2. As controls, we used PDCA-1⁺ cells isolated from LN of naïve mice.

(A and B) Sorted cells from naïve or infected mice were stained with antibodies to CD11c and PDCA-1. Numbers represent the frequency of cells in each gate.

(C and D) PDCA-1⁺ cells were stained with antibodies to IA^b or CD80.

(E) Purified PDCA-1⁺ cells from naïve or infected mice were used in quadruplicate cultures to stimulate specific CD8⁺ cells. Asterisk denotes the that number of SFCs of CD8⁺ cells stimulated with PDCA-1⁺ cells purified from infected mice were significantly higher the one stimulated with PDCA-1⁺ cells from naïve animals ($P < 0.01$).

infections [34–36]. The precise reasons for these divergent responses are not clear but probably reflect differences in the priming sites as well as, the immunopathologies caused by the different infectious agents.

In addition to the role of S1P1-dependent circulation during protective immunity acquired during *T. cruzi* infection, we also

Fig. 6. Administration of FTY720 increases the number of peptide-specific cells in the LN of mice infected with *T. cruzi*.

C57BL/6 mice were infected s.c. with 10^5 *T. cruzi* blood-borne parasites. Mice were administered on the same day of challenge and every 2 days thereafter 20 μ g of FTY720 or diluent i.p. Two weeks later, the frequency of IFN- γ -producing cells were estimated *ex vivo* by using ELISPOT assay in the presence of peptides VNHRFTLV or TsKb-20. The results are presented as means $\pm$ SD of 4 mice per group and are representative of experiments performed at least twice with similar results. Asterisks denote the number of SFCs from infected mice given FTY720 were significantly higher than infected mice treated with the diluent only ($P < 0.01$).

observed that previously vaccinated mice became more susceptible to infection when subjected to FTY720 exposure. For vaccination, we used a heterologous prime-boost regimen consisting of an initial immunization with plasmid DNA and a booster immunization with a replication-defective recombinant human adenovirus type 5 (HuAd5), both encoding the *asp-2* gene. Immunity elicited by this vaccination protocol is long lived and mediated by Th1 CD4⁺ as well as CD8⁺ Tc1 cells [25,31,37].

The heterologous prime-boosting regimen of vaccination using plasmid DNA and replication-defective recombinant HuAd5 provides protective immunity in some other important pre-clinical experimental models such as SIV, malaria, Ebola, and Marburg viruses [38–45]. Based on these pre-clinical experimental models, human trials have been initiated [46–49]. Our observation that S1P1 is important for protective activity of T cells in previously vaccinated animals is completely new and should be studied further in these experimental models.

Although we measured only CD8 T-cell mediated immune responses only, it is highly possible that the same pattern would happen to specific CD4⁺ T cells. This T-cell sub-population is very important for protective immunity during to *T. cruzi* infection [25]. The absence of re-circulation of both types of lymphocytes probably account for the sub-optimal protective immunity observed after administration of FTY720. Possibly, both cells promote the processes required for parasite elimination on the tissue.

The fact that FTY720 interfere with S1P1 activation makes it theoretically capable of act on other cells types that express this receptor. However, the effect on other cell types is poorly known at present. It has been previously described that FTY720 administration may increase or reduce the activity of regulatory T cells [50,51]. A recent study indicated that this drug act on astrocytes S1P1 to reduce experimental allergic encephalomyelitis clinical scores [52]. Whether these or other cell types play a role in our system is currently unknown.

Fig. 7. Administration of FTY720 reduces the number of peptide-specific cells in the spleen of mice infected with *T. cruzi*

C57BL/6 mice were infected s.c. with 10^4 *T. cruzi* blood-borne parasites. Mice were administered on the same day of challenge and every 2 days thereafter 20 μ g of FTY720 or diluent i.p. Two weeks later, the frequency of IFN- γ -producing cells were estimated *ex vivo* by using ELISPOT assay in the presence of peptides VNHRFTLV or TsKb-20 (Panels A and B).

Alternatively, splenocytes from these mice were cultured in the presence of anti-CD107a and anti-CD28, with or without the peptides VNHRFTLV or TsKb-20. After 12 h, the cells were stained to detect CD8, IFN- γ , and TNF- α (Panels C and D). The results are presented as means $\pm$ SD of 4 mice per group and are representative of experiments performed at least twice with similar results. Asterisks denote that the number of SFCs from infected mice receiving FTY720 were significantly lower than the infected mice treated with the diluent alone ($P < 0.01$).

A current limitation of this experimental model for *T. cruzi* infection is the lack of information on where CD8⁺ T cells encounter and eliminate parasite-infected cells; this is an aspect that may be critical to fully understand immune responses. Considering

Fig. 8. Administration of FTY720 increases susceptibility to *T. cruzi* infection. C57BL/6 mice were infected s.c. with 10^5 *T. cruzi* blood parasites. Mice were treated on the same day of challenge and every 2 days thereafter with 20 μ g of FTY720 i.p. or diluent only. (A) Parasitemia levels for each mouse group is presented as the mean $\pm$ SD ($n = 6$). Asterisks denote that mice from groups injected with diluent only exhibited significantly lower parasitemia ($P < 0.01$) than animals receiving FTY720. (B) Kaplan–Meier survival curves for the mouse groups treated as described above ($n = 6$). Mice from groups injected with diluent only survived longer than animals receiving FTY720 ($P < 0.01$, Logrank test). No animals died after the 30th day until the termination of the experiment. The results are representative of 2 independent experiments.

that *T. cruzi* can infect many cell types and cause systemic infection, it is plausible that many tissues may serve as sites of infection and for parasite/T-cell encounters. Supporting this hypothesis, we observed that vaccinated animals were resistant to *T. cruzi* challenge by different routes of infection (i.p. and s.c. [25,37]).

The finding that the administration of FTY720 significantly reduces protective immunity against *T. cruzi* infection and impairs the protective immunity afforded by vaccination may also have clinical implications for the use of this immunosuppressive drug. Certainly, its use in regions where Chagas disease is endemic should be done with caution considering the potential increase in susceptibility of treated individuals. Finally, treatment of organ-transplanted patients with FTY720 may interfere with immunity elicited by previous vaccination.

In conclusion, our study provides useful information on the importance of S1P1 for resistance against experimental infection with human protozoan parasites.

Fig. 9. Administration of FTY720 increases susceptibility to *T. cruzi* infection in previously vaccinated A/Sn mice. A/Sn mice were primed i.m. with 100 µg of plasmids, pcDNA3 or plgSPCL.9. Three weeks later, these mice were boosted i.m. with 2×10^8 pfu Adβ-gal or AdASP-2. All mice were challenged s.c. with 150 bloodstream trypomastigotes. Half of the vaccinated mice received 20 µg of FTY720 every other day, and the other half received diluent only. (A) Parasitemia levels for each mouse group is presented as the mean $\pm$ SD ($n=5$). Asterisks denote that mice from groups immunized with plgSPCL.9/AdASP-2 and who received diluent only had significantly lower parasitemia than other mouse groups ($P<0.01$). (B) Kaplan–Meier survival curves for the mouse groups immunized and challenged as described above ($n=10$). Mice immunized with plgSPCL.9/AdASP-2 and receiving diluent only survived significantly longer than those immunized with plgSPCL.9/AdASP-2 and administered FTY720 ($P<0.01$). The results shown are combined from 2 experiments. No animals died after the 50th day of challenge.

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Relevance of long-lived CD8⁺ T effector memory cells for protective immunity elicited by heterologous prime-boost vaccination

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Owing to the importance of major histocompatibility complex class Ia-restricted CD8⁺ T cells for host survival following viral, bacterial, fungal, or parasitic infection, it has become largely accepted that these cells should be considered in the design of a new generation of vaccines. For the past 20 years, solid evidence has been provided that the heterologous prime-boost regimen achieves the best results in terms of induction of long-lived protective CD8⁺ T cells against a variety of experimental infections. Although this regimen has often been used experimentally, as is the case for many vaccines, the mechanism behind the efficacy of this vaccination regimen is still largely unknown. The main purpose of this review is to examine the characteristics of the protective CD8⁺ T cells generated by this vaccination regimen. Part of its efficacy certainly relies on the generation and maintenance of large numbers of specific lymphocytes. Other specific characteristics may also be important, and studies on this direction have only recently been initiated. So far, the characterization of these protective, long-lived T cell populations suggests that there is a high frequency of polyfunctional T cells; these cells cover a large breadth and display a T effector memory (TEM) phenotype. These TEM cells are capable of proliferating after an infectious challenge and are highly refractory to apoptosis due to a control of the expression of pro-apoptotic receptors such as CD95. Also, they do not undergo significant long-term immunological erosion. Understanding the mechanisms that control the generation and maintenance of the protective activity of these long-lived TEM cells will certainly provide important insights into the physiology of CD8⁺ T cells and pave the way for the design of new or improved vaccines.

Keywords: memory, vaccines, CD8, adenovirus

GENETIC VACCINATION USING THE HETEROLOGOUS PRIME-BOOST REGIMEN

Genetic vaccination using naked DNA or recombinant viruses is being pursued as an alternative to traditional vaccines. This strategy could be particularly important in the case of intracellular pathogens and neoplastic cells, where the effectiveness relies heavily on the capacity of the vaccine to elicit specific immune responses mediated by cytotoxic CD8⁺ T cells (reviewed in Rice et al., 2008; Lasaro and Ertl, 2009; Bassett et al., 2011; Gómez et al., 2011; Mudd and Watkins, 2011; Saade and Petrovsky, 2012).

Whereas the use of single-vector delivery for priming and boosting is usually the initial option, one of the most prolific areas of genetic vaccine development is the strategy known as the heterologous prime-boost regimen. This strategy uses two different vectors, each carrying a gene that encodes the same antigenic protein for priming and boosting immunizations. The utility and importance of this strategy was first proposed in the early 1990s using a combination of recombinant viruses (influenza and vaccinia) to induce protective immunity against

malaria (Li et al., 1993; Rodrigues et al., 1994). Subsequently, this approach was extended and simplified by incorporating naked DNA for priming followed by a booster injection of a recombinant poxviral vector (i.e., modified vaccinia Ankara, MVA); this was also used to generate sterile protective immunity against rodent malaria (Schneider et al., 1998; Sedegah et al., 1998). Collectively, these studies demonstrated that the heterologous prime-boost regimen generated significantly higher immune responses and proved more effective than the use of any of these genetic vectors individually. The initial use of rodent malaria parasites as a model system may have delayed the development of the field, because malaria is not a conventional model system for developing antiviral or antibacterial vaccines. Nevertheless, this fortuitous fact established the important concept that the difficulty in generating immunity to malaria, and perhaps to other intracellular parasites, in many ways recapitulates the struggles encountered to elicit protective immunity against viruses and bacteria that cause chronic infections such as HIV and tuberculosis (TB).

In subsequent years, the heterologous prime-boost vaccination regimen was adopted worldwide as a powerful means to elicit strong type 1 effector CD8⁺ T cell-mediated immune responses (Tc1) against viral, parasitic, and neoplastic antigens in rodents and non-human primates (NHP; Irvine et al., 1997; Amara et al., 2001; McShane et al., 2001; Zavala et al., 2001; Moore and Hill, 2004; Ellenberger et al., 2006; Gilbert et al., 2006; Aidoo et al., 2007; Nigam et al., 2007; Martinon et al., 2008; Sadagopal et al., 2008). Based on pre-clinical studies, a number of human clinical trials have also been initiated. However, to our knowledge, heterologous prime-boost regimens using plasmid DNA and recombinant poxviruses have not yet provided meaningful protective immunity in humans (McConkey et al., 2003; Moorthy et al., 2004; Keating et al., 2005; Vuola et al., 2005; Cebere et al., 2006; Dunachie et al., 2006; Goonetilleke et al., 2006). The precise reason for such failures is not yet clear. It may be due to the target antigens chosen or to the possibility that the combination of vectors may elicit a type of effector CD8⁺ T cells in humans that are not functionally and/or phenotypically related to mice, as discussed below.

A number of possible vector combinations that significantly improved cell-mediated immunity, particularly the generation of specific CD8⁺ T cells, have been described in parallel. Among them, heterologous prime-boost vaccination using naked plasmid DNA for priming followed by a booster injection of recombinant replication-deficient human adenovirus 5 (AdHu5) has recently received significant attention. This strategy has proved successful in some relevant experimental models such as simian immunodeficiency virus (SIV), malaria, Marburg, and Ebola virus infection, and Chagas disease (American trypanosomiasis), providing a considerable degree of protective immunity (Gilbert et al., 2002; Casimiro et al., 2003, 2005; Santra et al., 2005; Acierno et al., 2006; Letvin et al., 2006; Mattapallil et al., 2006; Sun et al., 2006; Wilson et al., 2006; de Alencar et al., 2009; Geisbert et al., 2010; Hensley et al., 2010; Martins et al., 2010; Dominguez et al., 2011; Rigato et al., 2011). These relative successes obtained in pre-clinical experimental models fueled human phase I trials (Freel et al., 2010; Jaoko et al., 2010; Koup et al., 2010; Schooley et al., 2010; Churchyard et al., 2011; De Rosa et al., 2011).

Very recently, improved vector combinations have yielded results (measured in terms of protective immunity) that are slightly better than the results obtained by using plasmid DNA followed by replication-deficient AdHu5 viruses. These new strategies include (i) prime with plasmid DNA in the presence of cytokine genes such as IL-12 or GM-CSF (Lai et al., 2011; Winstone et al., 2011), (ii) genes encoding multimeric proteins (Lakhashe et al., 2011), (iii) boost of adenovirus-immunized animals with an optimized plasmid DNA (Hutnick et al., 2012), (iv) prime with rhesus cytomegalovirus (Hansen et al., 2011), and (v) prime with a different heterologous strain of adenovirus (Barouch et al., 2012).

The precise reason for the superior performance of the heterologous prime-boost vaccination compared to the sequential use of the same vector is still a matter of controversy. Some evidence indicates the possibility that the intense immunity to epitopes present on the priming vector prevents the boosting effect. For example, a recent study in humans shows that a second dose of a recombinant AdHu5 does not provide significant boosting.

In parallel, recombinant AdHu5 boosting of DNA-primed individuals resulted in significantly higher immune responses (De Rosa et al., 2011). The anti-vector immunity can be either antibody mediated or independent (Cockburn et al., 2008; Schirmbeck et al., 2008; Frahm et al., 2012). Haut et al. (2011) found that in B cell-deficient mice transgene-specific CD8⁺ T cell responses were significantly higher in systemic compartments. In contrast, recent studies in humans showed that neutralizing antibodies titers to AdHu5 did not correlate with the magnitude of specific CD8⁺ T cell of priming after immunization with a recombinant AdHu5. In these experiments, the frequency of specific CD4⁺ T cells negatively correlated with the intensity of specific CD8⁺ T cells priming (Frahm et al., 2012).

In spite of the clear evidences that pre-existing immunity may interfere with the use of viral vectors, still, the heterologous prime-boost regimen of immunization is described as a possible solution to this problem. This can be achieved by strong priming with cytokine genes (for example, see Barouch et al., 2003).

Independent of the reasons why the heterologous prime-boost vaccination regimen is superior to the sequential use of the same vector, the main purpose of this review is to examine the characteristics of the protective CD8⁺ T cells generated by this vaccination regimen.

CHARACTERISTICS OF PROTECTIVE CD8⁺ T CELLS ELICITED AFTER HETEROLOGOUS PRIME-BOOST VACCINATION

HIGH FREQUENCIES OF SPECIFIC CD8⁺ T CELLS

One hallmark of the heterologous prime-boost regimen is the elicitation of a higher frequency of epitope-specific CD8⁺ T cells across multiple models. This high number of effector T cells was initially estimated by the presence of epitope-specific IFN- γ -producing cells using the *ex vivo* ELISPOT assay (Murata et al., 1996; Schneider et al., 1998; Sedegah et al., 1998; Bruña-Romero et al., 2001). Subsequently, the hypothesis was further validated by intracellular staining for IFN- γ (Pinto et al., 2003) and tetramer staining of epitope-specific CD8⁺ T cells (Tao et al., 2005). More recently, intracellular staining for TNF, IL-2, MIP1- β , T cell surface mobilization of CD107a, and *in vivo* cytotoxicity provided extended evidence (for examples, see Masopust et al., 2006; Mattapallil et al., 2006; Cox et al., 2008; de Alencar et al., 2009; Freel et al., 2010; Reyes-Sandoval et al., 2010; Rigato et al., 2011).

Because most studies are performed with T cells collected from the spleen or peripheral blood lymphocytes (PBL) of mice or NHP, respectively, it is not clear whether these results reflect an overall increase in every tissue. The presence of a large number of epitope-specific T cells in several tissues has been documented in the case of mouse lung, liver, intraepithelial lymphocytes, and PBL (Masopust et al., 2006; Reyes-Sandoval et al., 2011); however, because parallel comparison was not performed with animals that were immunized with a single vector, it is not clear whether these levels were particularly higher than the other vaccination protocols. Conversely, the frequency of epitope-specific CD8⁺ T cells seems to decrease in mouse lymph nodes (Masopust et al., 2006). This may be due to the lack of CD62L expression on the surface of these activated T cells, as discussed below. In addition, the pattern of circulation and recirculation of these lymphocytes has

been poorly explored. A single study, however, demonstrated that, after a recombinant plasmid DNA prime-AdHu5 boost, CD8⁺ T cells need to recirculate in order to exert protective immunity against an infectious challenge with the protozoan parasite *Trypanosoma cruzi*. In these vaccinated mice, treatment with the drug FTY720 significantly reduced the efficacy of the protective immunity by interfering specifically with signaling through sphingosine-1-phosphate receptor-1, thereby inhibiting the egress of T cells from the lymph nodes (Dominguez et al., 2012). This observation is important because immunity to certain pathogens is not dependent on the recirculation of T lymphocytes (Pinschewer et al., 2000; Kursar et al., 2008; Jiang et al., 2012).

The importance of recirculation is likely dependent on factors such as (i) the host, (ii) the vectors used for prime and/or boost, and (iii) the route of administration of each vector (for examples, see Mattapallil et al., 2006; Kaufman et al., 2010). These are important characteristics that will certainly influence the protective immunity, as pathogens can cause either tissue-specific or systemic infections.

The T cell protective immunity elicited by heterologous prime-boost regimen is not only significantly higher than the immunity elicited by the traditional vaccine regimen but is also longer-lived. Several experimental models have shown that the immunity lasts for significant periods of time (Amara et al., 2001; Bruña-Romero et al., 2001; de Alencar et al., 2009; Reyes-Sandoval et al., 2010; Rigato et al., 2011).

POLYFUNCTIONALITY OF SPECIFIC CD8⁺ T CELLS

Based on the assays described above, it became clear that the specific CD8⁺ T cells elicited by the heterologous prime-boost regimen could exert different immunological functions, as measured by *ex vivo* or *in vivo* assays. Confirmation of the polyfunctionality of these cells was made possible through FACS analyses coupling intracellular cytokine staining and cell surface mobilization of the degranulation marker CD107a.

Accordingly, a number of studies confirmed that distinct heterologous prime-boost regimens elicited polyfunctional CD8⁺ T cells as defined by the cells' capability to exert two or more functions at the same time. The most frequent example of this across different models is specific CD8⁺ T cells producing IFN- γ and TNF simultaneously. High frequencies of polyfunctional specific CD8⁺ T cells were described in (i) mice (Masopust et al., 2006; Duke et al., 2007; de Alencar et al., 2009; Elvang et al., 2009; Reyes-Sandoval et al., 2010; Rigato et al., 2011; Rodríguez et al., 2012; Vijayan et al., 2012), (ii) NHP (Mattapallil et al., 2006; Sun et al., 2006; Cox et al., 2008; Liu et al., 2008; Magalhaes et al., 2008; Cayabyab et al., 2009; Wilks et al., 2010; Hutnick et al., 2012), and (iii) humans (Beveridge et al., 2007; Harari et al., 2008; Winstone et al., 2009; Freel et al., 2010; Jaoko et al., 2010; Koup et al., 2010; Schooley et al., 2010; Churchyard et al., 2011; De Rosa et al., 2011).

It is noteworthy that although these T cells have a high frequency of polyfunctionality, their ability to mediate multiple immunological functions has never been clearly linked to their protective capacity. It is possible that this characteristic aids in but is not critical for their effector functions, depending on the mechanism necessary for pathogen elimination. In a single study

performed using genetically deficient mice, in the absence of either IFN- γ or perforin, heterologous prime-boost vaccination failed to mediate protective immunity against infection with the intracellular parasite *T. cruzi*. In the case of perforin-deficient mice, the lack of protective immunity was associated mainly with a significant decrease in the induction of polyfunctional T cells (de Alencar et al., 2009). A second study correlated the presence of higher frequencies of polyfunctional T cells to protective immunity against liver stages of malaria parasite (Reyes-Sandoval et al., 2010). Although these studies may suggest a role for polyfunctional T cells during protective immunity, it is still too early to conclude that they are critical for the protective immunity exerted by CD8⁺ T cells elicited following the heterologous prime-boost vaccination regimen.

BREADTH OF SPECIFIC CD8⁺ T CELLS

T cell immune responses are often restricted to a few immunodominant epitopes, a phenomenon termed immunodominance (Akram and Inman, 2012). The precise reason for such a restriction is not clear; however, it may have evolved to maximize the immune response, while at the same time reducing the risk of autoimmunity. For the purpose of vaccine development, having only a narrow number of recognized epitopes may be dangerous, as the pathogens will rapidly select for escape mutants to avoid effector immune responses (reviewed in Streeck and Nixon, 2010; Chopera et al., 2011).

Although it has been possible to increase the frequency of T cells specific for immunodominant epitopes, it is still a challenge to broaden the vaccine-induced CD8⁺ T cell response to a number of subdominant T cell epitopes. There is evidence that heterologous rAdHu5 boosting improved not only the magnitude but also the breadth of specific CD8⁺ cells (Liu et al., 2008). However, the impact of this response on protective immunity is not clear. Two recent studies indicated that immunity to subdominant epitopes might participate in vaccine-induced protective immunity following a DNA prime-AdHu5 boost vaccination regimen. The first study provided a correlation between the breadth of the immune response and the protective immunity observed in individual rhesus monkeys vaccinated with SIV genes (Martins et al., 2010). A second study formally demonstrated that heterologous prime-boost vaccination with plasmid DNA followed by recombinant AdHu5 elicited strong immune response to two subdominant epitopes that were not recognized during infection (Dominguez et al., 2011). Based on these observations, mutant genes were generated in which the dominant epitope was removed. Heterologous prime-boost vaccination with these mutant genes induced CD8⁺ T cell immune responses only to the subdominant epitopes. Most importantly, strong CD8⁺ T cell-mediated immunity was still observed (Dominguez et al., 2011). These results unequivocally demonstrate the importance of immunity to the subdominant epitopes and the ability of the heterologous prime-boost to elicit them. Nevertheless, other groups still have difficulty improving the immune response to subdominant epitopes following heterologous prime-boost vaccination of NHP (Vojnov et al., 2011). Therefore, new strategies to improve the breadth of the immune response might be developed in order to potentiate vaccine formulation.

TEM PHENOTYPE OF SPECIFIC LONG-LIVED CD8⁺ T CELLS

The current immunological paradigm divides antigen-experienced CD8⁺ T cells into three main types: (i) T effector (TE), (ii) T effector memory (TEM), and (iii) T central memory (TCM). These populations of T cells can be distinguished by the presence of activation markers, as well as by differences observed in their localization and recirculation patterns and their ability to proliferate and present certain effector functions/molecules (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Ahmed and Akondy, 2011; Sheridan and Lefrançois, 2011). More recently, other subpopulations of CD8⁺ T cells have also been described (Wakim et al., 2010; Sheridan and Lefrançois, 2011; Jiang et al., 2012). The concept of TE/TEM/TCM was primarily established by experimental infections with self-curing pathogens. In these cases, TE are short-lived effector cells (CD44^{High} CD11a^{High} CD62L^{Low} CD127^{Low} KLRG1^{High}); TCM are long-lived memory cells (CD44^{High} CD11a^{High} CD62L^{High} CD127^{High} KLRG1^{High}); and TEM are transitional cells that exist for a short period of time and have distinct surface marker expression (CD44^{High} CD11a^{High} CD62L^{Low} CD127^{High} KLRG1^{High}). However, even by using models of self-curing infections, the existence of other memory T cell subpopulations that might be more relevant for long-term T cell immunity have been proposed (Hikono et al., 2007).

In addition, very recently, considerable interest has been placed on tissue-resident memory T (TRM) cells. These cells are potent long-lived effector cells present in a variety of peripheral tissues including the skin and sensory ganglia, gut, brain, lung, etc. (Wakim et al., 2008; Gebhardt et al., 2009; Masopust et al., 2010; Purwar et al., 2011). They mediate protective immunity against brain infection with vesicular stomatitis virus or skin infection with vaccinia or herpes simplex virus (Wakim et al., 2010; Jiang et al., 2012; Mackay et al., 2012). Their phenotype is CD44^{High}, CD62L^{Low}, CD103^{High}, CD69^{High}, CD127^{Low}, and PD-1^{Low}. The relationship of TRM cells with TEM or TCM is yet to be determined. Some authors propose that these cells constitute an independent lineage primed within the tissue (Wakim et al., 2010, 2012).

Very recently, the development of a methodology to perform transcriptional profiling at the single cell level has detected further differentiation profiles among TEM and TCM. The analysis of specific splenic CD8⁺ T cells from mice immunized with distinct vaccination protocols yields significant differences among these subpopulations (Flatz et al., 2011). For example, while 74% of the specific TEM elicited by the heterologous DNA prime-recombinant AdHu5 boost vaccination were Klrk1[−] Klrp1[−] Ccr5[−], only 20% of the cells generated by HuAd5-prime-recombinant LCMV boost had a similar phenotype (Flatz et al., 2011). This type of analysis further highlights the heterogeneity still being uncovered within these memory cells.

It has been observed on multiple occasions that after heterologous prime-boost vaccination, the boosting immunization drives not only an expansion of the T cells but also a different phenotype in the long-lived memory T cells. Different doses of the vaccine also caused a significant increase in the frequency of specific CD8⁺ T cells with a TEM phenotype (CD44^{High} CD11a^{High} CD62L^{Low} CD127^{High} KLRG1^{High}; Masopust et al., 2006). TEM cells were described initially as highly protective against certain viral and

bacterial infections (Bachmann et al., 2005a,b; Huster et al., 2006). Likewise, protective immunity afforded by the different heterologous prime-boost vaccination protocols has been associated with the presence of this type of T cell (Hansen et al., 2011; Reyes-Sandoval et al., 2011; Rigato et al., 2011; Xiao et al., 2011; Barouch et al., 2012; Yamamoto et al., 2012).

Based on the relatively poor knowledge of the surface activation markers present on long-lived specific TEM CD8⁺ T cells, we performed a detailed analysis of the different T cells markers following intramuscular DNA prime-adenovirus boost immunization. We identified transgenic epitope-specific T cells in the spleen of immunized mice 14 or 98 days after the boost vaccination. **Figure 1** summarizes the surface marker phenotype of these epitope-specific T cells compared to the phenotype of the naïve cells.

PROLIFERATIVE CAPACITY OF SPECIFIC CD8⁺ T CELLS

The proliferative capacity of specific T cells elicited by heterologous prime-boost vaccination has not been thoroughly studied to date. In general, after resolution of experimental self-curing infections, the frequency of total CD8⁺ T cells declines to less than 10% of the maximal number of specific T cells observed during the peak of the immune response; this is known as the contraction phase. The decrease in the number of specific T cells occurs mainly among the short-lived effector cells (Angelosanto and Wherry, 2010; Cui and Kaech, 2010; Ahmed and Akondy, 2011; Sheridan and Lefrançois,

Naïve CD8 ⁺ T cells	Specific CD8 ⁺ T cells 14 days	Specific CD8 ⁺ T cells 98 days
CD11a Low	CD11a High	CD11a High
CD11c Low	CD11c Int.	CD11c Low
CD25 Low	CD25 High	CD25 Low
CD27 High	CD27 Low/High	CD27 Low/High
CD31 High	CD31 Low	CD31 Low
CD43 Low	CD43 High	CD43 High
CD44 Low	CD44 High	CD44 High
CD49d Low	CD49d High	CD49d High
CD69 Low	CD69 Low	CD69 Low
CD62L High	CD62L Low	CD62L Low
CD95 Low	CD95 Low	CD95 Low
CD122 Low	CD122 High	CD122 Int.
CD127 High	CD127 Low	CD127 High
CCR5 Low	CCR5 Low	CCR5 Low
KLRG-1 Low	KLRG-1 Low/High	KLRG-1 Low/High
T Naïve	TE	TEM

FIGURE 1 | Phenotype of specific CD8⁺ T cells elicited by heterologous prime-boost vaccination using recombinant plasmid DNA and AdHu5. Prime-boost regimen was performed as detailed described by Rigato et al. (2011). Mice were primed i.m. with plasmid DNA (100 µg) and boosted 21 days later with AdHu5 (2 × 10⁹) both expressing the gene encoding the amastigote surface protein-2 of *T. cruzi*. Expression of distinct adhesion/activation receptors on the surface of splenic CD8⁺ specific T cells is shown at day 14 or day 98 after the boost immunization.

2011). However, this does not seem to be the case for the TE cells elicited by the heterologous prime-boost vaccination regimen (Jameson and Masopust, 2009). In this case, the cells expand and are maintained at a high frequency in the spleen for a long period of time (Masopust et al., 2006; Vezys et al., 2009; Rigato et al., 2011). This is a somewhat unexpected finding and might be of great relevance for the success of this vaccination protocol. Because this expansion and maintenance contrasts with the retraction observed after self-curing infections, it opens a number of questions that should be studied in depth: (i) How can cells expand out of control to reach frequencies higher than the primary immune response? (ii) How do T cells avoid the apoptotic process observed for the short-lived effector cells during the contraction phase? (iii) How are T cells maintained for these long periods? These questions are keys to understanding the physiology of CD8⁺ T cells and developing better T cell vaccines.

After an infectious challenge, specific T cells proliferate. However, it is still unknown whether this proliferative response occurs among the antigen-primed TE(M) cells, rare TCM or naïve T cells. To address this question, it is now possible to use the *gzm-BCreERT2/Rosa26EYFP* transgenic mouse line (Bannard et al., 2009). Specific TE CD8⁺ T lymphocytes can be indelibly labeled with enhanced yellow fluorescent protein (EYFP). Following an infectious challenge, it will be possible to follow the expansion of EYFP-labeled TE cells. This result will confirm or not whether the cells that expand after the infectious challenge are indeed mainly antigen-experienced TE. Also, by the adoptive transference of green-fluorescent naïve specific T cells prior to the infectious challenge it will be possible to determine whether these cells proliferate or not together with the specific TE cells. Whether this is true for all different protocols of heterologous prime-boost vaccination strategies is still unknown and might be relevant for the development of vaccines to be used in individuals primed with conventional vaccines such as *Bacillus Calmette–Guérin* (BCG; Hoft et al., 2012).

REFRACTORINESS TO APOPTOSIS OF SPECIFIC CD8⁺ T CELLS

As mentioned above, after an intense immune response and pathogen elimination, the number of specific CD8⁺ T cell is drastically reduced during a period called the contraction phase. In that period, TE or short-lived effector cells are eliminated by mechanisms that involve apoptosis mediated by BCL-2-interacting mediator of cell death (Bim) and CD95 (also known as FAS; Green, 2008; Hughes et al., 2008; Weant et al., 2008; Bouillet and O'Reilly, 2009). We observed that following prime-boost immunization with recombinant plasmid DNA and AdHu5, TEM cells do not upregulate surface CD95 expression and are refractory to anti-CD95-induced apoptosis *in vitro* (Rigato et al., 2011). Because CD95 is an important initiator of the intrinsic pathway of apoptosis in T lymphocytes, low levels of expression may protect T cells from apoptotic death.

Decreased levels of expression or resistance to activation of other receptors that control T cell death, such as TNF receptor and TRAIL, might also play a role during the survival of specific TEM cells. In contrast, increased expression of other receptors may act to protect these specific T cells. Recently, expression of PDL-1 (B7-H1) on antigen-activated CD8⁺ T cells made these

cells more resistant to Ca²⁺-dependent and Fas ligand-dependent killing by cytotoxic T lymphocytes (Pulko et al., 2011). However, more details are necessary to understand the expression levels of many of these molecules controlling T cell survival.

It is plausible that Bim is poorly activated, or not activated at all, on these cells. Because Bim is activated in response to the lack of certain external stimuli, these activation signals might be maintained for long-term. One such mechanism is antigen presentation by dendritic cells. Using a replication-deficient adenoviral vector, Tatsis et al. (2007) demonstrated that the transgene product remains available for antigen presentation for as long as 16 weeks after a single immunizing dose. The importance of continuous transgene expression in the maintenance of the specific CD8⁺ T cells after AdHu5 vaccination was also demonstrated by a causal relationship between both using an inducible system to turn off the transgene expression (Finn et al., 2009). Bassett et al. (2011) showed that both hematopoietic and non-hematopoietic antigen-presenting cells are necessary for the maintenance of CD8⁺ T cells following immunization with recombinant adenovirus. However, it is not clear whether antigen persistence is also observed with other vectors.

We tested whether the IFN- γ or IL12/IL23 signaling pathways might be important for maintaining these long-lived CD8⁺ TEM cells. The absence of either pathway individually made little difference in the generation of specific CD8⁺ T cells. The absence of the IL12/IL23 pathway, but not the IFN- γ pathway, was important for the long-term survival of these cells (Rigato et al., 2011). Further details regarding the relevance of these signaling pathways in maintaining a high frequency of CD8⁺ T cells are unknown. In addition, little is known about the impact of the lack of the IL12/IL23 pathway in the maintenance of specific CD8⁺ T cells after other heterologous prime-boost vaccination regimens. We consider this area of critical importance in understanding how T cells can be maintained at high frequencies for long periods of time. A possible explanation that remains to be tested is whether the lack of contraction could be due to the fact that many specific long-lived CD8⁺ T cells express low levels of the chemokine receptors CXCR3 and CCR5 (Flatz et al., 2011). This possibility is supported by recent observations that genetically deficient CD8⁺ T cells that do not express both these receptors were refractory to contraction and accumulated in higher numbers in the spleen (Kohlmeier et al., 2011).

Recently, we described a new and potentially very important aspect of CD8⁺ TE cells. After recombinant AdHu5 vaccination, CD8⁺ TE cells do not undergo apoptosis, as well as prevent the development of a pro-apoptotic phenotype that occurs during experimental infection with the protozoan parasite *T. cruzi*. This phenomenon was observed when the administration of the recombinant AdHu5 vaccine was provided before (as part of a prime-boost regimen or alone) or at the time of the infectious challenge. AdHu5 treatment modulated specifically the CD8⁺ TE cells to express lower levels of CD95 (FAS) and become resistant to CD95-induced apoptosis. The determination of the distinct adhesion/activation receptors on the surface of the CD8⁺-specific T cells elicited by either infection or recombinant AdHu5 immunization showed very limited differences that were almost exclusively confined to the apoptotic receptor CD95 (Figure 2).

Naive CD8 ⁺ T cells	Specific CD8 ⁺ T cells Infected	Specific CD8 ⁺ T cells Immunized
CD11a Low	CD11a High	CD11a High
CD11c Low	CD11c Int.	CD11c Low
CD25 Low	CD25 Low	CD25 Low
CD27 High	CD27 Low	CD27 Low
CD43 Low	CD43 High	CD43 High
CD44 Low	CD44 High	CD44 High
CD49d Low	CD49d High	CD49d High
CD69 Low	CD69 Low	CD69 Low
CD62L High	CD62L Low	CD62L Low
CD95 Low	CD95 High	CD95 Low
CD122 Low	CD122 Low	CD122 Int.
CD127 High	CD127 Low	CD127 Low
CCR5 Low	CCR5 Low	CCR5 Low
KLRG-1 Low	KLRG-1 High	KLRG-1 High
PD1 Low	PD1 Low	PD1 Low/Int
CTLA-4 Low	CTLA-4 Low	CTLA-4 Low
BTLA Low	BTLA Low	BTLA Low
T Naive	TE	TE

FIGURE 2 | Expression of distinct adhesion/activation receptors on the surface of specific CD8⁺ T cells elicited by either *T. cruzi* infection or recombinant AdHu5 immunization (Vasconcelos et al., 2012). Mice were infected s.c. with *T. cruzi* (150 blood stream trypomastigotes) or immunized i.m. with AdHu5 (2×10^8 pfu) expressing the gene encoding the amastigote surface protein-2 of *T. cruzi* 19 days earlier.

Despite the above results, we have not ruled out that other pro-apoptotic signaling pathways might also be altered. In these immunized and challenged mice, the CD8⁺ T cell population expanded largely and protected mice against an otherwise lethal infection (Vasconcelos et al., 2012). An important mechanism that controls the expression of CD95 on the surface of specific CD8⁺ T cells has just been described during development of immune responses to viral infections. TCR activation led to an increase of the RIG-I-like receptor LGP2 expression which down-regulated CD95 expression. In KO mice, the absence of this molecule significantly increased the apoptosis reducing the effectiveness of the immune response and resistance to the viral infection (Suthar et al., 2012). We consider this a promising area of study because it suggests that perhaps the control of T cell survival might be the key element for the development of new or improved vaccines. The proposed pathway of specific CD8⁺ T cell activation following prime-boost vaccination and infection is shown in **Figure 3** (based on Vasconcelos et al., 2012).

REDUCED IMMUNOLOGICAL EROSION OF SPECIFIC CD8⁺ T CELLS

Memory CD8⁺ T cells are not a stable pool; subsequent infections may cause attrition and erosion of the memory T cell pool (Selin et al., 1996, 1999; Dudani et al., 2008; Huster et al., 2009; Schmidt and Harty, 2011). However, different heterologous

prime-boost regimens generated a pool of CD8⁺ T cells that was not eroded by subsequent viral infections (Vezys et al., 2009; Rigato et al., 2011). After a vaccination regimen consisting of recombinant plasmid DNA prime-AdHu5 boost, we observed that viral infections had limited impact on the number or quality of the TEM cells as measured by different functional immunological assays. Most importantly, protective immunity mediated by these CD8⁺ TEM cells was not altered in these mice (Rigato et al., 2011).

In contrast, using a different prime-boost vaccination protocol consisting of dendritic cells coated with circumsporozoite protein peptide and a booster immunization with recombinant *actA-inlB*-deficient *Listeria monocytogenes* expressing the same epitope elicited an immune response that could be eroded by multiple subsequent infections (Schmidt and Harty, 2011). The discrepant results highlight the importance of determining the characteristic of each TEM elicited by the distinct regimen of vaccination, as suggested earlier (Flatz et al., 2011).

The resistance to immunological erosion is one more interesting characteristic of these long-lived TEM cells that has been poorly explored and may have an important impact in the development of efficient vaccines. As mentioned above, this high-resistance immunological erosion can be linked to the differential expression of surface molecules (death receptors such as CD95/FAS) or apoptosis mediators (such as Bim).

UNKNOWN FATE OF THE SPECIFIC CD8⁺ TEM CELLS

Although some studies described that these TEM can be long-lived in some mouse models, it is not clear what will be the fate of these cells on extended periods of time. Will they become TCM by upregulating CD62L? Will they simply die? Can they be boosted? Perhaps these questions can be addressed by using the *gzmBCreERT2/Rosa26EYFP* transgenic mouse line (Bannard et al., 2009). Labeling specific TE CD8⁺ T lymphocytes will allow us to follow these cells for longer periods of time.

Also, their long-term fate in NHP or humans is even more important for the purpose of development of practical vaccines. Due to the obvious constraints, few studies so far have addressed this issue.

CONCLUDING REMARKS AND PERSPECTIVES

Genetic vaccination using heterologous prime-boost regimen protocols has the potential to serve as the basis for the development of new vaccines against many pathogens. In spite of the progress over the past 20 years, much work is required to improve the relevance of vaccine research, considering that human immune response is still at least one order of magnitude lower than that observed in mouse or even NHP models.

Several areas can be pursued for this matter. So far, protective immunity is clearly associated with the presence of a high number of long-lived specific polyfunctional TEM cells. Nevertheless, several points should be considered. First, TEM may vary from one protocol to the other. It is also important to improve the number of specific TCM cells, for example, by using pharmacological modulators (Li et al., 2012; Takai et al., 2012). However, it will be a challenge to increase the number of TCM cells without reducing the number of TEM cells. Fine tuning the TEM

FIGURE 3 | The proposed pathway of activation of specific CD8⁺ T cells following prime-boost vaccination and infection (based on Vasconcelos et al., 2012). Prime-boost regimen was performed as detailed described by Rigato et al. (2011). Mice were primed i.m. with

plasmid DNA (100 µg) and boosted 21 days later with AdHu5 (2×10^8) both expressing the gene encoding the amastigote surface protein-2 of *T. cruzi*. Mice were infected s.c. with *T. cruzi* (150 blood stream trypomastigotes).

subpopulations and increasing the number of TCM cells could further improve and prolong protective immunity beyond the levels currently achieved. Furthermore, new strategies to broaden the epitopes recognized by protective T cells would also improve both the quantity and the quality of the immune response. Finally, the circulation of these cells should be further studied for the purpose of personalizing the vaccination regimen for different types of infections, considering that the site of entry of each pathogen will vary, as will the localization of the T cells at the time of the infection.

In summary, the study of the control of memory T cell generation, maintenance, quality, and recirculation after distinct heterologous prime-boost vaccination regimens will provide important clues regarding the physiology of lymphocytes

and the immune system, with potential applications in public health.

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Adenovirus Vector-Induced CD8⁺ T Effector Memory Cell Differentiation and Recirculation, But Not Proliferation, Are Important for Protective Immunity Against Experimental *Trypanosoma cruzi* Infection

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Abstract

Heterologous prime-boost vaccination using plasmid DNA followed by replication-defective adenovirus vector generates a large number of specific CD8⁺ T effector memory (TEM) cells that provide long-term immunity against a variety of pathogens. In the present study, we initially characterized the frequency, phenotype, and function of these T cells in vaccinated mice that were subjected to infectious challenge with the human protozoan parasite *Trypanosoma cruzi*. We observed that the frequency of the specific CD8⁺ T cells in the spleens of the vaccinated mice increased after challenge. Specific TEM cells differentiated into cells with a KLRG1^{High} CD27^{Low} CD43^{Low} CD183^{Low} *T-bet*^{High} *Eomes*^{Low} phenotype and capable to produce simultaneously the anti-parasitic mediators IFN γ and TNF. Using the gzmBCreERT2/ROSA26EYFP transgenic mouse line, in which the cells that express Granzyme B after immunization, are indelibly labeled with enhanced yellow fluorescent protein, we confirmed that CD8⁺ T cells present after challenge were indeed TEM cells that had been induced by vaccination. Subsequently, we observed that the *in vivo* increase in the frequency of the specific CD8⁺ T cells was not because of an anamnestic immune response. Most importantly, after challenge, the increase in the frequency of specific cells and the protective immunity they mediate were insensitive to treatment with the cytostatic toxic agent hydroxyurea. We have previously described that the administration of the drug FTY720, which reduces lymphocyte recirculation, severely impairs protective immunity, and our evidence supports the model that when large amounts of antigen-experienced CD8⁺ TEM cells are present after heterologous prime-boost vaccination, differentiation, and recirculation, rather than proliferation, are key for the resultant protective immunity.

Introduction

GENETIC VACCINATION USING the heterologous prime-boost regimen is being actively pursued to elicit specific immune responses mediated by cytotoxic CD8⁺ T cells. This strategy employs two different vaccine vectors, both of which

carry the same foreign gene encoding the target antigen for priming and boosting immunizations. A number of different vector combinations have been tested, and the application of this strategy has been shown to successfully provide immunity against various infections, such as simian immunodeficiency virus malaria, Ebola virus, leishmaniasis,

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tuberculosis, Chagas disease, and toxoplasmosis (reviewed by Lasaro and Ertl, 2009; Ranasinghe and Ramshaw, 2009; Hill *et al.*, 2010; Rollier *et al.*, 2011; Vasconcelos *et al.*, 2012b). The knowledge accumulated using preclinical experimental models is now being translated into a number of ongoing phase I/II trials in humans (Freel *et al.*, 2010; Jaoko *et al.*, 2010; Koup *et al.*, 2010; De Rosa *et al.*, 2011; O'Connell *et al.*, 2012; Casazza *et al.*, 2013; Chuang *et al.*, 2013; Hammer *et al.*, 2013).

Although the heterologous prime-boost regimen is being increasingly used for vaccine development, the precise reason for its success is still not fully understood. Distinct, but not mutually exclusively, explanations may account for its enhanced vaccination efficacy. This regimen elicits a high number of T effector (TE) cells (CD11a^{High}, CD44^{High}, CD127^{Low}, and CD62L^{Low}), which subsequently become long-lived T effector memory (TEM) cells (CD11a^{High}, CD44^{High}, CD127^{High}, and CD62L^{Low}) (reviewed by Vasconcelos *et al.*, 2012b). It is conceivable that the large number of TE(M) cells might be the most important factor contributing to the efficacy of this immunization strategy. Their high frequency becomes even more important because in many cases, they are multifunctional T cells that are capable of lysing target cells and secreting multiple effector cytokines (reviewed by Vasconcelos *et al.*, 2012b). In addition, other reasons that might explain the high efficacy of this regimen include the capacity of these cells to proliferate and/or to recirculate after infectious challenge.

Within the past few years, we reported that the heterologous prime-boost strategy successfully vaccinates highly susceptible A/Sn mice against systemic infection with the human intracellular protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease (American trypanosomiasis) (De Alencar *et al.*, 2009; Haolla *et al.*, 2009; Dominguez *et al.*, 2011, 2012; Rigato *et al.*, 2011). In these experiments, we used a recombinant plasmid DNA for priming and the human replication-defective recombinant adenovirus type 5 for boosting, both expressing the amastigote surface protein-2 (ASP-2) of *T. cruzi*. Our vaccination protocol elicited a strong immune response mediated by CD8⁺ TE cells (CD11a^{High}, CD44^{High}, CD127^{Low}, and CD62L^{Low}). These cells developed to form a stable pool of functional long-lived CD8⁺ TEM cells (CD11a^{High}, CD44^{High}, CD127^{High}, and CD62L^{Low}). Classical TCM (CD11a^{High} CD44^{High} CD62L^{High} CD127^{High}) are not seen in our system until 180 days after immunization. Their long-term survival required a functional IL-12/IL23 signaling pathway, but not the presence of CD4⁺ T cells. These cells were resistant to immunological erosion and, most important for vaccine development, they mediated strong protective immunity against experimental systemic infection with *T. cruzi* (Rigato *et al.*, 2011).

In a subsequent study, we hypothesized that the T cells elicited by heterologous prime-boost vaccination must recirculate to mediate their antiparasitic effects. We approached this subject by administering the immunosuppressive drug FTY720 to vaccinated mice. This drug reduces lymphocyte recirculation by interfering with T cell signaling via sphingosine-1-phosphate receptor-1 (S1Pr1). This interference results in sustained inhibition of S1Pr1 signaling, effectively trapping T cells within the secondary lymphoid without inhibiting T cell activation. FTY720 administration signifi-

cantly impaired protective immunity, supporting the hypothesis that T cell recirculation is critical for the protective immunity they mediate (Dominguez *et al.*, 2012).

Because it is not well established whether in addition to recirculation, TE(M) cells must differentiate and proliferate to eliminate the parasite, the purpose of the present study was to determine the frequencies, phenotypes, and functions of the TE(M) cells that are present after infectious challenge and whether protective immunity can be inhibited by treatment with the cytostatic toxic agent hydroxyurea (HU).

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (www.cobea.org.br/). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of Sao Paulo (Id No. CEP 0426/09).

Mice and parasites

Female 5–8-week-old C57BL/6 and A/Sn mice were purchased from CEDEME (Federal University of São Paulo). gzmBCreERT2/ROSA26EYFP or green fluorescent protein (GFP) transgenic mouse line C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ was generated as described by Bannard *et al.* (2009) or Okabe *et al.* (1997) and bred in our own animal facility. Bloodstream trypomastigotes of the Y strain of *T. cruzi* were obtained from mice infected 7 days earlier (De Alencar *et al.*, 2009). The concentration of parasites was estimated and adjusted to 10⁵ or 750 parasites/ml. Each mouse was inoculated with 10⁴ (C57BL/6 or gzmBCreERT2/ROSA26EYFP) or 150 (A/Sn) trypomastigotes diluted in 0.1 ml phosphate buffered saline (PBS) and administered subcutaneously in the base of the tail. HU (Sigma) was administered as cycle of 2 intraperitoneal injections of 1 gr/kg/day, 7 hr apart. Mice received four cycles every 2 days.

Peptides

Synthetic peptides were purchased from Genscript. Peptide purity was higher than 90%. Peptide identities were confirmed by a Q-TOF Micro equipped with an electrospray ionization source (Micromass). The immunodominant epitopes of ASP-2 were represented by AA VNHRFTLV. The pentamer H2K^b-VNHRFTLV was purchased from ProImmune Inc.

Genetic vaccination

Plasmid pIgSPclone9 and human replication-deficient adenovirus type 5 expressing the ASP-2 gene (AdASP-2) were generated, grown, and purified as described earlier (Boscardin *et al.*, 2003; Machado *et al.*, 2006). Control mice were immunized with pcDNA3 and human replication-deficient adenovirus type 5 expressing β -galactosidase (Ad β -gal). Mice were inoculated intramuscularly in each *tibialis anterioris* muscle with 50 μ g of plasmid DNA.

Twenty-one days later, these mice received 50 μ l of a viral suspension containing 2×10^8 plaque forming units of adenovirus in the same locations. Immunological assays were performed at the days indicated in each figure.

Immunological assays

For flow cytometry analyses, we used mouse splenocytes treated with ACK buffer (NH_4Cl , 0.15 M; KHCO_3 , 10 mM; $\text{Na}_2\text{-EDTA}$ 0.1 mM; pH 7.4) for lysing the erythrocytes. Single-cell suspensions were washed in PBS, stained for 10 min at room temperature with biotinylated MHC I multimer H-2K^b-VNHRFTLV, and stained for 20 min at 4°C with avidin-APC- or avidin-APC-Cy7-labeled and PerCP- or Pacific Blue-labeled anti-CD8 antibodies (BD Pharmingen). For the analyses of other cell-surface markers, single-cell suspensions from spleens of mice were stained with multimers and anti-CD8 antibody as described above. Then, cells were incubated with PE-labeled anti-CD27 (clone LG.3A10) and FITC-labeled anti-KLRG1 (clone MAFA) purchased from eBioscience. Alternatively, cells were incubated with PercP-labeled anti-CD43 (1B11) and PE-Cy7-labeled anti-CD183 (CXCR3-173) purchased from Biolegend. Isotype control antibodies were PE-labeled hamster IgG1k and rat IgG1k, and FITC-labeled IgG2ak (BD Pharmingen). At least 200,000 cells were acquired on a BD FACS Canto flow cytometer and then analyzed with FlowJo (Tree Star).

For the surface intracellular expression of cytokines (IFN γ and TNF; ICS), splenocytes collected from C57BL/6 mice were treated with ACK buffer. ICS were evaluated after *in vitro* culture of splenocytes in the presence or absence of the antigenic stimulus. Cells were washed 3 times in plain RPMI and resuspended in cell culture medium consisting of RPMI 1640 medium, pH 7.4, supplemented with 10 mM HEPES, 0.2% sodium bicarbonate, 59 mg/liter of penicillin, 133 mg/liter of streptomycin, and 10% Hyclone fetal bovine sera (Hyclone). The viability of the cells was evaluated using 0.2% trypan blue exclusion dye to discriminate between live and dead cells. Cell concentration was adjusted to 5×10^6 cells/ml in cell culture medium containing anti-CD28 (2 μ g/ml), BcGolgiPlug, and monensin (5 μ g/ml). In half of the cultures, a final concentration of 10 μ M of the VNHRFTLV peptide was added. The cells were cultivated in flat-bottom 96-well plates (Corning) in a final volume of 200 μ l in duplicate, at 37°C in a humid environment containing 5% CO_2 . After 12 hr incubation, cells were stained for surface markers with PerCP- and PE-labeled anti-CD8, on ice for 20 min. To detect IFN γ and TNF by intracellular staining, cells were then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA, fixed, and permeabilized with BD perm/wash buffer. After being washed twice with BD perm/wash buffer, cells were stained for intracellular markers using APC-labeled anti-IFN γ (Clone XMG1.2) and PE-labeled anti-TNF (clone MP6-XT22), and surface markers FITC-labeled anti-KLRG1, FITC-labeled CD27 (LG.7F9), Alexa-fluor-labeled CD43, and PercP-labeled CD183 for 20 min on ice. Finally, cells were washed twice with BD perm/wash buffer and fixed in 1% PBS-paraformaldehyde. At least 300,000 cells were acquired on a BD FACS Canto flow cytometer and then analyzed with FlowJo.

For detection of bromodeoxyuridine (BrdU), mice were injected intraperitoneally with 2 mg of BrdU (Sigma) 7 times for 14 days. The cells were treated according to the manufacturer's instructions and stained with anti-BrdU-FITC (BD Pharmingen). At least 200,000 cells were analyzed by FACS as described above.

Statistical analysis

The values of parasitemia were log transformed before being compared using one-way ANOVA followed by Tukey's HSD tests. The log-rank test was used to compare mouse survival rates after challenge with *T. cruzi*. The differences were considered significant when the *p*-value was < 0.05.

Results

Frequencies, phenotypes, and functions of specific CD8⁺ T cells in vaccinated mice after infectious challenge with *T. cruzi*

Protective immunity against *T. cruzi* infection can be achieved by genetic vaccination with the ASP-2 gene following a heterologous plasmid DNA priming-recombinant adenovirus boosting regimen (De Alencar *et al.*, 2009; Haolla *et al.*, 2009; Dominguez *et al.*, 2011, 2012; Rigato *et al.*, 2011). In C57BL/6 mice, protective immunity is mediated by CD8⁺ T cells that are specific for the immunodominant epitope VNHRFTLV (De Alencar *et al.*, 2009; Rigato *et al.*, 2011). Fourteen days after boosting with AdASP-2, specific CD8⁺ cells expressed the TE cell phenotype (CD11a^{High}, CD44^{High}, CD127^{Low}, and CD62L^{Low}). Later, 98 days after boosting, their phenotype developed into that of TEM cells (CD11a^{High}, CD44^{High}, CD127^{High}, and CD62L^{Low}) (Rigato *et al.*, 2011).

To study the frequencies of the specific CD8⁺ T cells, the vaccinated mice were challenged at different times after the last immunizing dose, as depicted in Fig. 1A. The frequencies of the CD8⁺ H-2K^b-VNHRFTLV⁺ cells in the spleen were compared among mice:

1. Naïve: nonmanipulated
2. β -Gal/*T. cruzi*: immunized with the control plasmid pcDNA3/Ad β -Gal and challenged with *T. cruzi*
3. ASP-2: immunized with pIGSP Cl.9/AdASP-2
4. ASP-2/*T. cruzi*: immunized pIGSP Cl.9/AdASP-2 and challenged with *T. cruzi*

After infectious challenge with *T. cruzi*, the frequency of the CD8⁺ H-2K^b-VNHRFTLV⁺ cells consistently increased among the splenic CD8⁺ T cells of the ASP-2/*T. cruzi* mice. This increase was always observed, irrespective of the day on which the challenge was performed after the last immunizing dose. The frequencies of the specific CD8⁺ T cells in the spleen of the ASP-2/*T. cruzi* mice were higher (from 2.46- to 7.83-fold) than those of the β -Gal/*T. cruzi* or ASP-2 mice (Fig. 1B).

Because we used pools of splenic T cells in the experiments described above, we reproduced the experiment in which we measured the individual frequencies of the specific CD8⁺ T cells using mice that were immunized according to the protocol described in Fig. 1C. The frequency of the CD8⁺ H-2K^b-VNHRFTLV⁺ cells was analyzed in

FIG. 1. Frequencies of specific splenic CD8⁺ T cells increase after infectious challenge with *T. cruzi* in mice vaccinated with the heterologous prime-boost vaccination regimen. **(A)** C57BL/6 mice were immunized and challenged as depicted. Priming and boosting immunizations were performed as described in the Materials and Methods section. Mouse groups: naïve, nonmanipulated; β -Gal/*T. cruzi*, immunized with the control vector pcDNA3/Ad β -Gal and challenged with *T. cruzi*; ASP-2, immunized with pIgSP C1.9/AdASP-2; ASP-2/*T. cruzi*, immunized pIgSP C1.9/AdASP-2 and challenged with *T. cruzi*. **(B)** H-2K^b-VNHRFTLV⁺CD8⁺ splenic cell frequencies were estimated in pools of splenic cells from 4 mice. **(C)** GzmBCreERT2/ROSA26YFP transgenic mice were immunized and challenged as depicted. **(D)** H-2K^b-VNHRFTLV⁺CD8⁺ cell frequencies were estimated in the spleen of individual mouse ($n=4$). **(E)** The total number of H-2K^b-VNHRFTLV⁺CD8⁺ cells was estimated in the spleen of each individual mouse ($n=4$). **(F)** H-2K^b-VNHRFTLV⁺CD8⁺ cell frequencies were estimated in the inguinal and popliteal lymph nodes of each individual mouse. **(G)** The total numbers of H-2K^b-VNHRFTLV⁺CD8⁺ cells were estimated in the inguinal and popliteal lymph nodes of each individual mouse ($n=4$). The dots represent each mouse, and the bars represent the means $\pm$ SD.

the spleen and draining lymph nodes (popliteal and inguinal) of each individual mouse. After challenge, we detected a significant increase in the frequency of the splenic H-2K^b-VNHRFTLV⁺ CD8⁺ T cells (Fig. 1D). This event was not observed in the lymph nodes of the same mice (Fig. 1F).

Because of the splenomegaly in the mice challenged with *T. cruzi*, we also calculated the total number of CD8⁺ H-

2K^b-VNHRFTLV⁺ cells per spleen and draining lymph nodes. We found that the total number of specific T cells was even higher in the ASP-2/*T. cruzi* mice than in the other mouse groups (Fig. 1E). In the case of the lymph nodes, we did not detect a difference between the ASP-2/*T. cruzi* mice and the β Gal/*T. cruzi* mice ($p=NS$). However, there was a difference between the β Gal/*T. cruzi* or ASP-2/*T. cruzi* mice

and the ASP-2 mice. It reflected that the total number of recovered cells was significantly higher in the *T. cruzi*-challenged mice (Fig. 1G).

Analyses of the frequencies of specific CD8⁺ T cells in the blood of the different mouse groups revealed that immune and/or infected mice had a higher frequency than control β Gal mice ($p < 0.001$). ASP-2/*T. cruzi* mice had a higher frequency-specific CD8⁺ T cells; however, they were not statistically different from β Gal/*T. cruzi* or ASP-2 mice ($p = \text{NS}$, data not shown).

To determine whether these specific CD8⁺ T cell subpopulations accounted for any relevant antiparasitic effector functions, we evaluated the pattern of IFN γ /TNF expression upon *in vitro* stimulation with the antigen. Splenic mouse cells were collected from mice challenged 14 days earlier, at different days after the booster injection, as described in the

timeline shown in Fig. 2A. These cells were cultured *in vitro* in the presence or absence of the cognate peptide VNHRFTLV. In the presence of the antigen, the CD8⁺ T cells from the β -Gal/*T. cruzi*, ASP-2, and ASP-2/*T. cruzi* mice expressed IFN γ and/or TNF (Fig. 2B). In contrast, the CD8⁺ T cells from the naïve control mice did not. Splenic T cells cultured *in vitro* in the absence of peptide presented very low frequencies of CD8⁺ cells that expressed either IFN γ or TNF (data not shown). The frequencies of the IFN γ ⁺/TNF⁺ cells were higher in the population of splenic CD8⁺ cells from the ASP-2/*T. cruzi* mice (Fig. 1B). Similarly, these mice also presented higher frequencies of the IFN γ ⁺ (Fig. 1C).

Because we used pools of splenic T cells in the experiments described above, we reproduced these experiments using individual mouse that were immunized and challenged

FIG. 2. Frequencies of specific cytokine-secreting splenic CD8⁺ T cells increase after infectious challenge with *T. cruzi* in mice vaccinated with the heterologous prime-boost vaccination regimen. **(A)** C57BL/6 mice were immunized and challenged as depicted. Priming and boosting immunizations were performed as described in the Materials and Methods section. The mouse groups were the same as those described in the legend of Fig. 1. **(B)** Pools of splenic cells from 4 mice were restimulated *in vitro* in the absence (Med) or presence of the peptide VNHRFTLV (Pep), anti-CD28, BdGolgiPlug, and monensin. After 12 hr, the cells were stained with anti-CD8, anti-IFN γ , and anti-TNF. **(C)** The same as above except that they represent only the cells stained with anti-CD8 and anti-IFN γ but not with anti-TNF. **(D)** C57BL/6 mice were immunized and challenged as depicted. **(E)** Splenic cells were restimulated *in vitro* in the presence of the peptide VNHRFTLV, anti-CD28, BdGolgiPlug, and monensin. After 12 hr, the cells were stained with anti-CD8, anti-IFN γ , and anti-TNF. The results are represented by medians (bars) from four individual mouse per group (dots). Two asterisks denote that the values of these groups were higher than those of the naïve control mice or all other groups ($p < 0.01$), respectively. **(F)** Pie charts show the fraction of peptide-specific cells expressing the indicated molecules. The results are expressed as the mean values for four mice per group.

according to the protocol described in Fig. 2D. The frequencies of specific CD8⁺ T cells expressing IFN γ and TNF in the spleen of each individual mouse after challenge were significantly higher than in the other groups (Fig. 2E). These results corroborated with our multimer staining results (Fig. 1B and D), confirming that the specific CD8⁺ T cells are indeed functional. The quality of the immune response was also compared by estimating the frequencies of cells expressing different cytokines. We did not find significant differences between the ASP-2 and ASP-2/*T. cruzi* mice in this analysis (Fig. 2F).

From our evaluation of the expression of the markers CD44, CD62L, and CD127, the specific CD8⁺ T cells always presented a TE(M) phenotype (Rigato *et al.*, 2011). Thus, we then searched for other surface markers that could subdivide this population. On the basis of a previous study by Hikono *et al.* (2007), we used antibodies against KLRG1, CD27, CD43 (glycosylated form), and CD183 (chemokine receptor CXCR3) to compare the phenotype of resting naïve splenic CD8⁺ T cells to that of H-2K^b-VNHRFTLV⁺ CD8⁺ T cells from the β -Gal/*T. cruzi*, ASP-2, and ASP-2/*T. cruzi* mice. When comparing the expression of KLRG-1 or CD27, the phenotype of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells varied dramatically in the different groups of mice (Fig. 3A). The ASP-2/*T. cruzi* mice had significantly higher frequencies of KLRG1⁺ and CD27⁺ cells in their H-2K^b-VNHRFTLV⁺ CD8⁺ T cell population. The expression of CD43 or CD183 on the H-2K^b-VNHRFTLV⁺ CD8⁺ T cells was low in all mouse groups and was comparable to the staining observed for naïve CD8⁺ T cells. We also found that these cells were all *T-bet*⁺ and *Eomes*⁺ (Fig. 3B).

To compare the different populations of H-2K^b-VNHRFTLV⁺ CD8⁺ T cells stained for the four different markers, we performed Boolean analyses. For these analyses, splenic cells were simultaneously labeled for CD8, H-2K^b-VNHRFTLV, KLRG1, CD27, CD43, and CD183. As depicted in Fig. 3C, the majority of the cells in the ASP-2/*T. cruzi* mice were KLRG1⁺CD27⁺CD43⁺CD183⁺ cells. A number of isotype control antibodies were used described in the Materials and Methods section. None of them stained the CD8⁺H-2K^b-VNHRFTLV⁺ cells (data not shown).

In addition to these markers, the H-2K^b-VNHRFTLV⁺ CD8⁺ from the ASP-2/*T. cruzi* mice were CD11a^{High}, CD44^{High}, CD62L^{Low}, CD127^{Low}, CD11c^{High}, CD49d^{High}, PD1^{Low}, CD95^{Low}, CD38^{High}, PDL-1^{Low}, CD134^{High}, LAG-3^{High}, TRAIL^{Low}, TRAIL-R2^{Low}, TIM3^{Low}, and TRAN-CE^{Low} (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hum).

To determine the phenotype of the IFN γ - and TNF-producing cells, we combined the ICS analysis with staining for the surface markers KLRG1, CD27, CD43, and CD183. The CD8⁺ T cells expressing IFN γ or TNF were not proportionally equal in the different mouse groups. Higher frequencies of IFN γ ⁺KLRG1⁺ and TNF⁺KLRG1⁺ were observed in the splenic CD8⁺ cell population from the ASP-2/*T. cruzi* mice compared with the β -Gal/*T. cruzi* and ASP-2 mice. Similar analyses were also performed using CD27, CD43, and CD183. In these cases, we found that the predominant phenotype for the CD8⁺ IFN γ ⁺ and CD8⁺ TNF⁺ cells was always CD27⁺, CD43⁺, and CD183⁺ (Supplementary Fig. S2).

The specific CD8⁺ T cells observed after infectious challenge with T. cruzi are immune TE(M) cells previously expanded by vaccination

The results described above that before and after an infectious challenge, specific CD8⁺ T cells differ with respect to their frequency and phenotype raised the possibility that cells other than the vaccination-induced CD8⁺ TE(M) cells accounted for a proportion of the cells observed after challenge. To examine this possibility, we made use of the *gzmBCreERT2/ROSA26EYFP* transgenic mouse line. After antigenic stimulation *in vivo* and treatment with tamoxifen, cells expressing Granzyme B are indelibly labeled with EYFP (Bannard *et al.*, 2009). Therefore, in these mice, antigen-specific CD8⁺ T cells with an effector function (TE cells) will become fluorescent (EYFP⁺). In Fig. 4A, the detailed protocol used for these experiments is shown. The tamoxifen treatment was performed over 7 days after the booster injection with the recombinant adenovirus (days 22–28).

The strategy used for quantifying the H-2K^b-VNHRFTLV⁺ CD8⁺ cells labeled with EYFP is depicted in Fig. 4B. We initially gated splenic H-2K^b-VNHRFTLV⁺ CD8⁺ cells, and the frequency of EYFP fluorescent cells was then estimated. The results are expressed as the proportion of EYFP⁺ cells within the total VNHRFTLV⁺ CD8⁺ cell population. As depicted in Fig. 4C, at day 52, 15–19% of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells in the ASP-2 mice were labeled with EYFP. In vaccinated and challenged mice (ASP-2/*T. cruzi*), the frequency of H-2K^b-VNHRFTLV⁺ CD8⁺ cells labeled with EYFP ranged from 9.9% to 14% (Fig. 4C). The EYFP-labeled activated specific cells never reached 20%. This partial labeling was described earlier as a result of inefficient Cre-mediated recombination (Bannard *et al.*, 2009).

Similar measurements made at day 102 detected 9.7% to 14% of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells in the ASP-2 mice labeled with EYFP (Fig. 4D). Similarly, in vaccinated and challenged mice (ASP-2/*T. cruzi*), the frequency of H-2K^b-VNHRFTLV⁺ CD8⁺ cells labeled with EYFP ranged from 9.2% to 15% (Fig. 4D). In the β -Gal/*T. cruzi* mice, the frequencies of H-2K^b-VNHRFTLV⁺ CD8⁺ cells labeled with EYFP ranged between 1.2% and 3.3% (Fig. 4C and D).

Analysis of these results showed that although there was a statistically significant difference ($p < 0.05$) between the frequencies of 2K^b-VNHRFTLV⁺ CD8⁺ EYFP⁺ cells observed in the ASP-2 and ASP-2/*T. cruzi* groups at day 52, we found no difference at day 102 ($p = \text{NS}$). Subsequent experiments confirmed that there was no significant difference between these groups. Thus, we concluded that the H-2K^b-VNHRFTLV⁺ CD8⁺ cells observed after the infectious challenge are the same antigen-specific effector CD8⁺ T cells that expressed Granzyme B after immunization with AdASP-2.

To further confirm that naïve T cells do not participate in the immune response after the infectious challenge of vaccinated mice, we performed experiments in which we transferred naïve GFP-transgenic splenic cells to vaccinate mice at the same day of the challenge (Supplementary Fig. S3A). After 20 days, we estimated the frequencies of specific T cells using *in vitro* restimulation and ICS. As shown

FIG. 3. Phenotype of specific splenic CD8⁺ T cells after infectious challenge with *T. cruzi* in mice vaccinated with the heterologous prime-boost vaccination regimen. **(A)** C57BL/6 mice were immunized and challenged as depicted. Priming and boosting immunizations were performed as described in the Materials and Methods section. The mouse groups were the same as those described in the legend of Fig. 1. The red and blue lines depict the naïve CD8⁺ cells and H-2K^b-VNHRFTLV CD8⁺ cells, respectively. Pools of splenic cells from 4 mice were stained with anti-CD8, H-2K^b-VNHRFTLV, and anti-KLRG1, anti-CD27, anti-CD43, or anti-CD183. The red and blue lines denote the naïve CD8⁺ cells and H-2K^b-VNHRFTLV CD8⁺ cells, respectively. Analysis of individual mouse in each group provided similar results. **(B)** Pools of splenic cells from four mice were stained with anti-CD8, H-2K^b-VNHRFTLV, and anti-*T-bet* or anti-*Eomes*. **(C)** Boolean analyses of pools of splenic cells from 4 mice that were simultaneously stained with anti-CD8, H-2K^b-VNHRFTLV, anti-KLRG1, anti-CD27, anti-CD43, and anti-CD183. The red and blue lines denote the naïve CD8⁺ cells and H-2K^b-VNHRFTLV CD8⁺ cells, respectively. Analysis of individual mouse in each group provided similar results.

earlier, the frequencies of specific CD8⁺ T cells were higher in the ASP-2/*T. cruzi* mice than those in the β -gal/*T. cruzi* mice (Supplementary Fig. S3B and E). Because the naïve cells were GFP⁺, we were able to localize these naïve CD8⁺ cells (Supplementary Fig. S3C). We then estimated

the frequencies of the CD8⁺ IFN γ ⁺ GFP⁺ cells (Supplementary Fig. S3D and F). A statistical comparison showed that the frequencies of the CD8⁺ IFN γ ⁺ GFP⁺ cells in the β -gal/*T. cruzi* mice were significantly higher than those in the ASP-2/*T. cruzi* mice (Supplementary Fig. S3F). In fact,

FIG. 4. The specific CD8⁺ T cells detected after challenge with *T. cruzi* are TE(M) cells. (A) GzmBCre ERT2/ROSA26EYFP transgenic mice were immunized, treated with tamoxifen, and challenged as depicted. The mouse groups were the same as those described in the legend of Fig. 1. (B) Splenocytes were stained with anti-CD8 and H-2K^b-VNHRFTLV. The EYFP⁺ TE cell frequency in each group was then estimated. (C) Splenocytes isolated at day 52 were stained with anti-CD8 and H-2K^b-VNHRFTLV. The frequency of EYFP⁺ cells (Granzyme B⁺ TE) in each group is shown for each individual mouse (dots). Bars represent the median of each mouse group. (D) The same as above except that the cells were obtained from mice at day 102. The asterisk denotes a higher frequency of EYFP⁺ H-2K^b-VNHRFTLV⁺ CD8⁺ cells ($p < 0.05$).

very few CD8⁺ IFN γ ⁺ GFP⁺ cells were detected in these mice, showing that the presence of specific CD8⁺ TE cells completely inhibited naïve T cell activation.

Proliferation plays a minor role in the increase in the frequency of specific CD8⁺ T cells in the spleen and the protective immunity resulting from vaccination

A plausible explanation for the increase in the frequencies of specific splenic CD8⁺ T cells after challenge is proliferation upon antigen restimulation (anamnestic response). Alternatively, this increase could be because of recirculation, for example. To evaluate these hypotheses, we immunized and challenged GzmBCreERT2/ROSA26EYFP mice line as depicted in Fig. 5A. We then analyzed the frequencies of specific cells (stained for H-2K^b-VNHRFTLV) within the total EYFP⁺ CD8⁺ T cells in the ASP-2 or ASP-2/*T. cruzi* mice. Our rationale was that if antigen-specific proliferation occurs after challenge, the frequency of the EYFP⁺ H-2K^b-VNHRFTLV⁺ CD8⁺ cells (within the EYFP⁺ CD8⁺ cells) should be much higher in the ASP-2/*T. cruzi* mice than in the ASP-2 mice because of the anamnestic proliferation.

As described above, when analyzed among total spleen cells, we found that the frequency of splenic H-2K^b-VNHRFTLV⁺ CD8⁺ cells was higher in the ASP-2/*T. cruzi*

mice compared with the ASP-2 mice (Fig. 5B). In contrast, when analyzed among the EYFP⁺ CD8⁺ cells, we found that there was little increase in the frequency of EYFP⁺ H-2K^b-VNHRFTLV⁺ CD8⁺ cells in the spleen of ASP-2/*T. cruzi* mice compared with the ASP-2 animals (Fig. 5C and D). Among the EYFP⁺ CD8⁺ cells, in the ASP-2 mice, the frequency of EYFP⁺ H-2K^b-VNHRFTLV⁺ CD8⁺ cells ranged from 39% to 49.3%. In the ASP-2/*T. cruzi* mice, this frequency ranged from 41.9% to 62.3% ($p = \text{NS}$). Thus, we concluded that the increase in these frequencies was not *T. cruzi* antigen specific.

The limited expansion of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells was further corroborated by experiments of *in vivo* BrdU incorporation. Vaccinated or control mice were challenged or not with *T. cruzi*. In the subsequent days, these animals were treated with BrdU. Fourteen days later, the frequencies of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells that incorporated BrdU or stained for KLRG1 were estimated. As shown in Supplementary Fig. S4B, there is a significant increase in the frequency of specific CD8⁺ T cells from ASP-2/*T. cruzi* mice when compared with the other mouse groups as described earlier.

We also observed that 22% of CD8⁺ T cells from control mice (β -gal) proliferated during the period of 2 weeks as estimated by BrdU incorporation. In contrast, as expected, a much higher frequency (66%) of H-2K^b-VNHRFTLV⁺

D

Frequencies of
EYFP⁺H-2K^b-VNHRFTLV⁺CD8⁺ / EYFP⁺CD8⁺

ASP-2	39.0%	43.3%	49.3%	47.2%
ASP-2/ <i>T. cruzi</i>	55.8%	41.9%	42.1%	62.3%

P=0.36 (NS)

FIG. 5. EYFP⁺ H-2K^b-VNHRFTLV⁺ and EYFP⁺ H-2K^b-VNHRFTLV⁻ CD8⁺ T cell frequencies increase after infectious challenge. (A) GzmBCreERT2/RO-SA26EYFP transgenic mice were immunized, treated with tamoxifen, and challenged as depicted. The mouse groups were the same those as described in the legend of Fig. 1. M1–M4 denote each individual mouse analyzed. (B) The frequency of the splenic H-2K^b-VNHRFTLV⁺ CD8⁺ cells was higher in the ASP-2/*T. cruzi* mice compared with the ASP-2 mice ($p < 0.01$). (C) Frequencies of the EYFP⁺ H-2K^b-VNHRFTLV⁺ and EYFP⁺ H-2K^b-VNHRFTLV⁻ CD8⁺ cells in the ASP-2 and ASP-2/*T. cruzi* mice. (D) Frequencies of EYFP⁺ H-2K^b-VNHRFTLV⁺ CD8⁺ T cells/total EYFP⁺ CD8⁺ T cells. No significant difference was found between the frequency of the EYFP⁺ H-2K^b-VNHRFTLV⁺ CD8⁺ cells in the ASP-2/*T. cruzi* mice compared with that of the ASP-2 animals ($p = \text{NS}$). Results are from one of three independent experiments performed.

CD8⁺ cells from *T. cruzi*-infected mice incorporated this marker. In mice that were vaccinated but not challenged (ASP-2), proliferation of specific CD8⁺ T cells was minor (14%). Finally, we observed that only 36% of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells from ASP-2/*T. cruzi* incorpo-

rated BrdU. We concluded that although a fraction of the specific T cells proliferated, the vast majority did not. Similar experiment is also shown in Supplementary Fig. S5C. In this case, the incorporation of BrdU was detected only in 12.1% of the specific CD8⁺ T cells. In this figure, we also

show that the BrdU incorporation in splenic cells in this mouse group (ASP-2/*T. cruzi*) is not altered and occurred in parallel (Supplementary Fig. 5SD).

To determine whether the increase in frequency of specific TE(M) cells and the protective immunity they mediate is dependent on proliferation, we treated the immunized mice with the cytostatic toxic agent HU. We followed the immunization and treatment schedule shown in Fig. 6A. Fifteen days after challenge, we estimated the frequency of H-2K^b-VNHRFTLV⁺ CD8⁺ cells in the spleens of ASP-2/*T. cruzi* mice that were treated or not with HU. We did not detect a significant difference when compared the frequency or total number of H-2K^b-VNHRFTLV⁺ CD8⁺ cells per spleen between these two groups (p =NS, Fig. 6B and C, respectively). The HU treatment in this experiment led to a significant decrease in the total number of spleen cells. On average, the spleen cell number of the untreated mice was 1.56-fold higher than that of the HU-treated mice (p <0.05). Also, we performed BrdU incorporation experiments to

FIG. 6. The increase in the specific splenic CD8⁺ T cell frequency after infectious challenge is not inhibited by treatment with hydroxyurea (HU). (A) C57BL/6 mice were immunized, infected, and treated with HU as depicted. The mouse groups were the same as those described in the legend of Fig. 1 except that β Gal denotes mice that were injected with pcDNA3 and Ad β -Gal. (B) Fourteen days after challenge, the H-2K^b-VNHRFTLV⁺ CD8⁺ cell frequency was estimated. Representative plot of each group. (C) Number of splenic H-2K^b-VNHRFTLV⁺ CD8⁺ cell expressed as mean $\pm$ SD (n =4). Results are from one of two independent experiments performed.

confirm whether the treatment indeed inhibited proliferation as we expected. As shown in Supplementary Fig. S5D, BrdU was incorporated by 69.4% or 41.1% of splenic cells from *T. cruzi*-infected mice (β Gal/*T. cruzi* or ASP-2/*T. cruzi*, respectively; Supplementary Fig. 5SD). In contrast, only 12.2% of the cells from ASP-2/*T. cruzi* mice treated with HU incorporated BrdU. From this experiment we could confirm that the treatment with HU severely inhibits the proliferation of all splenic cells. Because our ultimate goal was to determine the importance of T cell proliferation in protective immunity, we used the highly susceptible mouse strain A/Sn. In these mice, protective immunity is mediated by CD8⁺ cells that are specific for the epitopes TE-WETGQI, PETLGHEI, and YIEVAGII (De Alencar *et al.*, 2009; Haolla *et al.*, 2009; Dominguez *et al.*, 2011, 2012). These mice were vaccinated and treated according to the protocol described in Fig. 7A. After challenge, the vaccinated mice were treated or not with HU. The mice treated with HU (Fig. 7D) displayed significantly higher parasitemia than the vaccinated mice that were not treated with HU (Fig. 7C; p <0.01). However, their parasitemia was significantly lower than that of the infected β Gal control mice (Fig. 7B; p <0.01).

Despite their higher parasitemia, the vaccinated mice treated with HU recovered, and 90% of them survived the lethal challenge (Fig. 7E). The increase in survival was not statistically significant between the vaccinated mice treated or not with HU. These results led us to conclude that neither the increase in frequency of splenic T cells nor the observed protective immunity is sensitive to treatment with the cytostatic toxic drug HU.

Discussion

In the present study, we described the changes in frequency, phenotype, and function of CD8⁺ T cells after infectious challenge with *T. cruzi* in mice vaccinated by heterologous prime-boost with plasmid DNA and an adenoviral vector. The most important observations made in this study were that after the infectious challenge, the frequency of splenic CD8⁺ specific T cells increased and that these cells differentiated into KLGR1^{High}CD27^{Low} cells. Most of the cells were multifunctional and produced IFN γ and TNF upon antigen restimulation. Using transgenic gzmBCreERT2/ROSA26EYFP mice, we confirmed that the specific T cells in the spleen were in fact CD8⁺ T cells that had been expanded after vaccination. Not only did these vaccine-induced T cells dominate the immune response after infectious challenge, but they also completely inhibited the expansion of specific naïve T cells (Supplementary Fig. S4). Although the mechanism that leads to inhibition of those specific naïve T cells was beyond the scope of our study, it can be hypothesized that the inhibition of parasite multiplication reduced the amount of antigen available for efficient priming. In addition, it has been described that antigen-specific activated CD8⁺ T cells are potent inhibitors of the priming of naïve cells, as they rapidly eliminate antigen-presenting cells (Hafalla *et al.*, 2003; Wong *et al.*, 2003).

Despite the increase in the frequency of specific T cells, we could not find evidence that this rise was because of antigen-specific proliferation. Indeed our results showed that

FIG. 7. The protective immunity elicited by heterologous prime-boost vaccination is not inhibited by treatment with HU. **(A)** A/Sn mice were immunized, infected, and treated with HU as depicted. **(B)** Parasitemia of each individual control mouse primed with pcDNA3 and boosted with Ad β -Gal ($n = 12$). **(C)** Parasitemia of each individual ASP-2-immunized mouse ($n = 12$). **(D)** Parasitemia of each individual ASP-2-immunized mouse treated with HU ($n = 10$). Although the day of peak parasitemia was different for each mouse, we used the maximal values for statistical comparison. The results of the statistical comparisons are below panel D. **(E)** Kaplan-Meier survival curves of the different groups were compared, and the results showed that ASP-2-immunized mice treated or not with HU survived significantly longer ($p < 0.01$) than control animals injected with β Gal. Results are from two experiments pooled.

the ratio of activated CD8⁺ EYFP-expressing cells that were specific for the *T. cruzi* epitope or the adenoviral carrier did not change in mice challenged with the parasite (Fig. 5). In addition, treatment with the drug HU did not significantly reduce the frequencies of specific T cells after challenge infection (Fig. 6 and Supplementary Fig. S5). These observations are in agreement with BrdU incorporation experiments. We did not observe significant labeling of specific CD8⁺ cells after the infectious challenge of vaccinated mice. In contrast, in nonvaccinated and challenged mice, the majority of T cells were labeled with BrdU, denoting an intense proliferation (Supplementary Figs. S4 and S5). The anamnestic potential of CD8⁺ T cells primed by adenoviral vectors has been long shown to be excellent when boosted with recombinant vaccinia viruses (Bruña-Romero *et al.*, 2001). However, more recently, using other antigenic stimuli, different groups have described a poor

anamnestic response of CD8⁺ T cells primed with AdHu5 vector when compared with other adenovirus vector serotypes (Penaloza-MacMaster *et al.*, 2013; Tan *et al.*, 2013). Perhaps, in our case, *T. cruzi* belongs to this class of poor AdHu5-induced TE(M) cell activators.

Secondary responses depend on the timing of the booster injection (Bruna-Romero *et al.*, 2001). This is probably because memory T cells need to rest before they can be restimulated with the antigen. In our case, we tested at different periods of time until 165 days and could not find remarkable differences in the increase in frequencies of specific T cells after challenge (Fig. 1B). These observations confirm that *T. cruzi* challenge at any timing after the immunization led to similar changes in frequencies of splenic specific CD8⁺ TE(M) cells. BrdU incorporation further corroborates with these observations. Also, these observations are important because they may indicate a path for

vaccine improvement. Other adenovirus vectors of distinct serotypes expressing *T. cruzi* antigens may provide better priming for parasite boosting.

The increase in frequencies of specific CD8⁺ T cells in the spleen after challenge infection alone does not seem to account for the observed protective immunity. In our previous study, we showed that vaccinated mice that were administered FTY720 were significantly more susceptible to infection than untreated, vaccinated control animals (Dominguez *et al.*, 2012). In FTY720-treated and vaccinated mice, we still observed a similar increase in the frequencies of specific CD8⁺ T cells after challenge (J.R.V., M.R.D., R.L., and M.M.R., unpublished results). Therefore, we concluded that the increase in these frequencies does not always correlate with or predict efficient protective immunity.

The increase in the frequency of the H-2K^b-VNHRFTLV⁺ CD8⁺ cells in the spleens of the ASP-2/*T. cruzi* mice possibly represents a major recirculation event of the activated CD8⁺ T cells. As mentioned above, the frequency of the splenic EYFP⁺ cells increases in a manner that is apparently independent of their specificity. This ability to rapidly recirculate might be associated with their phenotype, as discussed below. Alternatively, it might be caused by the infection. These mice display visible signs of splenomegaly. The factors that cause this splenomegaly and the influx of activated T cells after an infectious challenge are still poorly understood and remain an interesting aspect to be investigated.

The predominant phenotype of the specific CD8⁺ T cells detected after challenge was KLRG1^{High} CD27^{Low} CD43^{Low} CD183^{Low} *T-bet*^{High} *Eomes*^{Low} (Fig. 3C and D). These cells were also CD62L^{Low} and CD127^{Low} (Supplementary Fig. S2). This phenotype resembles the recently described phenotype of effector-like memory CD8⁺ T cells, which persist to the memory phase, providing efficient control of infection with *Listeria monocytogenes* or vaccinia virus (Olson *et al.*, 2013). These authors described that the number of these effector-like memory CD8⁺ T cells is greatly increased in the blood and splenic red pulp and is greatly reduced in the lymph nodes (Olson *et al.*, 2013). Although we showed that these cells are reduced in the draining lymph nodes, we have yet to determine if our T cells also share other characteristics that could explain the observed rapid recirculation event.

In addition to their phenotype, these two protective CD8⁺ T cell types share functional similarities. Both of them express Granzyme B (Figs. 4 and 5) (Olson *et al.*, 2013). They also require the expression of perforin to mediate efficient protective immunity (De Alencar *et al.*, 2009; Olson *et al.*, 2013).

The CD8⁺ TEM cells described herein also resemble CD8⁺ TEM cells that have been previously detected after heterologous prime-boost vaccination using different viral vectors (Fraser *et al.*, 2013). However, it is important to note that in our case, the expression of PD1 is much lower. In fact, the expression of molecules that control cell survival/apoptosis, such as PD1, PDL-1, CD95, TRAIL, TRAIL2, and TIM3, is always low (Supplementary Fig. S1). In contrast, high levels of the activation markers CD44, CD11a, CD11c, CD38, and CD49d are a hallmark (Supplementary Fig. S1). A similar phenotype (PD1^{Low}) was described for the specific CD8⁺ T cells elicited by heterologous prime-

boost vaccination with plasmid DNA and recombinant, replication-defective adenovirus (AdHu5 and AdHu35) (Steffensen *et al.*, 2013). Therefore, PD1^{Low} phenotype may represent a general characteristic of the CD8⁺ T cells elicited by adenovirus vector immunization, as we have previously described (Vasconcelos *et al.*, 2012a).

Treatment with HU after challenge did not significantly modify the frequency of splenic specific CD8⁺ T cells or impair the observed protective immunity. Earlier studies using HU treatment have shown exactly the opposite effects. Treatment with HU was found to completely abolish memory responses to lymphocytic choriomeningitis virus antigens (LCMV) (Bellier *et al.*, 2003). These authors postulate that memory T cells must continue to proliferate to maintain immunological memory. The discrepancies in these results may reflect the fact that the memory T cells elicited by LCMV infection are mostly TCM cells (CD62L^{High}CD127^{High}) (Wherry *et al.*, 2003). TCM cells display high homeostatic proliferation. In contrast, TEM cells have limited homeostatic and antigen-driven proliferation (Wherry *et al.*, 2003). Therefore, TCM cells might be more susceptible to HU treatment than TEM cells. The possibility that the HU treatment acts upon parasite elimination is unlikely. C57BL/6 mice infected with *T. cruzi* and treated HU from day 7 to day 13 have been found to uniformly succumb to infection (M.R.D. and M.M.R., unpublished results). The result that the T cells from vaccinated mice do not need to proliferate supports the concept that the high frequency of TE(M) cells present after the prime-boost immunization regimen in our model is sufficient to mediate protective immunity. Because our experiments were performed only 2 weeks after boost immunization (Figs. 5–7), it is possible that at longer times, when the frequency of specific T CD8⁺ T cells declines (more than 120 days), they may need to proliferate to mediate protective immunity. Alternatively, protective immunity may be lost as the number of cells decline. Although we consider these experiments important, because of their long duration, we plan to study this issue in the future.

These results complement our previous observation that CD8⁺ T cells must recirculate to mediate protective immunity against *T. cruzi* infection (Dominguez *et al.*, 2012). To corroborate this observation, the molecular mechanisms used by these T cells to recirculate are currently being explored. We observed that in addition to FTY720, antibodies against integrins and chemokines drastically impact the protective immune response without impairing the specific immune response (F.V., M.M.R., and J.R.V., unpublished results). In addition to integrins and chemokines, a molecular mechanism that controls the ability of the CD8⁺ T cells to recirculate has been very recently described. Recirculating CD8⁺ T cells express the transcription factor KLF2. This factor increases the expression of the S1Pr1 receptor on CD8⁺ TE cells, allowing them to continue to recirculate (Skon *et al.*, 2013). Our protective T cells certainly express the S1Pr1 receptor because they are targets of the drug FTY720.

In summary, our evidence supports the model that when large amounts of antigen-experienced CD8⁺ TEM cells are present after heterologous prime-boost vaccination, differentiation and recirculation, rather than proliferation, are key for the resultant protective immunity.

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Author Disclosure Statement

R.T.G., M.M.R., A.V.M., and O.B.-R. are named inventors on patent applications covering *Trypanosoma cruzi*-vectored vaccines and immunization regimens. No competing financial interests exist for the remaining authors.

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Research Article

Genetic Vaccination against Experimental Infection with Myotropic Parasite Strains of *Trypanosoma cruzi*

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In earlier studies, we reported that a heterologous prime-boost regimen using recombinant plasmid DNA followed by replication-defective adenovirus vector, both containing *Trypanosoma cruzi* genes encoding *trans*-sialidase (TS) and amastigote surface protein (ASP) 2, provided protective immunity against experimental infection with a reticulotropic strain of this human protozoan parasite. Herein, we tested the outcome of genetic vaccination of F1 (CB10XBALB/c) mice challenged with myotropic parasite strains (Brazil and Colombian). Initially, we determined that the coadministration during priming of a DNA plasmid containing the murine IL-12 gene improved the immune response and was essential for protective immunity elicited by the heterologous prime-boost regimen in susceptible male mice against acute lethal infections with these parasites. The prophylactic or therapeutic vaccination of resistant female mice led to a drastic reduction in the number of inflammatory infiltrates in cardiac and skeletal muscles during the chronic phase of infection with either strain. Analysis of the electrocardiographic parameters showed that prophylactic vaccination reduced the frequencies of sinus arrhythmia and atrioventricular block. Our results confirmed that prophylactic vaccination using the TS and ASP-2 genes benefits the host against acute and chronic pathologies caused by *T. cruzi* and should be further evaluated for the development of a veterinary or human vaccine against Chagas disease.

1. Introduction

Chagas disease is an acute and chronic illness caused by *Trypanosoma cruzi*, an obligatory intracellular protozoan parasite that is endemic in the Americas. The disease currently affects millions of people who are chronically infected. Thousands of new cases are also estimated to occur every year [1, 2]. Prophylactic measures aimed at eliminating transmission by the vector (kissing bugs) have been very successful in many countries [3]. Considering their success and the highly neglected state of Chagas disease research, vaccine development has often been considered a difficult and expensive strategy for disease control and eradication.

Despite this general consideration, recent theoretical studies have shown a divergent perspective on the problem, indicating that the development of a vaccine against Chagas disease would be cost effective even if the efficacy is not high and the transmission is low [4, 5]. In moving toward the development of vaccines against Chagas disease, several laboratories have described a number of antigens and delivery systems capable of providing some degree of protective immunity against experimental infections. These systems include genetically attenuated parasites, recombinant protein in adjuvant systems, and genetically modified bacteria or viral vectors (reviewed in [6–12]). The antigens pursued for recombinant vaccines include *T. cruzi* trans-sialidase, cruzain (a cysteine proteinase), and the amastigote surface proteins 2, 3, 4, TcG1, TcG2, TcG4, TSA-1, and Tc24 (reviewed in [6–12]).

Our group in particular has been working for many years on the development of genetic vaccines against *T. cruzi* infection. We initially used the gene encoding the catalytic domain of the parasite trans-sialidase (TS, [13, 14]). More recently, we have described studies using a heterologous prime-boost regimen consisting of priming with plasmid DNA followed by a booster injection with replication-defective recombinant human adenovirus type 5. Both the prime and the boost contained the gene encoding the amastigote surface protein (ASP)-2 antigen of *T. cruzi*. This regimen successfully vaccinated highly susceptible A/Sn and resistant C57BL/6 mice against infection with the reticulotropic Y strain of *T. cruzi* [15–18]. Protective immunity was mediated by long-lived CD4⁺ and CD8⁺ T Effector or effector memory cells [15, 19, 20].

T. cruzi infection in mammalian hosts leads to diverse clinical manifestations. Among the most relevant factors influencing this diversity is the existence of biologically different parasite strains. *T. cruzi* intraspecific nomenclature was established in 2009, and the isolates and strains are assigned to one of six genetic groups or discrete typing units, named TcI to TcVI [21]. Among domestic transmission cycles, TcI occurs predominantly in northern South America, while TcII, TcV, and TcVI are more often observed in the Southern Cone countries [22]. Considering this genetic variability, it is important that studies of vaccination or chemotherapy are conducted in different experimental models using distinct parasite strains. Because our previous studies of vaccination have been performed with the reticulotropic Y strain (TcII), it was our intention to extend these studies using experimental

infections with two myotropic strains of the parasites belonging to TcI (Colombian) and TcII (Brazil).

2. Materials and Methods

2.1. Ethics Statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (<http://www.cobea.org.br/>). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institutional Animal Care and Use Committee at the Federal University of Sao Paulo (Id # CEP 0426/09).

2.2. Mice and Parasites. Female or male 5- to 8-week-old F1 (CB10XBALB/c) mice were purchased from CEDEME (Federal University of São Paulo). Bloodstream trypomastigotes of the Colombian or Brazil strain of *T. cruzi* were obtained from mice infected 21–28 days earlier. The concentration of parasites was estimated and adjusted to 10^4 parasites/mL. Each mouse was inoculated with 10^3 trypomastigotes diluted in 0.1 mL PBS and administrated subcutaneously (s.c.) in the base of the tail.

2.3. Peptides. Synthetic peptides (Genscript, Piscataway, New Jersey) were higher than 90% pure. The immunodominant epitopes of ASP-2 or TS were represented by AA VNHRFTLV or IYNVGQSVI, respectively.

2.4. Genetic Vaccination. The plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of murine (m) IL-12 was generated as earlier described [23]. Plasmids p154/13 (TS gene) or pIgSPclone9 (ASP-2 gene) were generated, grown, and purified as described earlier [13, 24]. Human replication deficient adenovirus type 5 expressing the ASP-2 gene (AdASP-2) or TS gene (AdTS) was generated and produced as described earlier [25]. Control mice were immunized with pcDNA3 and human replication deficient adenovirus type 5 expressing β -galactosidase (Ad β -gal). F1 (CB10XBALB/c) mice were immunized i.m. in each *tibialis anterioris* muscle with plasmid DNA. For priming, control mice received plasmid pcDNA3 (300 μ g), a second group was immunized with a mixture of pIgSPC19 and p154/13 (200 μ g), and the third, a mixture of pIL12, pIgSPC19, and p154/13 (300 μ g). Twenty-one days later, these mice received in these same spots 100 μ L of viral suspension containing a total of 2×10^8 plaque forming units (pfu) of rec. adenovirus. For boosting, control mice received adenovirus Ad β gal; other mouse groups were immunized with a mixture of rec. adenovirus AdASP-2 and Ad-TS. Immunological assays were performed 14 days after viral inoculation.

Therapeutic vaccination was performed as follows. F1 (CB10XBALB/c) female mice were challenged s.c. with 1,000 trypomastigotes of the indicated parasite strain. Thirty days later, control mice received a mixture of plasmid pcDNA3 and of pIL12 (300 μ g) and second mice group was immunized with a mixture of pIL12pIgSPC19 and p154/13 (300 μ g). Twenty days later, these mice received in these same spots

100 μ L of viral suspension containing a total of 2×10^8 pfu of rec. adenovirus. For boosting, control mice received adenovirus Ad β gal and the other mice were immunized with a mixture of rec. adenovirus AdASP-2 and Ad-TS.

2.5. Immunological Assays. For the surface intracellular expression of cytokines (IFN γ and TNF, ICS) or translocation of CD107a to the membrane, splenocytes collected from immune mice were treated with ACK buffer. ICS and surface mobilization of CD107a were evaluated after *in vitro* culture of splenocytes in the presence or absence of the antigenic stimulus. Cells were washed 3 times in plain RPMI and resuspended in cell culture medium consisting of RPMI 1640 medium, pH 7.4, supplemented with 10 mM Hepes, 0.2% sodium bicarbonate, 59 mg/L of penicillin, 133 mg/L of streptomycin, and 10% Hyclone fetal bovine sera (Hyclone, Logan, Utah). The viability of the cells was evaluated using 0.2% trypan blue exclusion dye to discriminate between live and dead cells. Cell concentration was adjusted to 5×10^6 cells/mL in cell culture medium containing FITC-labeled anti-CD107a (2 μ g/mL), anti-CD28 (2 μ g/mL), BdGolgiPlug, and monensin (5 μ g/mL). In part of the cultures, a final concentration of 10 μ M of the VNHRFTLV or IYNVGQSVI peptides was added. The cells were cultivated in flat-bottom 96-well plates (Corning) in a final volume of 200 μ L in duplicate, at 37°C in a humid environment containing 5% CO₂. After 12 h incubation, cells were stained for surface markers with PerCP-labeled anti-CD8, on ice for 20 min. To detect IFN γ and TNF by intracellular staining, cells were then washed twice in buffer containing PBS, 0.5% BSA, and 2 mM EDTA, fixed and permeabilized with BD perm/wash buffer. After being washed twice with BD perm/wash buffer, cells were stained for intracellular markers using APC-labeled anti-IFN γ (Clone XMGI.2) and PE-labeled anti-TNF (clone MP6-XT22). Finally, cells were washed twice with BD perm/wash buffer and fixed in 1% PBS-paraformaldehyde. At least 300,000 cells were acquired on a BD FACSanto flow cytometer and then analyzed with FlowJo.

2.6. Electrocardiogram (ECG). Mice were i.p. tranquilized with diazepam (20 mg/kg) and transducers were carefully placed under the skin in accordance with chosen preferential derivation (DII). Traces were recorded using a digital system (Power Lab 2/20) connected to a bioamplifier in 2 mV for 1 s (PanLab Instruments). Filters were standardized between 0.1 and 100 Hz and traces were analyzed using the Scope software for Windows V3.6.10 (PanLab Instruments). We measured heart rate (beats per minute—bpm), duration of the PR, QRS, and QT intervals, and P wave in ms (millisecond) on 222 dpi. The relationship between the QT interval and RR interval was individually assessed to obtain physiologically relevant values for the heart rate-corrected QT interval (QTc) through Bazze's formula.

2.7. Morphometric Analyses. Analyses were performed essentially as described in [26]. Briefly, heart sections were analyzed by light microscopy after paraffin embedding, followed by standard hematoxylin and eosin staining. Inflammatory

cells infiltrating heart tissue were counted using a digital morphometric evaluation system. Images were digitized using a color digital video camera (CoolSnap, Photometrics, Montreal, QC, Canada) adapted to a BX41 microscope (Olympus, Tokyo, Japan). Morphometric analyses were performed using the software Image-Pro Plus v.7.0 (Media Cybernetics, San Diego, CA, USA). The inflammatory cells were counted in 10 fields ($\times 400$ view)/heart. All of the analyses were performed in a blinded fashion.

2.8. Statistical Analysis. For the purpose of comparing the different mouse groups, we used the statistical analyses suggested by [27]. The values were compared using One Way ANOVA followed by Tukey's HSD tests or Fisher exact probability test (<http://faculty.vassar.edu/lowry/VassarStats.html/>). The Logrank test was used to compare mouse survival rates after challenge with *T. cruzi* (<http://bioinf.wehi.edu.au/software/russell/logrank/>). The differences were considered significant when the *P* value was <0.05 .

3. Results

3.1. Addition of pIL-12 during Priming Improved the Immune Response Mediated by CD8⁺ T Cells and Increased Protective Immunity against Acute Infection. A previous study has reported that the coadministration of plasmid DNA expressing IL-12 during priming improves the efficacy of a heterologous prime-boost vaccination regimen, as estimated by protective immunity against SIV infection [28]. We, therefore, decided to determine whether this strategy could also improve the immune response and protective immunity against *T. cruzi* infection in the mouse model. Mice were immunized with the following: (i) pcDNA3 followed by a booster injection of Ad β gal (G1, control); (ii) p154/13 and pIgSPC19 followed by a booster injection of AdASP-2 and AdTS (G2); (iii) pIL-12, p154/13 and pIgSPC19 followed by a booster injection of AdASP-2 and AdTS (G3).

To compare the different mouse groups, we stained CD8⁺ spleen cells following *in vitro* peptide stimulation for the surface mobilization of CD107a, a marker for T cell degranulation, and intracellular effector cytokines (IFN γ and TNF, ICS). Figure 1(a) depicts examples of the frequency estimates of peptide-specific CD8⁺ spleen cells that mobilized CD107a to their surface and expressed IFN γ . The frequencies of CD8⁺ cells that mobilized CD107a to the cell surface and expressed IFN γ were higher in G3. Control mice (G1) had a negligible number of specific CD8⁺CD107a⁺IFN γ ⁺ cells. These frequencies were dependent on the presence of peptide in culture because the frequency of CD8⁺CD107a⁺IFN γ ⁺ cells was very small in the absence of peptide in all groups.

We made a similar observation when we estimated the frequencies of splenic peptide-specific CD8 cells that expressed IFN γ or IFN γ and TNF. These frequencies were higher in G3 mice (Figure 1(b)). As described above, control mice (G1) had a negligible number of specific CD8⁺IFN γ ⁺ or CD8⁺IFN γ ⁺TNF⁺ cells. The numerical differences and the statistical significances are depicted in Figures 1(c)–1(f). As shown in Figures 1(d) and 1(f), following peptide

FIGURE 1: Frequencies of specific cytokine-secreting splenic CD8⁺ T cells in mice vaccinated with the heterologous prime-boost vaccination regimen. F1 (CB10XBALB/c) mice were immunized i.m. in each *tibialis anterior* muscle with plasmid DNA. For priming, G1 received control plasmid pcDNA3 (300 μ g), G2 was immunized with a mixture of pIgSPCL9 and p154/13 (200 μ g), and G3 received a mixture of pIL-12, pIgSPCL9, and p154/13 (300 μ g). Twenty-one days later, these mice received, in these same spots, 100 μ L of a viral suspension containing a total of 2×10^8 pfu of rec. adenovirus. For boosting, G1 received control adenovirus Ad β -gal, and G2 and G3 were immunized with a mixture of rec. adenovirus AdASP-2 and Ad-TS. Immunological assays were performed 14 days after viral inoculation. Spleen cells were restimulated *in vitro* in the presence or absence of the peptides VNHRFTLV or IYNVGQSVI, anti-CD107a, anti-CD28, BdGolgiPlug, and monensin. After 12 h, the cells were stained with anti-CD8, anti-IFN γ , and anti-TNF. (a) The histograms show FACS analysis of CD8⁺ cells. The numbers represent the frequencies of cells stained for CD107a and/or IFN γ . The results are a representative of 4 mice per group (Median). (b) Frequencies of CD8⁺ cells stained for IFN γ and/or TNF. The results are a representative of 4 mice per group (Median). (c) Total frequencies of CD8⁺ cells specific for the peptide VNHRFTLV and stained for CD107a or IFN γ or TNF. The results are representative of 4 mice per group (Mean $\pm$ SD). (d) Frequencies of CD8⁺ cells specific for the peptide VNHRFTLV and stained for each marker (CD107a, IFN γ , or TNF). The results are representative of 4 mice per group (Mean $\pm$ SD). (e) Same as in (c) for CD8⁺ cells specific for the peptide IYNVGQSVI. The results are representative of 4 mice per group (Mean $\pm$ SD). (f) Same as in (d) for CD8⁺ cells specific for the peptide IYNVGQSVI. All three groups were compared statistically by one-way ANOVA followed by Tukey HSD (c–e). The values of G2 and G3 mice were always higher than those of G1 mice ($P < 0.01$ in all cases). Asterisks denote that the values of G3 mice were higher than those of G2 mice ($P < 0.05$). The results are representative of three independent experiments.

stimulation *in vitro*, most of the CD8⁺ cells were either CD107a⁺IFN γ ⁺TNF⁺ or CD107a⁺IFN γ ⁺.

To determine whether this improvement in the frequencies of specific CD8⁺ T cells would impact protective immunity, we vaccinated susceptible male mice and challenged them with trypanosomes of either the Brazil or Colombian strain. As shown in Figures 2(a) and 2(c), mice vaccinated with the TS and ASP-2 genes (G2 and G3) presented a statistically significant reduction in peak parasitemia. A comparison between these two groups also showed that the parasitemia in G3 was significantly lower than that in G2. Not only did mice from G3 display lower parasitemia, but vaccination also significantly reduced mouse mortality

(Figures 2(b) and 2(d)). While all G3 mice survived the experimental challenge, most control (G1) and G2 mice died after challenge with the Brazil or Colombian parasite strain. Based on these results, we concluded that the use of pIL-12 for priming significantly improved the immune response and the protective immunity against acute infection with the myotropic Brazil and Colombian strains.

3.2. Impact of Genetic Vaccination against Chronic Infection. To evaluate the impact of genetic vaccination on the chronic symptoms of the experimental infection, resistant female mice were treated according to the following protocols: (i) naïve (G1); (ii) pIL-12 and pcDNA3 followed by a booster

FIGURE 2: The protective immunity elicited by heterologous prime-boost vaccination in susceptible mice. F1 (CB10XBALB/c) male mice were immunized i.m. exactly as described in the legend of Figure 1. Fourteen days later, mice were challenged s.c. with 1,000 trypomastigotes of the indicated parasite strain. (a) Mean parasitemia $\pm$ SD of each mouse group ($n = 6$) challenged with parasites of the Brazil strain. The values of G2 and G3 mice at days 21, 25 and 35 were lower than those of G1 mice ($P < 0.01$ in all cases, one-way ANOVA, Tukey HSD). Asterisks denote that the values of G3 mice were lower than those of G2 mice ($P < 0.05$); (b) The Kaplan-Meier survival curves of the different groups were compared, and the results showed that G3 survived significantly longer ($P < 0.01$) than the two other groups; (c) Mean parasitemia $\pm$ SD of each mouse group ($n = 6$) challenged with parasites of the Colombian strain. The values of G2 and G3 mice at days 25 and 31 were lower than those of G1 mice ($P < 0.01$ in all cases). Asterisks denote that the values of G3 mice were lower than those of the G2 mice ($P < 0.05$); (d) The Kaplan-Meier survival curves of the different groups were compared, and the results showed that G3 survived significantly longer ($P < 0.01$) than the two other groups. The results were obtained from one of two independent experiments.

TABLE 1: Summary of the ECG records.

Days after challenge	Strain	Sinus arrhythmia mouse groups			P values		
		G1	G2	G3	G1 × G2	G1 × G3	G2 × G3
80	Brazil	0/6	0/6	0/6	NS	NS	NS
150	Brazil	0/6	19/6	0/6	0.001	NS	0.001
240	Brazil	0/6	20/6	3/6	0.001	NS	0.026
80	Colombian	0/6	0/6	0/5	NS	NS	NS
150	Colombian	0/6	1/6	1/5	NS	NS	NS
240	Colombian	0/6	9/6	1/5	0.017	NS	0.093
Days after challenge	Strain	Atrioventricular block			P values		
		G1	G2	G3	G1 × G2	G1 × G3	G2 × G3
80	Brazil	0/6	0/6	0/6	NS	NS	NS
150	Brazil	0/6	4/6	2/6	0.03	NS	NS
240	Brazil	0/6	59/6	7/6	0.001	0.004	0.003
80	Colombian	0/6	19/6	0/5	0.001	NS	NS
150	Colombian	0/6	14/6	6/5	0.001	0.002	NS
240	Colombian	0/6	39/6	10/5	0.001	0.002	NS

ECG record of mice genetically vaccinated and challenged as described in the legend of Figure 5(a). Values represent the number of episodes (sinus arrhythmia or atrioventricular block) recorded during one minute per total of mice analyzed and were compared by Fisher exact probability test.

injection of Ad β gal (G2, control); (iii) pIL-12, p154/13 and pIgSPC19 followed by a booster injection of AdASP-2 and AdTS (G3).

G2 and G3 mice were subsequently challenged with parasites of either the Brazil or the Colombian strain, as depicted in Figure 3(a). We estimated the level of parasitemia for each mouse group. G3 mice presented a lower peak parasitemia at day 27 ($P = 0.02$) after infection with parasites of the Brazil strain (Figure 3(b)). In the case of mice challenged with parasites of the Colombian strain, there was a statistically significant reduction in the levels of parasitemia at days 13, 20, 24, 28, and 31 ($P < 0.05$ in all cases, Figure 3(c)).

Individual ECGs were evaluated at days 80, 150, and 240 following infection. As shown in Table 1, the primary abnormalities we detected were sinus arrhythmia and atrioventricular block. Significantly lower frequencies of sinus arrhythmia were observed in G3 mice when compared with G2 mice following infection with the Brazil or Colombian strains. These differences were observed at later days (150 or 240 days) following challenge. Similarly, significantly lower frequencies of atrioventricular block were observed in G3 mice when compared with G2 mice following infection with parasites of the Brazil strain. In the case of mice challenged with parasites of the Colombian strain, the differences were smaller and not statistically significant.

At day 240 after challenge, the mice were euthanized, and morphometric analyses of the heart and skeletal muscle were performed. The number of inflammatory cells within the skeletal muscle, but not the heart, of G3 mice challenged with parasites of the Brazil strain was significantly lower (Figure 4(b)). In G3 mice challenged with parasites of the Colombian strain, we found significantly lower numbers of inflammatory cells within the skeletal muscle and heart when compared to G2 mice (Figure 4(c)).

We then evaluated the impact of therapeutic genetic vaccination on the chronic symptoms of experimental infection. Resistant female mice were infected and then treated

according to the protocols described in Figures 5(a) and 6(a). Mice received the following treatments: (i) pIL-12 and pcDNA3 followed by a booster injection of Ad β gal (G2, control); (ii) pIL-12, p154/13 and pIgSPC19 followed by a booster injection of AdASP-2 and AdTS (G3).

The estimation of parasitemia until day 50 did not reveal any difference between G2 or G3 mice infected with parasites of the Brazil or Colombian strains (Figures 5(b) and 5(c), resp.). We concluded that the plasmid administration at day 30 after challenge did not elicit immunity that could reduce the ongoing parasitemia. Individual ECGs were evaluated at days 90, 120, 150, and 180 following infection. As shown in Table 2, we detected differences in the frequencies of sinus arrhythmia, but not atrioventricular block, in G3 mice when compared with G2 mice following infection with the Brazil strain of *T. cruzi*. However, this difference was observed only on days 90 and 120. Later (150 and 180 days), G3 mice also developed sinus arrhythmia. G3 mice infected with parasites of the Colombian strain had significantly lower frequencies of sinus arrhythmia at days 120 and 180, when G2 mice developed a significant number of events. However, the occurrence of atrioventricular block in G3 mice was similar to that in G2. At day 180 after challenge, the mice were euthanized, and morphometric analyses of the heart and skeletal muscle were performed. The numbers of inflammatory cells within the skeletal muscle and heart of G3 mice challenged with parasites of the Brazil or Colombian strains were significantly lower than the frequencies in the tissues of G2 mice (Figures 6(b) and 6(c), resp.).

4. Discussion

The present study evaluated the outcome of experimental infection with two myotropic *T. cruzi* strains in F1 (CB10XBALB/c) mice genetically vaccinated with TS and ASP-2 following a heterologous DNA prime-adenovirus boost regimen. Prophylactic vaccination reduced the acute

FIGURE 3: The protective immunity elicited by heterologous prime-boost vaccination in resistant mice. (a) F1 (CB10XBALB/c) female mice were immunized i.m. as depicted. For priming, G2 mice received a mixture of control plasmid pcDNA3 and of pIL-12 (300 μ g), and G3 mice were immunized with a mixture of pIL-12, pIgSPCL9 and p154/13 (300 μ g). Twenty-one days later, these mice received, in these same spots, 100 μ L of a viral suspension containing a total of 2×10^8 pfu of rec. adenovirus. For boosting, G2 mice received control adenovirus Ad β gal, and G3 mice were immunized with a mixture of rec. adenovirus AdASP-2 and Ad-TS. Fourteen days later, mice were challenged s.c. with 1,000 trypomastigotes of the indicated parasite strain. (b) Mean parasitemia $\pm$ SD of each mouse group ($n = 6$) challenged with parasites of the Brazil strain. Asterisk denotes that at day 37, the values of G3 mice were lower than those of G2 mice ($P < 0.05$, one-way ANOVA, Tukey HSD); (c) Mean parasitemia $\pm$ SD of each mouse group ($n = 6$) challenged with parasites of the Colombian strain. Asterisks denote that at days 13 to 31, the values of G3 mice were lower than those of G2 mice ($P < 0.05$).

FIGURE 4: Frequencies of inflammatory cells in the hearts and skeletal muscles of resistant mice vaccinated by the heterologous prime-boost regimen. (a) F1 (CB10XBALB/c) female mice were immunized i.m., as depicted. Details on the dose are shown in Figure 3(a). (b) At 240 days, heart and skeletal muscle sections of mice challenged with the Brazil strain were stained with hematoxylin and eosin, and the number of inflammatory cells was quantified. Bars represent the mean of 6 mice/group $\pm$ SD. Asterisks denote that there was a significant difference between the indicated groups (* P < 0.05 or ** P < 0.01). (c) The same as above, except that the mice were challenged with parasites of the Colombian strain. Bars represent the mean of 5 mice/group $\pm$ SD.

phase symptoms, as estimated by parasitemia and mortality. In addition, ECG analysis demonstrated that prophylactic vaccination also reduced some of the chronic phase signs. These results essentially confirm and extend our previous observations in mice challenged with a reticulotropic *T. cruzi* strain [15–20].

In contrast to prophylactic vaccination, the therapeutic use of our vaccine had a much lower impact on the chronic phase signs evaluated by ECG. The treatment reduced sinus

arrhythmia earlier in mice challenged with the Brazil strain. Additionally, most mice challenged with the Colombian strain never developed episodes of sinus arrhythmia. Despite this observation, no differences were detected in the frequencies of episodes of atrioventricular block. It is important to highlight that these ECG alterations occurred in mice with a reduced number of inflammatory cells in their heart. This dichotomy most likely reflects the successful control by vaccination of the inflammatory reactions later during

FIGURE 5: Impact of therapeutic vaccination in the immunity of resistant mice. (a) F1 (CB10XBALB/c) female mice were challenged s.c. with 1,000 trypomastigotes of the indicated parasite strain. Thirty days later, G2 mice received a mixture of control plasmid pcDNA3 and of pIL-12 (300 μ g), and G3 mice were immunized with a mixture of pIL-12, pIgSPCL9, and p154/13 (300 μ g). Twenty days later, these mice received, in these same spots, 100 μ L of a viral suspension containing a total of 2×10^8 pfu of rec. adenovirus. For boosting, G2 mice received control adenovirus Ad β gal, and G3 mice were immunized with a mixture of rec. adenovirus AdASP-2 and Ad-TS. (b) Mean parasitemia $\pm$ SD of each mouse group ($n = 6$) challenged with parasites of the Brazil strain. (c) Mean parasitemia $\pm$ SD of each mouse group ($n = 6$) challenged with parasites of the Colombian strain.

FIGURE 6: Frequencies of inflammatory cells in the hearts and skeletal muscles of resistant mice treated by the heterologous prime-boost regimen. (a) F1 (CB10XBALB/c) female mice were immunized i.m., as depicted. Details on the dose are shown Figure 5(a). (b) At 180 days, heart and skeletal muscle sections of mice challenged with the Brazil strain were stained with hematoxylin and eosin, and the number of inflammatory cells was quantified. Bars represent the mean of 6 mice/group $\pm$ SD. Asterisks denote that there was a significant difference between the indicated groups (* P < 0.05 or ** P < 0.01). (c) The same as above, except that the mice were challenged with parasites of the Colombian strain.

infection, when the mice were euthanized. However, the damage to the heart had been established earlier and could no longer be reversed.

The reason why the T cells elicited by therapeutic genetic vaccination are not capable of reducing the symptoms is unknown at present. One possibility is that following infection, specific T cells are already committed to express the death receptor CD95, as we have recently described [29]. Once these cells express CD95, their viability is impaired, and they can no longer expand properly. It will be important to test this possibility because it has implications for the use of

T cell vaccines for therapeutic purposes during this or other chronic diseases [30–36].

The protective immune mechanisms mediating the effects of our new vaccination regimen were not addressed in the present study. However, it is plausible that CD4⁺ and CD8⁺ T cells participate in this immune response, as we have previously described [15]. Corroborating this hypothesis is the fact that the addition of pIL-12 during priming led to a significant increase in the frequencies of multifunctional CD8⁺ T cells and a parallel increase in the protective immunity against acute infection.

TABLE 2: Summary of the ECG records.

Days after challenge	Strain	Sinus arrhythmia mouse groups				P values	
		G1	G2	G3	G1 × G2	G1 × G3	G2 × G3
90	Brazil	0/6	10/6	0/6	0.001	NS	0.012
120	Brazil	0/6	10/6	1/6	0.001	NS	0.044
150	Brazil	0/6	11/6	5/6	0.001	0.007	NS
180	Brazil	0/6	4/6	4/6	0.03	0.03	NS
90	Colombian	0/6	3/6	1/5	NS	NS	NS
120	Colombian	0/6	3/6	0/5	NS	NS	NS
150	Colombian	0/6	12/6	0/5	0.001	NS	0.013
180	Colombian	0/6	16/6	1/5	0.001	NS	0.022
Days after challenge	Strain	Atrioventricular block mouse groups				P values	
		G1	G2	G3	G1 × G2	G1 × G3	G2 × G3
90	Brazil	0/6	36/6	46/6	0.001	0.001	NS
120	Brazil	0/6	19/6	22/6	0.001	0.001	NS
150	Brazil	0/6	5/6	6/6	0.007	0.001	NS
180	Brazil	0/6	5/6	3/6	0.007	NS	NS
90	Colombian	0/6	20/6	30/5	0.001	0.001	NS
120	Colombian	0/6	27/6	15/5	0.001	0.001	NS
150	Colombian	0/6	6/6	2/5	0.001	NS	NS
180	Colombian	0/6	10/6	10/5	0.001	0.001	NS

ECG record of mice challenged and treated as described in the legend of Figure 6(a). Values represent the number of episodes (sinus arrhythmia or atrioventricular block) recorded during one minute per total of mice analyzed and were compared by Fisher exact probability test.

In conclusion, this study reinforces and extends our previous work by demonstrating the improvement in acute and chronic symptoms after prophylactic genetic vaccination against *T. cruzi* infection using a vector expressing the TS and ASP-2 antigens.

Conflict of Interests

Ricardo Tostes Gazzinelli, Mauricio Martins Rodrigues, Alexandre Vieira Machado, and Oscar Bruna-Romero are named inventors on patent applications covering *Trypanosoma cruzi* vectored vaccines and immunization regimens. The other authors have no conflict of interests.

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Neglected Tropical Diseases, Bioinformatics, and Vaccines

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(See the major article by Teh-Poot et al on pages 258–66.)

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More than 2.5 billion individuals living in the tropics are estimated at risk of contracting at least 1 neglected tropical disease. Half of these individuals may be exposed to or have concomitantly ≥ 2 neglected tropical diseases, including those caused by helminths, schistosomes, parasitic protozoans, and viruses. Although the death toll caused by all neglected tropical diseases is not as high as for AIDS or tuberculosis, neglected tropical diseases still affect more than a million individuals, most of whom live in the poorest regions of Africa, Asia, and the Americas. In addition to the high mortality, most neglected tropical diseases are due to chronic infections and, therefore, have an enormous impact on childhood growth, disability-adjusted life-years, and productivity-associated economic losses targeting mainly the rural and poorest urban areas of developing countries [1].

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a prototypical example of an neglected tropical disease of Latin America, with an estimated 10 million people chronically infected, causing a large burden of disability-adjusted life-years and billions of annual costs [2]. Chagas disease is endemic in 21 countries, and, in some instances, the number of individuals coming in contact with the parasite (estimated on the basis of seroprevalence) can be as high as 6.75% of the population, as in Bolivia [2, 3]. In addition, because of the migration of chagasic individuals, the disease has also been considered a health problem in developed countries where the disease is not endemic, such as the United States and Spain [4].

Upon contact with the parasite, human hosts may develop a patent parasitemia (acute phase) that, in most cases, will resolve after a few weeks.

Two-thirds of individuals will become serologically positive for *T. cruzi* antigens but will never develop symptoms (the indeterminate form of the disease); a decade or more later, one-third of patients will develop chronic forms of the disease, which can be either cardiac (most common), digestive (megaesophagus and megacolon), or cardiodigestive.

The pathogenesis of chronic Chagas disease is still a matter of intense debate. Some researchers initially proposed that

there is an autoimmune response supporting the chronic inflammatory process [5, 6]. More recently, the identification of parasite DNA in the lesions has led to the hypothesis that parasite persistence is the main driving force leading to tissue inflammation and destruction [6, 7]. However, very recent observations have described heart tissue pathology in the absence of living parasites [8].

Treatment for Chagas disease is effective for acute cases and for children up to 14 years old, with cure rates as high as 100%, and is therefore recommended by the World Health Organization [9]. In adults, treatment success depends on the type of evaluation, using clinical and/or serologic indicators. Although definitive results of large randomized clinical trials (TRAENA and BENEFIT) are still forthcoming, based on previous non-randomized studies, the Latin American Network for Chagas Disease proposes that treatment should be mandatory [10].

Similar to other neglected tropical diseases, prevention needs to be inexpensive, considering the poor economic situation of the regions where much of the disease is transmitted. The most-effective traditional methods for the prevention of Chagas disease are based on control of the triatomine vector, using insecticides and blood screening prior to transfusions. Southern Cone countries (Argentina, Brazil, Chile, and Uruguay) have developed

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successful programs of vector control/elimination and blood screening before transfusion, leading to a significant reduction in transmission of the infection in these countries in the past 30–40 years [11].

Vaccine development as part of the strategy for disease control and eradication was once commonly not considered for Chagas disease [2], and the reasons for this are many. First, there are the successes of vector control and blood bank screening programs. Second, despite numerous efforts, no human vaccine against any parasitic disease has been successfully developed. These factors, combined with the shortage of resources for Chagas disease research, have kept vaccine development off priority lists.

Against this historical trend, recent studies have added a different perspective of the problem. These studies have indicated that vaccination can be cost-effective and economically feasible for a wide range of scenarios, even when the risk of infection is as low as 1% and vaccine efficacy is as low as 25% [12]. These studies support the concept that, despite all of the difficulties, the development of a vaccine against Chagas disease should be pursued. These studies have raised the interest of a nonprofit foundation devoted to the development of vaccines against neglected tropical diseases [13].

Because parasites have multiple developmental forms, which transiently express distinct antigens, immunity to them is very complex. Not only do parasites shift their antigens during their life cycles, they also strongly modulate the immune response in their favor. The complex balance between immune response and parasite escape mechanisms allows the survival of both parasite and host and the establishment of chronic infection [14]. The development of a successful vaccine will depend on how this equilibrium can be shifted in favor of the host, leading to elimination of the parasite.

More than 20 years ago, CD8⁺ T cells were described as being critical for naturally acquired resistance against experimental infection with *T. cruzi* [15].

Accordingly, the epitopes recognized by CD8⁺ T cells from mice and humans have been identified, and immunodominant epitopes have been shown to be present on proteins of the *trans*-sialidase family [16–19]. The immunodominant CD8 epitopes are certainly important targets for protective immunity. Nevertheless, the fact that *trans*-sialidases are a family of highly diverse polypeptides may complicate their use as part of broad subunit vaccines.

On the other hand, several proteins also have subdominant CD8 epitopes [20, 21]. These subdominant epitopes elicit weaker immune responses that can, nonetheless, promote host resistance [22, 23]. Most relevant for the purpose of vaccine development is the fact that subdominant epitopes may be more conserved across distinct parasite strains.

Corroborating the studies on the importance of CD8⁺ T cells during naturally acquired resistance against experimental infection with *T. cruzi*, studies of vaccination using recombinant proteins, plasmid DNA, recombinant viruses, bacteria, and synthetic peptides have provided strong evidence that host protection can only be achieved in the presence of CD8⁺ T cells. Protective immunity is simply not observed in gene-deficient animals without CD8⁺ T cells or upon depletion of these cells by use of treatment with specific antibodies [24–31]. These findings reinforce the concept that the development of a successful vaccine against Chagas disease should, indeed, stimulate CD8⁺ T cells.

CD8⁺ T cells recognize short peptides bound to major histocompatibility complex (MHC) class I molecules. Because humans are highly polymorphic for MHC I alleles, a number of peptides will have to be discovered and assembled to generate a subunit vaccine to elicit broad immunity in a large, heterogeneous population. These multiple peptides must bind to a variety of MHC I alleles and be present in a vast number of parasite strains. To search and identify these short epitopes, a number of computer

programs are being developed, and these bioinformatics tools are used for the identification of CD8⁺ T cell epitopes within viruses and other pathogens [32]. Parasites, however, have large genomes, and some, as in the case of *T. cruzi*, have recently been sequenced [33]. In the current issue of *The Journal of Infectious Diseases*, Teh Poot et al use such bioinformatics tools to search for CD8⁺ T-cell epitopes in the large genome of *T. cruzi* [34].

Using this approach, the authors identified a number of candidate CD8⁺ T-cell epitopes within hypothetical proteins and proteins with putative functions. These epitopes as synthetic peptides were then tested for their ability to stimulate interferon γ production by specific T cells obtained from mice previously infected with *T. cruzi*. Because of their ability to restimulate T cells that were primed during infection, they were selected to be part of a subunit therapeutic vaccine against *T. cruzi* infection.

The authors' prediction was that these peptides would constitute important targets for the development of a CD8⁺ T cell-based therapeutic vaccine against this parasitic disease. The prediction was confirmed, as therapeutic vaccination with a mixture of these peptides in the presence of the Toll-like receptor 4 agonist monophosphoryl lipid A led to a significant control of the infection in vaccinated mice. This disease control was estimated by significant reductions in parasitemia, parasite burden in the tissue of the infected mice, and cardiac tissue damage, as well as an increase in mice survival.

Similar strategies of reverse vaccinology have been proposed in the past for parasites with large genomes [32]. However, to date, the use of bioinformatics has never been as successful as in the study by Teh Poot et al. This success opens new avenues to a general strategy that may greatly facilitate the development of vaccines against parasites and provides a new tool for the prevention and treatment of neglected tropical diseases.

Notes

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Potential conflict of interest. M. M. R. is a named inventor on patent applications covering *T. cruzi*-vectored vaccines and immunization regimens. J. E. certifies no potential conflicts of interest.

Both authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Review Article

CD8⁺ T Cell-Mediated Immunity during *Trypanosoma cruzi* Infection: A Path for Vaccine Development?

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MHC-restricted CD8⁺ T cells are important during infection with the intracellular protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease. Experimental studies performed in the past 25 years have elucidated a number of features related to the immune response mediated by these T cells, which are important for establishing the parasite/host equilibrium leading to chronic infection. CD8⁺ T cells are specific for highly immunodominant antigens expressed by members of the *trans*-sialidase family. After infection, their activation is delayed, and the cells display a high proliferative activity associated with high apoptotic rates. Although they participate in parasite control and elimination, they are unable to clear the infection due to their low fitness, allowing the parasite to establish the chronic phase when these cells then play an active role in the induction of heart immunopathology. Vaccination with a number of subunit recombinant vaccines aimed at eliciting specific CD8⁺ T cells can reverse this path, thereby generating a productive immune response that will lead to the control of infection, reduction of symptoms, and reduction of disease transmission. Due to these attributes, activation of CD8⁺ T lymphocytes may constitute a path for the development of a veterinarian or human vaccine.

1. Relevance of Chagas Disease

Trypanosoma cruzi is the etiologic agent of Chagas disease, also known as American trypanosomiasis. Infection with this protozoan parasite currently affects approximately 10 million people (WHO 2013) and is the major cause of nonischemic heart disease worldwide. In Latin America, Chagas disease causes an annual loss of 750,000 years of productivity in addition to a loss of 1.2 billion dollars. Despite the fact that most infected individuals are present in endemic areas of Latin America, an increasing number of cases have been reported outside these areas in recent decades, mainly due to the migratory flux of infected people to other regions. Thus, it is estimated that there are approximately 300,000 currently infected people in the United States, 5,500 in Canada, 80,000 in Europe, 1,500 in Australia, and 3,000 in Japan.

Furthermore, there is no effective chemotherapeutic treatment against this disease. Drugs conventionally used for the treatment of infected individuals were developed in the 1960s (nifurtimox and benznidazole). Being outdated, these drugs are highly toxic, have variable efficacy and weak activity in immunocompromised patients, and require long treatments, and the cost of treatment is high, starting from USD 1,000 per year per patient.

Currently, vectorial control using insecticides is the main method to prevent the transmission. Although vectorial transmission has been controlled in some Southern Cone countries [1] there are reports of insect vector resistance to insecticides in other regions, such as the Gran Chaco [2]. Still, some insect vector species are particularly difficult to control by insecticides; thus, it is likely that control by insecticides

will only keep transmission at low levels without eradicating the disease [2].

It should be also noted that vector control does not block transmission by secondary routes, which are the main modes of transmission outside of endemic areas or where the insect vector has been controlled or is absent. The secondary routes are (i) mother to child transmission and blood transfusion and (ii) organ transplantation from infected people. In order to reduce infections by blood or organ transplants, it is critical to analyze donated blood and organs for the presence of parasites [3].

Considering the number of affected people, the economic impact in endemic areas, the recent dispersion of infected people, the inefficiency and cost of treatment, and the lack of effective methods to control transmission, the difficult task of developing a vaccine against infection by *Trypanosoma cruzi* has been resurrected.

2. Relevance of the Development of a Vaccine for Chagas Disease

In a recent study, Lee et al. generated a computer model to simulate the economic impact of a prophylactic vaccine for Chagas disease in Latin America [4]. This study demonstrated that a vaccine would be beneficial even if it had low protective efficacy (25%), even in places where the risk of becoming infected is less than 1%, and even if the vaccine cost is USD 20.00. A vaccine costing more than USD 200.00 would only be economically advantageous if it had efficacy greater than 50% and was used in places where the risk of becoming infected was greater than 20%. Based on these studies, several groups have been focusing on the development of a vaccine against Chagas disease.

3. Clinical Data of Chagas Disease

Chagas disease has two distinct clinical phases: acute and chronic. In the acute phase, individuals exhibit patent parasitemia that begins one to two weeks after infection. In this phase, the infection has variable symptoms from asymptomatic to intensely symptomatic and includes risk of death. Except Romaña sign that is characterized as a swelling at the site of infection, signs and symptoms related to the acute phase of Chagas disease symptoms are most of the times nonspecific and consequently it is difficult to detect the infection in the initial stage. In the acute phase, there is strong activation of the immune system with high levels of plasma cytokines, intense activation of B and T lymphocytes, and inflammatory reactions in the infected tissues (WHO 2013).

In most cases, the acute phase parasitemia is controlled gradually, presumably due to the host adaptive immune responses, and it is reduced to subpatent levels. After controlling acute parasitemia, the chronic phase is initiated. In this phase, it is possible to detect specific antibodies by different serological tests and, in many patients, the parasites can be isolated by blood culture or by xenodiagnosis. These tests are routinely used for parasitological exams (WHO 2013). The chronic phase can last for the entire life of the patient and is initially asymptomatic. After years of initial contact with the

parasite, 40% of infected persons develop symptomatology, while the remaining 60% remain asymptomatic throughout life.

In approximately 30% of cases, patients develop cardiac symptoms (associated with severe myocarditis), and in the remaining 10%, there is damage to the digestive system with infiltration of lymphocytes and neuronal degeneration, leading to the development of megacolon or megaesophagus [5]. Despite the fact that the causes of the immunopathology are not precisely known, several evidences indicate that parasite persistence is a critical factor for the development of Chagas disease [5, 6]. Accordingly, extensive destruction and fibrosis of the heart would be a result of the inflammatory reaction required to eliminate the parasites in the tissues [7]. Nevertheless, whether the parasite has to be present within the heart tissue is still a debatable question. Although most studies have found parasites in this tissue by using detection of DNA by PCR, live parasite has not been detected [8].

4. Immune Response During *T. cruzi* Infection

4.1. Patients with Chagas Disease. In order to establish a successful chronic infection, there must be a balance between parasite multiplication in the tissues and host immune control that will keep the host alive. In this regard, the host immune response is part of the parasite life cycle. Without proper control of the immune response, the host may die, and the parasite life cycle is broken.

During human infection, protective antibodies are mainly directed towards the Gal epitope containing the α Gal α 1,3-Gal β 1,4-GlcNAc, which is present in the majority of mucin glycoproteins on the surface of *T. cruzi* trypomastigotes [9]. These antibodies are present in high concentrations during the patent infection and the entire chronic phase and are markers for active human infection [10]. These antibodies kill the parasite independently of the presence of complement or cells, and their activity is facilitated by the presence of specific antibodies for the catalytic domain of the TS enzyme that inhibit sialic acid addition to the surface of trypomastigotes [11]. Whether antibodies directed to other parasite targets are also important in immunity against infection remains to be determined.

Specific T lymphocytes against *T. cruzi* antigens can be detected in most individuals during chronic infection [12]. There is no direct evidence that these cells are important for host resistance. However, it is possible that they exert antiparasitic activities similar to those observed during experimental infection (see below). Events leading to the depression of the immune response, such as acquired immunodeficiency or immunosuppressive treatments (e.g., AIDS), cause relatively rapid onset of symptomatic forms of the disease in some patients with Chagas disease [13]. In some cases, there is reappearance of patent parasitemia, that is, characteristic of the acute stage of infection [14]. Therefore, it seems clear that the adaptive immune response maintains the low levels of parasitemia observed in patients with Chagas disease during the chronic phase.

Comparative studies of the cellular immune responses in patients who develop clinical symptoms, such as chronic

chagasic cardiomyopathy (CCC), have led to conflicting results. Some researchers have associated the high production of IFN- γ with CCC [15, 16]. Based on this, polymorphisms in the promoter gene regions that encode BAT-1 and IL-10 anti-inflammatory proteins suggest that production of IFN- γ is more frequent in patients with CCC [17, 18]. Additionally, Tregs have been correlated with clinical CCC improvement [19]. Thus, patients able to control the inflammatory response in the myocardium may have less tissue damage and milder disease. In contrast, other groups have suggested the opposite: higher production of proinflammatory cytokines, such as IFN- γ and IL-17, correlates with an improvement in the clinical picture of chronic Chagas disease [20, 21]. In these cases, CCC would develop due to increased expression of molecules that inhibit the activation of specific T lymphocytes, leading to exhaustion of the specific immune response [21, 22].

4.2. Experimental Murine Models. In an attempt to identify the molecules and mechanisms associated with resistance to *T. cruzi* infection, immunologists have employed a number of genetically modified mice. It has been described that resistance to *T. cruzi* infection requires activation of the innate and adaptive immune responses directed at different stages of parasite development. In the case of the innate immune response, several mechanisms operate in order to partially control multiplication of the parasite. Accordingly, mice that are genetically deficient in Toll-like receptors (TLRs) and their adaptors (MyD88 and TRIF), the NOD-like receptors (NLR), or inflammasome activators are more susceptible to experimental *T. cruzi* infection [5, 23, 24]. These molecules provide signals for the secretion of mediators, such as TNF- α , IFN- γ , and IL-12, which in turn are critical for resistance against experimental infection [25]. Upon activation of the innate immune response, there is intense activation of CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes, as well as B cells. Likewise, in innate immunity, mice genetically deficient in CD4 or CD8 are also extremely susceptible to infection [26]. These T lymphocytes act by producing type 1 cytokines, such as IFN- γ . However, in the case of CD8 $^{+}$ T lymphocytes, direct cytotoxicity mediated by perforin may be important for resistance against infection with some strains of *T. cruzi* [27–29]. Finally, in addition to classical innate and adaptive immune responses, NK cells are also activated and produce IFN- γ [30].

5. Role of CD8 $^{+}$ T Cells during *T. cruzi* Infection

5.1. Patient with Chagas Disease. In the cardiac lesions of chronically infected individuals, the inflammatory infiltrate is largely dominated by CD8 $^{+}$ T cells with a minor proportion of CD4 $^{+}$ T cells, B cells, plasma cells, macrophages, eosinophils, and mast cells [22]. This predominance suggests an important role for CD8 $^{+}$ T cells during infection [31–33]. Given the difficulty of obtaining cardiac tissue from chagasic individuals, analysis of the specificity of intracardiac CD8 $^{+}$ T lymphocytes from patients with Chagas cardiomyopathy is limited to a single study involving a small group of patients with HLA-A0201 [34]. In this study, it was shown that patients had specific CD8 $^{+}$ T lymphocytes to some of the 26 peptides

tested from FL-160 or cruzipain antigens in the peripheral blood. In the same study, cells specific for one of the cruzipain peptides were isolated from the cardiac tissue of a patient. Cells with this same specificity were also present in the peripheral circulation [34].

The frequency of specific CD8 $^{+}$ T lymphocytes was lower in the peripheral blood of chagasic patients with severe disease than in asymptomatic or mildly symptomatic patients. Furthermore, the stage of differentiation of these T cells was different between these two groups. Patients with severe disease had fewer early memory cells (CD45RA $^{-}$, CD27 $^{+}$, and CD28 $^{+}$) and a higher number of late memory cells (CD45RA $^{-}$, CD27 $^{-}$, and CD28 $^{-}$). These results suggest that protection against this disease may be associated with an increased frequency of highly competent CD8 $^{+}$ T lymphocytes in the early state of differentiation [35]. Infected people in the chronic phase without cardiac symptoms or with mild symptoms had a higher frequency of specific CD8 $^{+}$ T lymphocytes producing IFN- γ than people who developed severe symptoms. Therefore, the severity of the disease may be related to the specificity and quality of the CD8 $^{+}$ T cell response generated during infection [20]. In addition, because it is a chronic infection and the antigen persists, it is possible that the cellular response is driven to exhaustion [35]. However, this is not the case of the mouse model. One possible explanation is that the murine model has a very short life span when compared to humans, and then, mice do not develop exhausted T cells.

The specificity of the CD8 $^{+}$ T lymphocyte response was analyzed, and several TS epitopes restricted to HLA-A0201 were identified. Due to the high degree of polymorphism of the human HLA plus the divergent specific response among different strains of *T. cruzi*, it is difficult to establish a correlation between the human immune response and resistance to infection [36]. To identify the target specificity of the CD8 $^{+}$ T lymphocyte response from patients in the chronic phase, Alvarez et al. (2008) carried out extensive screening of TS-derived peptides predicted to bind common HLA molecules. The most prevalent HLAs in the population were divided into six supertypes comprising one or more HLA alleles present in 95% of the human population irrespective of ethnic origin. The results of this study indicated that promiscuous TS epitopes, which were able to bind to HLA-A02, HLA-A03, and HLA-A24, are the main targets of memory CD8 $^{+}$ T lymphocytes in chronic chagasic patients. Responses to these epitopes were detected in 13 of 25 asymptomatic individuals. This approach suggests that a specific set of HLAs restricted to these peptides can be used to measure the functional activity and frequency of CD8 $^{+}$ T lymphocytes specific for *T. cruzi* in a wide variety of patients with Chagas disease without having to perform HLA typing. Furthermore, it was noted by the authors that the frequency of specific CD8 $^{+}$ T lymphocytes producing IL-2 was significantly reduced in chronically infected patients [36]. Considering that the parasite load is extremely low in these individuals, the authors proposed that long-term parasite persistence can result in a specific T lymphocyte population with low capacity for self-renewal. While this work was only focused on the response to TS proteins, thus only examining a fraction of all the possible

CD8⁺ T lymphocytes specific for *T. cruzi*, it is significant that more than 50% of the evaluated subjects responded to at least one TS epitope.

5.2. Experimental Murine Models. A few hours after penetrating the host cell, *T. cruzi* escapes from the parasitophorous vacuole and enters the cytosol, where it differentiates into amastigotes and begins its replication. Once the parasite is in the cytosol, its secreted antigens become available to be processed and presented via MHC-I [69]. The relevance of CD8⁺ T lymphocytes in immunity against experimental infection with *T. cruzi* has been demonstrated by different methods. Studies have used β 2-microglobulin-deficient mice, which lack cells restricted to MHC-I molecules (such as NK cells and CD8⁺ T lymphocytes), mice deficient in the CD8 molecule, or mice depleted of CD8⁺ T cells using monoclonal antibodies. In all cases, the absence of CD8⁺ T cells leads to extreme susceptibility to experimental infection [5]. Specific CD8⁺ T cells promote resistance to infection by *T. cruzi* through IFN- γ , but direct perforin-mediated cytotoxic activity can also play an important role depending on the strain of *T. cruzi* [5, 27, 28].

The mechanisms involved in the processing and presentation of these antigens to CD8⁺ T cells are unknown. There is evidence that CD11c⁺ dendritic cells are important for the initiation of the immune response that occurs in the draining lymph nodes [65, 70]. Afterwards, the CD8⁺ T lymphocytes recirculate and enter the spleen. This requires the expression of the chemotactic receptor S1PR1 (sphingosine 1 phosphate receptor) on the surface of lymphocytes, as egress can be blocked by use of the drug FTY720 [65].

The response of CD8⁺ T lymphocytes during experimental infection with *T. cruzi* has kinetics distinct from those observed with viruses or bacteria that cause self-limiting infections, such as influenza, LCMV, or *Listeria*. In these cases, the peak of the immune response measured by the frequency of specific CD8⁺ T lymphocytes is 7 to 15 days [71]. After *T. cruzi* infection, we and others have observed that the peak of the immune response occurs after approximately 30 days [27, 39, 72, 73]. These delayed kinetics of the immune response most likely allow the parasite to establish tissue infection before they can be eliminated by CD8⁺ T lymphocytes. The precise mechanisms that control this altered kinetics are not known, but we have observed that the parasite load is an important factor controlling the timing of the immune response. Thus, increasing the parasite inoculum leads to acceleration of the parasitemia/infection onset, which in turn triggers acceleration of specific CD8⁺ T lymphocyte generation [73].

In contrast to what is seen in common viral or bacterial infections, we do not observe a marked decrease (contraction) in the immune response. As shown in Figure 1, the frequencies of specific CD8⁺ T cells remain high for long periods of time. Furthermore, the phenotype of these CD8⁺ T lymphocytes is maintained as T effector memory (TEM) cells through this period, and this is linked to parasite persistence. Effective treatment with an antitrypanocidal drug led to the contraction of the number of CD8⁺ T cells and a change in their phenotype to CD62L^{Low} [74].

FIGURE 1: Kinetics of the immune response mediated by CD8⁺ lymphocytes after experimental infection with viruses or with *T. cruzi*.

Importantly, the immune response mediated by CD8⁺ T cells generated after experimental infection does not always allow host survival. Mice from susceptible strains, such as A/Sn, uniformly die after infection with few parasites [75]. However, after infection of resistant mice, such as C57BL/6, the immune response mediated by CD8⁺ T lymphocytes protects the animals from death. Nevertheless, these animals are unable to completely eliminate the parasites, which drives them to the chronic phase of infection. Thus, under no circumstances can the immune response mediated by CD8⁺ T lymphocytes eliminate *T. cruzi*. In contrast, genetic vaccination with a DNA plasmid and recombinant adenovirus expressing an immunodominant *T. cruzi* antigen amastigote surface protein 2 (ASP-2) is able to induce a potent immune response mediated by CD8⁺ T lymphocytes that protects highly susceptible A/Sn mice [28, 76]. These animals not only survive the acute infection but also do not develop chronic Chagas disease and have no detectable circulating parasites. This observation has created a paradox, since both experimental infections and genetic immunizations induce CD8⁺ T cells that are capable of secreting the antiparasitic mediator IFN- γ and mediating potent cytotoxic lymphocyte activity *in vivo*. To explain this paradox, we hypothesize that the specific CD8⁺ T lymphocytes induced by the adenoviral vector expressing the ASP-2 antigen (AdASP-2) or by experimental infection present distinct properties that either confer immunoprotective ability in the former or prevent an effective response in the latter. In order to identify this factor, we made a detailed comparison of the two immune responses. We observed that there was a high degree of similarity in the immune responses induced by AdASP-2 and by infection with *T. cruzi*. After 19 days, the immune responses had similar magnitudes and were mediated by multifunctional lymphocytes capable of translocating the CD107a molecule to the cell surface as well as accumulating IFN- γ and TNF- α after *in vitro* stimulation with cognate peptide [75]. Moreover, these specific lymphocytes were highly cytotoxic *in vivo*. Among the few differences, there was a markedly accelerated expansion of the CD8⁺ T cell immune response induced by AdASP-2 compared to *T. cruzi* infection [75]. When we evaluated the proliferation of specific CD8⁺ T

lymphocytes, we found that a higher frequency of cells proliferated following infection when compared to immunization with AdASP-2 [75]. However, as mentioned above, their frequencies were similar. This discrepancy was explained by a significant increase in the frequency of specific CD8⁺ T lymphocytes with a proapoptotic phenotype in infected animals compared to immunized animals. Therefore, the main difference observed when comparing the two immune responses was the viability (fitness) of specific CD8⁺ T lymphocytes. Whereas immunization with AdASP-2 induced highly viable specific CD8⁺ T cells, infection led to intense expansion and apoptosis. In order to determine the molecular basis for this phenomenon, we analyzed a total of 28 surface molecules, including adhesion molecules, functional markers, and receptors involved in T lymphocyte survival (Figure 2). The main difference observed was that specific CD8⁺ T cells induced by experimental infection showed a higher expression of apoptotic CD95 (Fas) receptor compared with specific CD8⁺ lymphocytes from mice vaccinated with AdASP-2 [75]. Not only these cells expressed high levels of CD95 on the surface, but signaling through this molecule *in vitro* with anti-CD95 mAb led to their death. In contrast, specific CD8⁺ T lymphocytes induced by vaccination with AdASP-2 were refractory to death induced by anti-CD95 [75].

These striking differences in the quality of the immune response induced by immunization with the AdASP-2 and by *T. cruzi* infection provide an important basis to explain the suboptimal immune response mediated by CD8⁺ T cells during infection. In addition, this difference may also explain the success of genetic vaccination with recombinant adenoviral vectors. Indeed, concomitant injection of AdASP-2 and *T. cruzi* led to (i) a strong specific immune response mediated by CD8⁺ T lymphocytes; (ii) lower expression of CD95, which led to resistance to anti-CD95-mediated apoptosis; (iii) protective immunity against experimental infection with *T. cruzi* [77] (Figure 3).

Another important factor that characterizes the immune response mediated by CD8⁺ T lymphocytes during experimental *T. cruzi* infection in mice is the fact that it is restricted to few epitopes. This occurs despite the great number of possible CD8 epitopes present in the parasite. This phenomenon is called immunodominance. All the immunodominant epitopes described thus far are expressed by members of the TS family of *T. cruzi*.

Several molecular mechanisms leading to the immunodominance of CD8⁺ T cells during infection are known, some of which are related to the formation of the peptide-MHC-I complex on the surface of APC. The following are important for the formation of these complexes: (i) abundance of the antigen; (ii) availability of APC; (iii) enzymatic processing by proteasomes and other proteolytic enzymes; (iv) translocation of peptides into the endoplasmic reticulum by transporter proteins; (v) binding of peptides to MHC-I. The second group of factors that influence immunodominance are related to the activation of CD8⁺ T lymphocytes: (i) frequency of cell precursors in the repertoire capable of recognizing the peptide-MHC-I complex; (ii) affinity of the T cell receptors; (iii) CD8⁺ T lymphocyte competition by the APC during priming. Other factors, such as regulation of

Naive CD8 ⁺ T cells	Specific CD8 ⁺ T infected	Specific CD8 ⁺ T immunized
CD11a Low	CD11a High	CD11a High
CD11c Low	CD11c Int.	CD11c Low
CD25 Low	CD25 Low	CD25 Low
CD27 High	CD27 Low	CD27 Low
CD43 Low	CD43 High	CD43 High
CD43I Low	CD43I High	CD43I High
CD44 Low	CD44 High	CD44 High
CD49d Low	CD49d High	CD49d High
CD69 Low	CD69 Low	CD69 Low
CD62L High	CD62L Low	CD62L Low
CD70 Low	CD70 Low	CD70 Low
CD95 Low	CD95 High	CD95 Low
CD95L Low	CD95L Low	CD95L Low
CD122 Low	CD122 Low	CD122 Int.
CD127 High	CD127 Low	CD127 Low
CD183 Low	CD183 Low	CD183 Low
CCR5 Low	CCR5 Low	CCR5 Low
CCR9 Low	CCR9 Low	CCR9 Low
KLRG-1 Low	KLRG-1 High	KLRG-1 Low/high
PD1 Low	PD1 Low/int.	PD1 Low/int.
CTLA-4 Low	CTLA-4 Low	CTLA-4 Low
BTLA Int.	BTLA Low	BTLA Low
PDL1 Low	PDL1 Low	PDL1 Low
TRAIL Low	TRAIL Low	TRAIL Low
TRANCE Low	TRANCE Low	TRANCE Low
LAG-3 Low	LAG-3 Low	LAG-3 Low
PNAD Low	PNAD Low	PNAD Low
OX-40 Low	OX-40 Low	OX-40 Low
T naive	TE	TE

FIGURE 2: Surface molecules expressed by specific CD8⁺ T lymphocytes following infection with *T. cruzi* and vaccination with AdASP-2.

specific immune responses, the ability of pathogens to mutate their recognized epitopes, expression of specific antigens on the different evolutionary stages, modulation of antigen expression at different stages of infection, and direct inhibition of mechanisms involved in the formation of the peptide-MHC-I complex could also theoretically influence immunodominance, but examples have not yet been described.

Due to the genetic restriction by MHC-I molecules in the case of CD8⁺ T cells, 90% of immunodominance is linked to the fact that only 1% of peptides can bind MHC-I with affinity sufficient to induce a response mediated by CD8⁺ T lymphocytes. This is related to the structure of MHC-I rather than the selective pressure by an organism to prevent the host CD8⁺ T lymphocyte response [78]. Because of the genomic complexity of many infectious agents, most studies seeking to define the molecular basis of immunodominance of CD8⁺ T cell-mediated immunity have used viral models. In a review article on immunodominance [79], they illustrate the evolution of immune restriction mediated by CD8⁺ lymphocytes during vaccinia virus infection. The vaccinia genome has the capacity to generate approximately 175,000 peptides of 8, 9, or 10 mer (without considering the possibility

FIGURE 3: Proposed model for activation of specific CD8⁺ lymphocytes during infection of *T. cruzi* in naive or AdASP-2 immunized mice. Specific CD8⁺ T cells activated with APC containing *T. cruzi* antigen induce higher levels of expression of CD95 and low viability of T cells. This poor viability leads to a suboptimal response and host pathology (death or chronic infection). In contrast, the contact of APC pulsed with recombinant ADASP-2 activates specific CD8⁺ T cells with low expression of CD95. In the second contact with APC pulsed with *T. cruzi* antigen, specific CD8⁺ T cells with CD95^{low} phenotype develop a strong immune response driving parasite elimination and promoting cure of the infection.

of alternative sequences of reads and posttranslational modifications). However, only 20% of these peptides are released after processing by the proteasome and other proteases (35,000 peptides). Although transport to the ER via TAP influences the selection of peptides that are made available to bind MHC-I, such transport has little effect on the restriction of epitopes during vaccinia infection, and approximately 30,000 peptides are transported. Even so, approximately 1% of these peptides bind MHC-I (150 peptides). Once the peptide-MHC-I complex is exposed on the surface of the APC, the repertoire of CD8⁺ T lymphocytes can recognize at least 50% of these epitopes (75 peptides). However, only 50 peptides are recognized, and these make up 90% of the response to vaccinia virus. Finally these 50 vaccinia peptides that are recognized during experimental infection, eight represent more than 50% of the CD8⁺ T lymphocyte response observed against infection.

As mentioned above, the antigen targets of the immune response mediated by CD8⁺ T cells during *T. cruzi* experimental infection are of TS family proteins [27, 72, 73, 80, 81] (Table 1). The molecular basis and biological significance of immunodominance are unknown. It is possible that the restriction of the number of known parasite epitopes may be important for establishing the necessary balance between the parasite and the host in order to keep the host alive without eliminating the pathogen, thus leading to chronic infection [5, 82]. Immunodominant epitopes vary by *T. cruzi* strain, such that an epitope can be immunodominant during

infection with one strain and subdominant with another in genetically identical hosts [39, 72].

Despite evidence of strong immunodominance during *T. cruzi* experimental infection, to date only one study has attempted to determine the cellular/molecular basis for this phenomenon. In this study, it was reported that infected homozygous C57BL/6 mice or heterozygous F1 mice (C57BL/6 X BALB/c) had similar CD8⁺ T lymphocyte responses to the MHC-I H-2K^b-restricted epitope VNHRFTLV. In contrast, the immune response to the MHC-I H-2K^d-restricted epitope IYNVGQVSI was significantly reduced in heterozygous animals when compared to homozygous BALB/c mice [72]. Such a discrepancy could not be attributed to the CD8⁺ T lymphocyte repertoire or to low MHC-I H-2K^d expression on the APC surface of heterozygous mice. This phenomenon, which restricted the immune response and generated strong immunodominance, could be reproduced in other heterozygote strains of mice after infection with *T. cruzi*. However, there was no difference in the immune responses of homozygote and heterozygote animals following immunization with the AdASP-2 expressing *T. cruzi* antigen [72]. Finally, in the same study, C57BL/6 mice infected with two different strains of *T. cruzi*, both the Y and CL-Brener strains, responded to the two immunodominant epitopes simultaneously. Based on these results, it was proposed that the strong immunodominance induced during infection with *T. cruzi* occurred by the competition of CD8⁺ T lymphocytes generated during priming for a limited number of infected APCs. This competition for APC is also called competitive priming or immunodomination and has been reported in some cases of experimental infection [72, 83].

These observations were independently confirmed in studies showing that CD8⁺ T cells of H-2^a infected mice recognized a single immunodominant epitope in the ASP-2 antigen. In contrast, CD8⁺ T cells from mice immunized with recombinant genetic vaccines expressing the same *T. cruzi* antigen recognized two other subdominant epitopes in addition to the immunodominant epitope [84].

Despite the immunoprotective role of CD8⁺ T lymphocytes during the acute phase of experimental *T. cruzi* infection, recent studies have proposed that a subpopulation of these lymphocytes is eventually responsible for further damage to heart tissue in the chronic phase. These lymphocytes produce perforin, but not IFN- γ , and have a greater propensity to migrate to the heart tissue, causing tissue damage leading to CCC [85].

5.3. CD8⁺ T Cell Mediated Immunity for Vaccine Development. Based on the proven antiparasitic activity of CD8⁺ T cells, several groups have tried to elicit specific CD8⁺ T cells for vaccine development against Chagas disease. For that purpose, subunit vaccines based on plasmid DNA, viral, and bacterial vectors were used. The antigens pursued for recombinant vaccines include *T. cruzi* trans-sialidase, cruzain (a cysteine proteinase), and the amastigote surface proteins 2, 3, 4, TcG1, TcG2, TcG4, TSA-1, and Tc24 (review references [23, 80, 86–90]). In addition to subunit vaccines, other groups are using attenuated parasites [90]. A more detailed list of

TABLE 1: Immunodominant epitopes recognized by murine CD8⁺ lymphocytes after infection with *T. cruzi*.

Antigen	Epitope	MHC	<i>T. cruzi</i> strain	Reference
TS	IYNVGQVSI	H-2K ^d	Y	[37]
ASP-2	VNHRFTLV	H-2K ^b	Y	[27]
ASP-2	TEWETGQI	H-2K ^k	Y	[38]
TSA	ANYDFTLV	H-2K ^b	Brazil	[39]
TSA	ANYKFTLV	H-2K ^b	Brazil	[39]

FIGURE 4: Proposed model for activation of specific CD8⁺ lymphocytes expanded by genetic vaccination after infection with *T. cruzi*.

the vaccination studies performed with subunit vaccines is shown in Table 2. Overall, the results of mouse vaccinations provide strong evidence that the induction of specific CD8⁺ T cells can elicit protective immunity against acute phase symptoms estimated by parasitemia and survival. In some cases, vaccination also reduced or eliminated chronic symptoms in these mouse models.

An important question is whether vaccination can achieve complete parasite elimination from the host. In most protocols of experimental vaccination, residual parasites are still present and are mostly within tissues rather than circulating in the blood. Therefore, vaccination may at least lead to a decrease or cessation in transmission. Vaccinated mice rarely show symptoms of acute or chronic disease. Therefore, even if they carry tissue parasites they will be asymptomatic. This picture resembles the ~60% of chagasic patients who also maintain residual parasites for their entire lives yet still remain asymptomatic (indeterminate form of the disease). Thus, it would be ethical to propose that a satisfactory vaccination goal could be to lower the frequencies of symptomatic individuals even if that means increasing the frequency of asymptomatic individuals.

During our recent studies of genetic immunization, we used the heterologous prime-boost vaccination regimen consisting of priming with plasmid DNA followed by a booster injection with replication defective human adenovirus type 5, both carrying the ASP-2 gene of *T. cruzi*. In these studies, we observed that our immunization protocol induced TE cells (CD11a^{High}, CD44^{High}, CD127^{Low}, and CD62L^{Low}). These cells formed a stable pool of functional long-lived CD8⁺ TEM cells (CD11a^{High}, CD44^{High}, CD127^{High}, and CD62L^{Low}). Their long-term survival required a functional IL-12/IL-23 signaling pathway. Most relevant for vaccine development was the fact that these cells were resistant to immunological

erosion and mediated strong protective immunity against experimental systemic infection with *T. cruzi* [76].

According to the current immunological paradigm of vaccines, protective immunity is mainly mediated by T central memory (TCM) cells, which proliferate intensely during recall responses. After an infectious challenge, specific TCM cells differentiate to TE cells, proliferate, and eliminate the pathogen. This concept established that, after vaccination, TCM cells act similarly to self-curing viral infections, such as acute LCMV [91].

Based on this paradigm, we evaluated the importance of CD8⁺ T cell differentiation, proliferation, and recirculation to mediate their antiparasitic effects. In our case, we only had a TEM pool to stimulate. We observed that specific TEM cells differentiated into cells with a KLRG1^{High} CD27^{Low} CD43^{Low} CD183^{Low} *T-bet*^{High} *Eomes*^{Low} phenotype and were capable of simultaneously producing the antiparasitic mediators IFN- γ and TNF- α . Subsequently, we observed that the specific CD8⁺ T cells did not participate in a strong anamnestic immune response, and the protective immunity they mediated was insensitive to treatment with the cytostatic toxic agent hydroxyurea, which drastically reduced their proliferation [92].

We also evaluated the importance of T cell recirculation by administering the immunosuppressive drug FTY720 to vaccinated mice after challenge with *T. cruzi*. This drug reduces lymphocyte recirculation by interfering with T cell signaling via sphingosine-1-phosphate receptor-1 (S1Pr1). This interference results in sustained inhibition of S1Pr1 signaling, effectively trapping T cells within the secondary lymphoid without inhibiting T cell activation. FTY720 administration significantly impaired protective immunity, supporting the hypothesis that T cell recirculation is critical for protective immunity [65].

TABLE 2: Summary of the different antigen delivery systems used for the induction of CD8⁺ T cells against experimental *T. cruzi* infection.

Delivery system	Adjuvant	<i>T. cruzi</i> antigen/gene	Reference
Rec. protein	Freund's adjuvant	TS	[40]
Rec. Protein	CpG ODN	TS	[41]
Rec. Protein	CpG ODN	Cruzipain	[42]
Rec. protein	Alum/CpG ODN	ASP-2	[38]
			[43]
Plasmid DNA	None	TSA-1	[44]
			[45]
			[46]
Plasmid DNA	IL-12	TSA	[47]
			[48]
Plasmid DNA	None	TS	[49]
			[50]
			[41]
Plasmid DNA	None	ASP-1	[51]
Plasmid DNA	None	ASP-2	[51]
			[52]
			[53]
			[54]
			[55]
Plasmid DNA	None	ASP-3	[56]
Plasmid DNA	None	ASP-4	[56]
Plasmid DNA	None	CRP	[57]
Plasmid DNA	None	KMP11	[58]
Plasmid DNA	None	Tc24	[45]
Plasmid DNA	IL-12/GM-CSF	TcG1	[59]
Plasmid DNA	IL-12/GM-CSF	TcG2	[59]
Plasmid DNA	IL-12/GM-CSF	TcG4	[59]
Rec. adenovirus	None	TS	[60]
		ASP-2	[60]
Rec. <i>Salmonella</i>	None	Cruzipain	[61]
Sendai virus	None	ASP-2	[62]
Yellow fever 17D	None	ASP-2	[63]
Rec. adenovirus + Rec. MVA	None	TSA	[64]
Plasmid DNA + Rec. Adenovirus	None	ASP-2	[28]
Plasmid DNA + Rec. Adenovirus	None	ASP-2	[65]
Rec. influenza + Rec. Adenovirus	None	ASP-2	[65, 66]
Plasmid + Rec. MVA	IL-12/GM-CSF	TcG2/TcG4	[67]
Plasmid + Rec. protein	IL-12/GM-CSF	TcG1/TcG2/TcG4	[68]

Based on these observations, we propose a model whereby large amounts of antigen-experienced CD8⁺ TEM cells present following heterologous prime-boost vaccination that differentiate and recirculate, rather than proliferate, are critical for protective immunity Figure 4. Recent studies using the murine LCMV infection model reported that CD8⁺ T cells that provide long-term protective immunity are a subpopulation of TEM cells with the same phenotype as our T cells (CD44^{High} CD62^{Low} KLRG1^{High} CD27^{Low} CD43

(1B1)^{Low} CD183^{Low} T-bet^{High} Eomes^{Low}) and not classical TCM. These cells have limited proliferative ability, recirculate, and are also critical in the resistance to infection with *Listeria monocytogenes* and vaccinia [93]. Together, these observations point to a novel CD8⁺ T cell population that provides long-term memory and can be used for T cell vaccine development. Additionally, these two observations challenge the paradigm that memory T lymphocytes need to proliferate in order to mediate protective immunity and

suggest that recirculation is indeed fundamental. This idea had not been properly explored and may have important implications in the development of new vaccines.

A current limitation of the experimental model of *T. cruzi* infection is the absence of information about the precise location where CD8⁺ T lymphocytes eliminate infected cells. Because *T. cruzi* can infect many cell types and cause systemic (or not) infection, it will be important to determine where CD8⁺ T lymphocytes encounter infected cells. The fact that our vaccinated animals were resistant to challenge with *T. cruzi* infection by different routes (i.p. and s.c.) suggests that it may also be useful for natural infections.

6. Concluding Remarks

Studies on the role of CD8⁺ T cells during *T. cruzi* infection have elucidated extremely important information regarding the interface of host/*T. cruzi* interactions and the equilibrium they achieve to establish chronic infection. In addition, they have opened new avenues for the development of a still elusive veterinarian or human vaccine against Chagas disease.

Conflict of Interests

M. M. Rodrigues is named inventor on patent applications covering *Trypanosoma cruzi* vectored vaccines and immunization regimens. The other authors have no conflict of interests.

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