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**Modulação da resposta anti-tumoral por agentes
infecciosos modificados geneticamente**

Tese apresentada ao Programa de Pós-Graduação
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Área de concentração: Imunologia

Orientador: Prof. Dr. João Gustavo Pessini
Amarante-Mendes

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**Modulation of anti-tumor immune response by
genetically modified infectious agents**

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of Immunology at the Institute of Biomedical
Sciences, University of São Paulo, in order to obtain
the degree of Doctor in Science

Area: Immunology

Supervisor: Prof. Dr. João Gustavo Pessini
Amarante-Mendes

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Orientador: João Gustavo Pessini Amarante-Mendes

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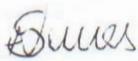
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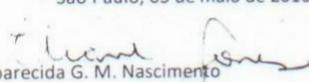
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<i>Mus musculus</i>	C57bl/6	Fêmea/female	6-12 semanas/weeks	810
	RIPK3 KO	Fêmea/female	6-12 semanas/weeks	270
	C57Bl/6 KO para caspase 1	Fêmea/female	6-12 semanas/weeks	270

São Paulo, 03 de maio de 2016.


Prof. Dr. Anderson de Sá Nunes
Coordenador CEUA-ICB/USP


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DECLARAÇÃO

Em adendo ao Certificado nº 28/2016/CEUA, de 27/04/2016, informo que os animais aprovados no projeto "*Modulação da resposta imune anti-tumoral por agentes infecciosos geneticamente modificados*" de responsabilidade do Prof. Dr. **João Gustavo P. Amarante-Mendes** devem constar como abaixo indicado, alterando-se o sexo de fêmea para macho:

Espécie	Linhagem	Sexo	Idade	Quant.
Mus musculus	C57bl/6	Macho	6-12 semanas	810
	RIPK3 KO	Macho	6-12 semanas	270
	C57Bl/6 KO para caspase 1	Macho	6-12 semanas	270

São Paulo, 17 de maio de 2016.

Luciane Valéria Sita

Profa. Dra. **Luciane Valéria Sita**
Vice-coordenadora da CEUA-ICB/USP

DEDICATION

I Dedicated this Humble Effort

To

“My Father and Mother”

*Who’s Prayers and Constant Encouragement Enabled Me to Do
this Task of Learning*

Acknowledgements

I start with the name of **Almighty ALLAH**, Whose monotheism is absolute, not relative or pluralistic in any sense of the word. He is without predecessor, perpetual of being without end. Everything other than Him is an originated thing that He created by His power from nothingness. All praises for Him, The most Merciful. Without Allah's divine help, I would not have been able to achieve anything in my life. Peace and blessings be upon the **Holy Prophet Hazrat Muhammad (S.A.W)**, who exhorted his followers to seek knowledge from cradle to grave.

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2. LIST OF SYMBOLS:

%	Percentage
>	Greater than
<	Less than
Δ	Delta
$^{\circ}\text{C}$	Degree Celsius
cm^3	Cubic centimeter
kDa	Kilodalton
ml	Milliliter
mm	Millimeter
ng	Nanogram
nM	Nanomolar
V	Volt
μg	Microgram
μl	Microliter
μM	Micromolar

3. LIST OF ABBREVIATIONS:

Ab	Antibody
APCs	Antigen presenting cells
ARID2	AT-rich interaction domain 2
ASC	Apoptosis-associated speck-like protein containing a CARD
BHI	Brain-heart infusion
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BTLA	Band T lymphocyte attenuator
Casp-1	Caspase 1
CCR7	C-C chemokine receptor type 7
CD8+ T	Cytotoxic T cell
CDKN2A	Cyclin dependent kinase inhibitor 2A
CFSE	Carboxy fluorescein succinimidyl ester
CFU	Colony forming unit
cIAPs	Cellular inhibitor of apoptosis proteins
CRT	Calreticulin
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CTV	Cell Trace Violet
CXCL-10	C-X-C motif chemokine 10
CYLD	Clindromatosis
DAMPs	Damaged associated molecular patterns
DCs	Dendritic cells
Drp1	Dynamin-related protein 1
ELISPOT	Enzyme-Linked ImmunoSpot
FACS	Fluorescence-activated cell sorting
FADD	FAS-associated death domain protein
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box protein P3
FSC-A	Forward scatter area

FSC-H	Forward scatter height
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gp100	Glycoprotein 100
GSDMD	Gasdermin D
HAMLET	Human alpha lactalbumin made lethal to tumor cells
HMGB1	High-mobility group box 1 protein
ICD	Immunogenic cell death
IDO	Indoleamine 2,3-dioxygenase
IFI16	Interferon- γ (IFN γ)-inducible protein 16
IFN- γ	Interferon gamma
IL-18	Interleukin-18
IL-2	Interleukin-2
InlA	Internalin A
iNOS	Inducible nitric oxide synthase
KIT	KIT proto-oncogene receptor tyrosine kinase
KLRG1	Killer cell lectin like receptor G1
LAG-3	Lymphocyte-activation gene 3
LAMP-1	Lysosomal-associated membrane protein 1
LCMV	Lymphocytic choriomeningitis virus
L-Glut	L-glutamine
LLO	Listeriolysin O
LM	<i>Listeria monocytogenes</i>
LM-OVA	Ovalbumin-expressing <i>Listeria monocytogenes</i>
MART-1	Melanoma-associated antigen recognized by T cells
MDSCs	Myeloid-derived suppressor cells
MHC-I	Major histocompatibility complex class-I
MLKL	Mixed lineage kinase domain-like
MyD88	Myeloid differentiation primary response 88
NEAs	Non essential amino acids
NFAT	Nuclear factor of activated T-cells
NKs	Natural Killer cells

NLR	NOD like receptor family
NLRC4	NLR family CARD domain containing 4
NLRP3	NLR family pyrin domain containing 3
NLRs	Nuclear oligomerization domain-like receptors
NRAS	NRAS (Asparaginyl-tRNA synthetase)
NRAS	NRAS proto-oncogene, GTPase
OD	Optical density
OVA	Ovalbumin
P2RX7	Purinergic receptor P2X7
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline-Tween 20
PD1	programmed cell death-1
Pen-Strep	Penicillin-Streptomycin
PFU	Plaque forming unit
PGAM5	Mitochondrial phosphatase phosphoglycerate mutase 5
PrfA	Positive regulatory factor A
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
qPCR	Quantitative Polymerase Chain Reaction
Rag1	Recombination activating gene 1
rhAd5.OVA	Recombinant human type-5 ovalbumin expressing adenovirus
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute (RPMI) 1640 medium
SDS	Sodium dodecyl sulfate
SMAC	Second mitochondria-derived activator of caspases
SOPs	Standard operating procedures
SSC-A	Side scatter area
TAA	Tumor associated antigen

TAP	Transporter associated with antigen processing
TBS	Tris Buffered Saline
TERT	Telomerase reverse transcriptase
TGF- β	Transforming growth factor- β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	Tumor infiltrating lymphocytes
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TLRs	Toll like receptors
TME	Tumor microenvironment
TNF- α	Tumor necrosis factor alpha
TP53	Tumor protein p53
TRADD	TNFRSF1A associated via death domain
TRAF2	TNF receptor associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
T _{reg}	Regulatory T cells
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WT	Wild Type

ABSTRACT:

Rana A. Modulation of anti-tumor immune response by genetically modified infectious agents. [Thesis (Doctor of Philosophy in Immunology)]. Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, 2019

An efficient induction of effector and long-term protective antigen-specific CD8⁺ T memory response by vaccination is essential to eliminate malignant and pathogen-infected cells. Intracellular infectious bacteria, including *Listeria monocytogenes*, have been considered potent vectors to carry multiple therapeutic proteins and generate antigen-specific CD8⁺ T cell responses. Although the role of molecules involved in inflammatory cell death pathways, such as necroptosis (RIPK3-mediated) and pyroptosis (Caspase-1/11-mediated), as effectors of immune response against intracellular bacteria are relatively well understood, their contribution to the adjuvant effect of recombinant bacterial vectors in the context of antigen-specific CD8⁺ T cell response remained obscure. Therefore, we evaluated the impact of RIPK3 and Caspase-1/11 (Casp-1/11) individual and combined deficiencies on the modulation of antigen-specific CD8⁺ T cell response during vaccination of mice with ovalbumin-expressing *Listeria monocytogenes* (LM-OVA). We observed that Casp-1/11 but not RIPK3 deficiency negatively impacts the capacity of mice to clear LM-OVA. Importantly, both RIPK3 and Casp-1/11 are necessary for optimal LM-OVA-mediated antigen-specific CD8⁺ T cell response, as measured by *in vivo* antigen-specific CD8⁺ T cell proliferation, target cell elimination and cytokine production. Furthermore, Casp-1/11 and Casp-1/11/RIPK3 combined deficiencies restrict the early initiation of antigen-specific CD8⁺ T cell memory response. Together our findings demonstrate that RIPK3 and Casp-1/11 influence the quality of CD8⁺ T cell responses induced by recombinant *L. monocytogenes* vectors. Interestingly, the reduction of OVA-specific CD8⁺ T cell response found in both RIPK3^{-/-} and Casp1/11^{-/-} mice infected with LM-OVA results in a deficiency to eliminate B16.OVA melanoma cells only in Casp1/11^{-/-} and Casp1,11^{-/-}/RIPK3^{-/-}. Furthermore, our findings may help to optimize the immunotherapeutic potential of LM- or other live vector-based vaccination strategies.

RESUMO

Rana A. Modulação da resposta anti-tumoral por agentes infecciosos modificados geneticamente. Tese (Doutor em Filosofia em Imunologia)]. Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2019

Uma indução eficiente de células T CD8⁺ antígeno-específicas efectoras e de longa duração, através de estratégias de vacinação, é essencial para eliminar células tumorais ou infectadas por patógenos. Bactérias intracelulares infectantes, tais como *Listeria monocytogenes*, têm sido consideradas potentes vetores para expressão de diversas proteínas terapêuticas, uma vez que tem a capacidade de gerar uma forte resposta mediada por células T CD8⁺ antígeno-específica. Embora o papel de moléculas envolvidas nas vias de sinalização de morte celular, tais como, RIPK3 (necroptose) e Caspase-1/11 (piroptose), na resposta imune efetora contra bactérias intracelulares, ser relativamente bem entendida, a contribuição, como efeito adjuvante, dos vetores bacteriano recombinantes no contexto da resposta T CD8⁺ antígeno-específica ainda não foi esclarecido. Dessa forma, foi avaliado o impacto da deficiência individual e combinada de RIPK3 e Caspase-1/11 (Casp-1/11) na modulação da resposta mediada por células T CD8⁺ antígeno-específica, durante a vacinação de camundongos com *Listeria monocytogenes* expressando ovalbumina (LM-OVA). Foi observado que a deficiência de Casp-1/11, mas não de RIPK3, impacta negativamente na capacidade dos camundongos de eliminar LM-OVA. De maneira importante, ambos RIPK3 e Casp-1/11 são necessários para uma resposta T CD8⁺ antígeno-específica de boa qualidade contra LM-OVA, conforme medido pela proliferação *in vivo* de células T CD8⁺ antígeno-específica, eliminação de células alvo e produção de citocinas. Ainda, deficiência combinadas de Casp-1/11 e Casp-1/11/RIPK3 restringe a estimulação de células T CD8⁺ antígeno-específicas de memória. Assim, nossos achados demonstraram que RIPK3 e Casp-1/11 influencia na qualidade das respostas de células T CD8⁺ antígeno-específicas induzidas por vetores recombinantes de *L. monocytogenes*. Interessantemente, a redução de células T CD8⁺ OVA-específicas encontradas em ambos os camundongos RIPK3^{-/-} e Casp-1/11^{-/-} infectados com LM-OVA resultou na deficiência em eliminar células B16.OVA de melanoma somente em Casp-1/11^{-/-} e Casp1/11^{-/-}/RIPK3^{-/-}. Assim, nossos achados podem ajudar a otimizar uma imunoterapia de LM-OVA ou outra estratégia terapêutica baseada em vetores vivos.

4. INTRODUCTION:

4.1. Cancer:

Cancer is a group of diseases characterized by uncontrolled growth and spread of malignant cells in any part of the body. Cancer is major cause of death worldwide and is estimated to account for more than 9.6 million deaths in 2018 worldwide.(1) Cancer incidents are expected to increase to 23.6 million by 2030 worldwide.(2) In 2018, approximately, 600,000 new cases of cancer have been reported in Brazil.(3) Cancer cell acquires a series of abilities, such as increased resistance to apoptosis, angiogenesis, metastasis, and extensively altered intracellular signaling which results in replicative immortality. Additionally, malignant cells modify or reprogram cellular metabolism and enable mechanisms responsible for cancer cell immune evasion.(4)

Immune system detects and eradicates nascent tumors, thereby gives primary protection during tumor progression. Immune cells protect from tumor progression by three ways. First, they protect the host from pathogen infection such as virus and bacteria, which may induce tumor. Second, the timely eradication of pathogens prevents inflammation-encouraging tumorigenesis. Third, immune cells specifically recognize and eliminate malignant cells via recognition of tumor-specific antigens, a phenomenon termed immune surveillance.(5) Despite the role of immune surveillance in antigen-specific tumor eradication, frequently tumor cells continue to proliferate within the host even in the presence of intact immune response. Tumor cells acquire genetic and/or epigenetic alterations, which increase cell diversity and favors immune cells to recognize them as foreign cells. However, as a mechanism of evasion of this recognition, tumor cells undergo the process of immune-editing, which makes the tumor cells less immunogenic and favors disease progression.(4)

Several factors such as tumor heterogeneity, tumor microenvironment and pharmacodynamics contribute to unresponsiveness of cancer treatment. Moreover, impaired apoptotic signaling and epigenetic modification promote tumor progression.(6) Cancer cells resist cell death mechanisms by upregulation of anti-apoptotic and down-regulation of pro-apoptotic proteins through epigenetic modifications. The intrinsic apoptotic pathway involves the sensing of internal stress to shift a balance of pro-apoptotic over anti-apoptotic proteins and their release through mitochondrial outer membrane.(7,8) Furthermore, irreversible DNA damage and matrix detachment induce apoptosis.(9) Both the activation of pro-apoptotic proteins and

irreversible DNA damage response converge to activate caspases and induce apoptosis.(7,8) Up-regulation of anti-apoptotic and down-regulation pro-apoptotic proteins inhibit the caspases activation and enhance tumor survival.(10) In contrast to the apoptotic cell death, in which the dying cells shrink into an invisible body that is rapidly engulfed by the neighboring cells, necrotic cells become bloated and finally explode to release their contents into the tissue microenvironment.(4) Necrotic cell death releases pro-inflammatory signals into tissue microenvironment and recruits inflammatory immune cells.(11,12) The recruitment of inflammatory cells into the tumor microenvironment initiate reactions that promote tumor progression by increasing proto-oncogenic or epigenetic alterations and induce angiogenesis and cancer cell invasiveness.(13,14)

4.2. Melanoma:

Skin cancer is one of the most prevalent cancer types, mainly due to the gradual increase of the world population's exposure to ultraviolet radiation. The most hostile and most fatal category of skin cancer is melanoma. Melanomas are derived from melanocytes, the pigment-producing cells of the skin.(15) Melanomas can occur in mouth, eyes, genitals, and anal area, but in comparison to the skin these sites are less common.(16) Melanoma accounts for less than 2% of skin cancer cases but results in a large majority of skin cancer deaths. Cutaneous melanoma is more common in Western world and causes 75% of the deaths related to skin cancer and its global incidence range from 15-25 per 100,000 individuals.(17) Cutaneous melanoma commonly occurs in white population, whereas the incidents of acral and mucosal melanomas are mostly develop in pigmented population of Africa and Asia.(18) Globally, incidence rate of cutaneous melanoma vary up to 100-folds among different populations, and the highest rates are being reported in Australia, where it reaches approximately 60 cases per 100,000 individuals per year. In United States, the rate is ~30 cases per 100,000 and in Europe, ~20 cases per 100,000 per year have been reported; by comparison, incidence rates of cutaneous melanoma in dark-skinned population from Asia and Africa are approximately one case per 100,000 per year.(19) According to American Society of Cancer, about 96,480 new melanoma cases will be diagnosed and more than 7,230 patients are expected to die with melanoma.(20) In 2019, 6,260 new cases of melanoma have been diagnosed and more than 1,794 patients are expected to die in

Brazil.(21) According to World Health Organization (WHO), every year more than 132,000 new case of melanoma are diagnosed worldwide.(22)

Cutaneous melanoma progression is associated with the different types of precursor lesions, ranging from benign and dysplastic nevi to melanoma *in situ* (Figure 1).(23) Melanomas are histologically into five distinct stages: common acquired and congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; radial and vertical growth phase melanoma and metastatic melanoma.(24) Melanoma progression is divided into five stages: stage zero refers to the *in situ* melanoma which means malignant cells are only present under the epidermis; Stage I refers to the thickness of melanoma but the malignant cells are still present epidermis; Stage II melanoma are thicker extending form epidermis to dermis; Stage III melanoma spread locally through lymphatic system celled in-transit metastasis; Stage IV melanoma spread through the bloodstream to the other parts of the body and is further based on the location of distant melanoma.(25)

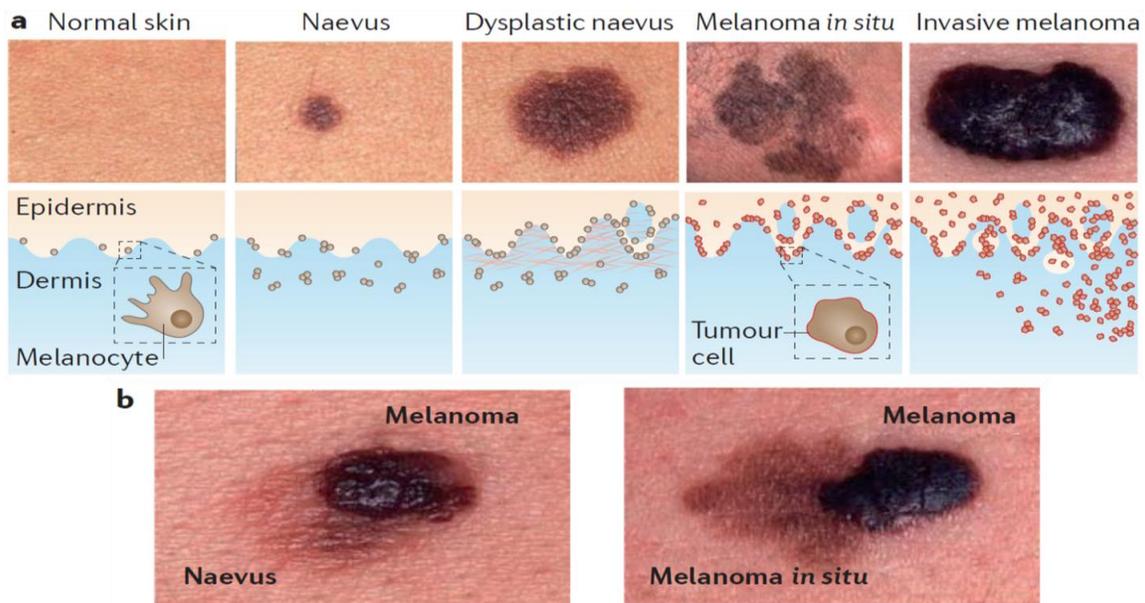


Figure 1: The morphological spectrum of melanocytic neoplasms. (a) Top row: clinical images showing a free-standing nevus, a dysplastic nevus, melanoma *in situ* and invasive melanoma. Second row: schematics illustrating the architectural features for each type of lesion. (b) Clinical images showing combined neoplasms. Adapted from Shain *et al.*, (2016), *Nature Reviews Cancer* (26)

Sun (ultraviolet) exposure is the main risk factor of cutaneous melanoma.(27) UV radiations damage melanocyte DNA and may result in hundreds of mutations, including genes

controlling signal transduction pathways and cell cycle progression. The most somatic mutation in chronically sun damaged (CSD) and non-CSD melanomas affect genes that are involved in key cell signaling pathways that governs cell proliferation (RAS, BRAF and NF1), metabolism and growth (PTEN and KIT cell identity (AT-rich interaction domain 2 (ARID2)), cell cycle control (cyclin-dependent kinase inhibitor 2A (CDKN2A), replicative lifespan (telomerase reverse transcriptase (TERT)) and resistance to cell death (TP53)).(28-31) Differential genes expression involved in cell cycle progression and signaling transduction between melanocytes and melanoma cells results in the somehow different surface expression of tumor associated antigens (TAAs) that may play an essential role for immune recognition and targeted elimination. In addition to the expression of TAAs, melanoma cells are known to express melanocytic lineage-related antigens such as glycoprotein 100 (gp100) and melanoma-associated antigen recognized by T cells (MART-1) that remain unrecognized by the immune system to generate some form of immune tolerance to self antigens.(32)

4.3. Tumor immune evasion:

Tumor cell evasion from the immune response is an important characteristic of cancer.(4) Tumor immunoediting is an important process whereby immune system restricts and promotes tumor growth, which proceed by three distinct phases termed as elimination, equilibrium and escape.(33) Elimination phase is based on immune surveillance, whereby both innate and adaptive immune cells cooperate to eradicate tumor growth.(34) In elimination phase immune system works in four different ways: (a) recognition of melanoma cells and their limited killing by innate immune cells such as natural killer cells (NKs) (b) maturation of antigen presenting cells (APCs) and cross-priming of T lymphocytes, (c) cytotoxic killing of antigen-specific melanoma cells by tumor infiltrating T lymphocytes, (d) and homing of tumor specific T-lymphocytes in the tumor microenvironment (TME) to kill tumor cells.(35) In equilibrium phase melanoma cells reduce their immunogenicity and enter into dormant state and harbor different mutations to avoid immune pressure. In the escapes phase some tumor clones evade immune detection by lack of tumor-antigen presentation through major histocompatibility complex (MHC) class-I and -II molecules to effector T-lymphocytes, resistance to cell death and release of cytokines.(34) Throughout these phases of immunoediting, tumor cells edit their immunogenicity and acquire immunosuppressive mechanisms that enable disease progression.

The mechanism of tumor resistance to immunotherapy broadly overlaps with tumor immunoediting to evade immune response.(33)

Tumor immune evasion depends on tumor intrinsic and extrinsic mechanism to avoid immune recognition and create tumor suppressive microenvironment. Tumor intrinsic mechanisms include defect or complete loss of MHC-I function, epigenetic silencing genes involve in antigen processing machinery such as endoplasmic reticulum aminopeptidases (ERAPs) and transporter associated with antigen processing (TAP), loss of tumor associated antigens (TAA), increasing resistance to apoptotic cell death by immune cells and expression of ligands for inhibitory receptor presents on T cells. Tumor extrinsic mechanisms include the recruitment of immune suppressive cells such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (T_{reg}) and macrophages. Furthermore, secretion of immune-suppressive cytokines such as vascular endothelial growth factor (VEGF), interleukin-10 (IL-10) and transforming growth factor- β (TGF- β) promote tumor immune evasion.(33)

T_{reg} produce immunosuppressive cytokines such as transforming growth factor- β (TGF- β) and interleukin-10 (IL-10) to impair antitumor CTLs function.(36) FOXP3⁺ T_{reg} reduce CD8⁺ T cell mediated target cell elimination by inhibiting the release and production of perforin and granzyme B.(37) Moreover, FOXP3 T_{reg} suppress the production of IFN- γ and TNF- α to restrict CD8⁺ T cell activation.(37,38) M2 macrophages release of inhibitory cytokines such as IL-10, reactive oxygen species (ROS) or prostaglandins in TME and inhibit lymphocyte functions.(39) MDSC promote tumor growth by the release of arginase 1, which synergizes with inducible nitric oxide synthase (iNOS) to enhance nitric oxide or superoxide production which interfere in the trafficking of T cells into tumor site.(40)

Tumor-specific CD8⁺ T are rapidly exhausted under immunosuppressive tumor microenvironment.(41) Exhausted CD8⁺ T cells express high level of inhibitory receptors, such as programmed cell death-1 (PD1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte-activation gene 3 (LAG-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT). They regulate effector function of CD8⁺ T cells and promote tolerance to self-antigen.(41-46) Melanoma induces and modulates adaptive immune response that restricts melanoma progression and is considered to be highly immunogenic.(47) During melanoma progression cell proliferation and apoptotic pathways are associated to induce immune editing to interconnect the phases of tumor

elimination, equilibrium and immune escape.(34) High mutational load, restricted T cell infiltration, secretion of inhibitory cytokines (TGF- β , IL-10, and IDO), and downregulation of antiapoptotic proteins facilitate melanoma to evade immune response.(48)

4.4. Cancer immunotherapy

Cancerous cells attain immune evasion response, uncontrolled proliferation and metastasis. Conventional therapeutic approaches such as radiation and chemotherapy can induce clinically positive response to treat different types of cancers. Despite the clinically positive outcomes these conventional therapies are not successful to eradicate advanced cancer even after a prolonged treatment.(49) In comparison to conventional therapies, immunotherapy elicits valuable systemic approach to deal with the different types of cancers by generating a strong antitumor immune response and was considered the breakthrough of science in 2013.(50) The main purpose of immunotherapy is to induce and maintain a long term adaptive immune response to achieve a prolonged tumor regression. Several therapeutic approaches including application of exogenous cytokines (IL-2), vaccination to induce a strong antigen specific T cell response, adoptive transfer of tumor specific immune effector cells (TILs), receptor (TLRs) agonists and the application of immune checkpoint inhibitors (CTLA-4, PD-1 and IDO) are being used.(51)

An efficient immunity against cancer needs a specific recognition and removal of malignant cells. Most of the present efforts to induce antitumor immunity are aimed to elicit effector CD8⁺ T cells response (cytotoxic T cell response), in which a single cell may share the functions of both antigen recognition and cytotoxicity. Tumor suppressor cells within the tumor microenvironment (TME) compete with the detection and killing of antigen-specific CTLs. Usually TME prevents efficient T lymphocytes priming, and infiltration of CTLs cells, which lead to tumor rejection. Tumor rejection in TME accounts for the lack of antigen-specific stimulation or short-lived activation and insufficient direct or indirect antigen presentation to T cells.(52) Activation of cytotoxic cells, including CTLs, $\gamma\delta$ T cells, NK T cells and NK cells, represents an important immunosurveillance mechanism.(53)

4.5. CD8⁺ T cells

CD8⁺ T cells are a subpopulation of major histocompatibility complex class-I (MHC-I) restricted T cells and play an integral role in adaptive immunity. Activated CD8⁺ T cells are also known as cytotoxic T lymphocytes (CTLs) and are important for targeted elimination of cancerous and pathogen infected cells.(54) During initial encounter with pathogen, naïve CD8⁺ T cells are activated to recognize pathogen specific cognate antigen presented by dendritic cells (DCs) in the spleen or lymph nodes. Upon activation, pathogen-specific CD8⁺ T cells undergo extensive clonal expansion and convert into effector CD8⁺ T cell (CTLs) population.(55-57) Cytotoxic T cells kill the target in an antigen specific manner and they produce effector cytokines such as interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) that contribute to host immunity. Cytotoxic T cells exert direct target cell killing upon TCR engagement with its cognate antigen in the context of MHC-class I. CTLs expel lytic granules proteins, such as perforin and granzymes, towards target cells by calcium dependent manner.(58) Once perforin and granzymes are expelled by CTLs into the interstitial space between CTLs and their target cells, perforin polymerize and generate transmembrane pores in the target cell membrane. Granzymes are serine proteases, enter through perforin pores and activate target cell apoptosis by inducing the activation of caspase 3.(59-61) CTL uses another mechanism to kill the target cells by engaging its Fas ligand (CD178) with Fas receptor (CD95) (also called death receptor) on target cells. This engagement initiates the formation of death-inducing signaling complexes (DISCs) that contains pro-caspases-8 and FAS-associated death domain protein (FADD) to activate caspase-8 mediated apoptosis (Figure 2).(59,61-63) Interestingly, FasL on tumor cells may bind to Fas receptor (CD95) on immune cells and initiates apoptosis of tumor infiltrating CD8⁺ T cell, which is one of the mechanisms of tumor immune evasion.(64) CTLs also produce effector cytokines such as IFN- γ and TNF- α . TNF- α after release from the CTLs engages with its receptors on the malignant or pathogen-infected cells, which can lead to the activation of caspase cascade and apoptosis of target cells. In addition, IFN- γ induces transcriptional activation of FAS receptor and MHC-I antigen presentation pathway, leading to enhanced FAS mediated apoptosis and endogenous antigen presentation by MHC-I.(65,66)

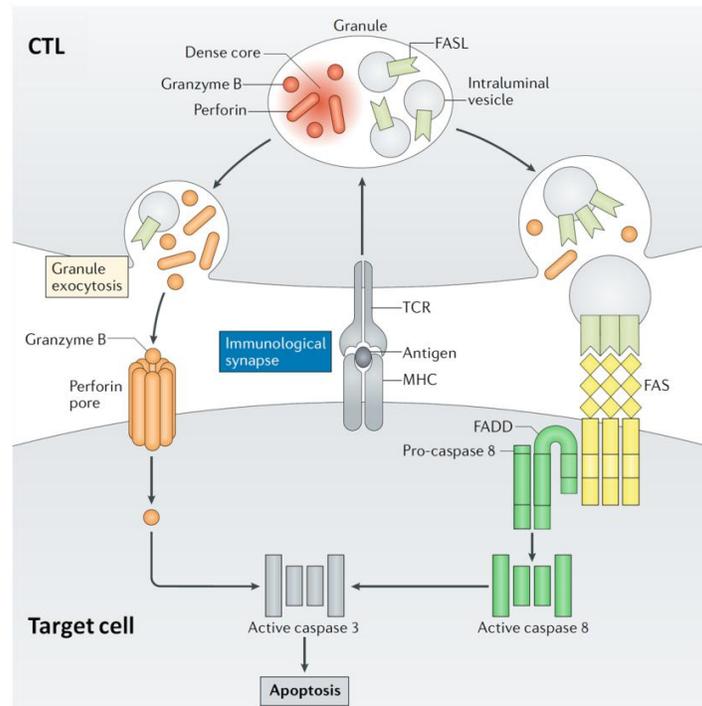


Figure 2: Mechanisms of CTL-mediated cytotoxicity. A cytotoxic T lymphocyte (CTL) recognizes an antigen-MHC complex on target cell through its T cell receptor (TCR). TCR-MHC binding triggers migration of granules containing FAS ligand (FASL), perforin and granzyme. Upon CTLs activation, granules release FAS ligand (FASL), perforin and granzyme into the intercellular space. FASL and FAS engagement activates caspase 8 through FAS-associated death domain protein (FADD), which in turn cleaves and activates caspase 3 to induce apoptosis. In the other mechanism, perforin generate pores in target cell membrane through which granzyme B enters the cytosol of target cell and activates caspase 3 to induce apoptosis. Adapted from Golstein and Griffiths, (2018), *Nature Reviews Immunology* (61)

To attain homeostasis and prevent autoimmunity, many effector $CD8^+$ T cells die and contract their population upto 5-20%. The remaining proportion of effector $CD8^+$ T cells converts into functional memory cells and survive for longer time through cytokine-dependent self-renewal.(67,68) During acute pathogen infection effector $CD8^+$ T cell population differentiate into subpopulations.(69,70) Memory $CD8^+$ T cells are divided into central memory (T_{CM}) and effector memory (T_{EM}) based on the relative expression of CD62L and C-C chemokine receptor type 7 (CCR7) (homing molecules).(71-73) T_{EM} cells do not express CD62L and CCR7 and since the lack of homing markers, they mostly localize in the peripheral organs and blood. In comparison to effector $CD8^+$ T cell (T_{EFF}), T_{EM} cell express higher level of IL-7R α (CD127) and show moderate expression of CD45RA.(74,75) T_{EM} cells sustain a high cytolytic

activity and anti-viral IFN- γ and TNF- α production. In contrast to T_{EM}, T_{CM} cells secrete less IL-2 and show poor proliferative capacity.(76) CD8⁺ T memory precursor cells express CD127^{hi}, which is functionally required for long term survival and differentiate them from short lived terminally differentiated short lived effector CD8⁺ T cells.(77,78) The development and function of CD8⁺ T cells depend on acute and/or chronic encounter of pathogen expressing specific antigen with CD8⁺ T cells. Following encounter with antigen naïve CD8⁺ T cells convert into short lived effector CD8⁺ T cells population expressing killer cell lectin like receptor G1 (KLRG1)^{hi} CD127^{lo} and KLRG1^{lo} CD127^{hi} memory precursor cells. After antigen clearance, memory precursor CD8⁺ T cells converts into memory CD8⁺ T cell population, which retains its ability to expand and convert into effector CD8⁺ T cell population upon secondary antigen encounter.(79) Memory precursor CD8⁺ T cell expressing CD127^{hi} develop into central memory CD8⁺ T cell and express homing receptor to reside in lymph nodes.(80) Table I shows the types of CD8⁺ T cells based on the surface expression markers and their phenotype. **Table I: Types of CD8⁺ T cells based on surface markers and observed phenotype.**

CD8 ⁺ T cell fate	Surface markers	Phenotype	Reference
Naïve	CD45RA ⁺ CCR7 ⁺ CD62L ⁺ CD45RO ⁻	i) Incapable of mediating effector response ii) Because of their role as precursors for CD8 ⁺ T-effector subsets, their loss is thought to be an important contributor to the immunosenescence	81
Effector	KLRG1 ⁺ CD43 ⁺ CD62L ⁻ CD69 ⁺ CD95 ⁺ CD137 ⁺	i) Direct cytotoxicity against malignant and pathogen infected cells ii) Induce cytotoxicity through Fas/FasL and perforin/granzyme	82-85
Central memory	CCR7 ⁺ CD44 ⁺ CD45RO ⁺ CD62L ⁺ CD122 ⁺ CD127 ⁺ IL15R ⁺	i) Less differentiated ii) Residing in lymph nodes, spleen, bone marrow and blood iii) No immediate effector function iv) Conversion into T _{EFF} upon antigen rechallenge v) Self-renewal capacity vi) IL-7/IL-15 dependence	86-91
Effector memory	CCR7 ⁻ CD44 ⁺ CD45RO ⁺ CD62L ⁻ CD127 ⁺ KLRG1 ⁺	i) Reside in lymphoid and peripheral tissues ii) Highly cytotoxic and rapidly release effector molecules iii) Rapidly conversion into T _{EFF} upon antigen rechallenge	86-91

4.6. CD8⁺ T cell exhaustion

T cell exhaustion is a dysfunctional state of T cells that arises during chronic pathogen infection and cancer. Main characteristics of exhausted T cells include poor effector function, distinct transcriptional state from the effector or memory T cells and continuous expression of inhibitory receptors. T cell exhaustion promotes pathogen infection and tumor progression.(92) Exhausted CD8⁺ T cell population was initially defined in a chronic lymphocytic choriomeningitis virus (LCMV) infection mouse model.(93) Exhausted CD8⁺ T cell loss their functions in an hierarchical manner, typically IL-2 production and *ex vivo* target killing and proliferative capacity lost first. Later exhausted CD8⁺ T cells completely lost their ability to produce effector cytokines such as TNF- α , IFN- γ , perforin and granzyme. Furthermore exhaustion results into the physical deletion of pathogen specific CD8⁺ T cells and prevents pathogen clearance.(94-96) During CD8⁺ T cell exhaustion, exhausted cells highly express the inhibitory receptors including CTLA-4, PD-1, TIM-3, LAG-3, TIGIT and BTLA.(97-102)

In tumor microenvironment (TME) most of the effector CD8⁺ T cells are differentiated into exhausted cells by expressing high levels of surface inhibitory receptors. Moreover, they lost their ability to eliminate malignant cells and produce less effector cytokines.(103) It has been reported that almost half of the tumor infiltrating CD8⁺ T cells become severely exhausted in proliferation and effector cytokine production by co-expressing CTLA-4 and PD-1.(104) Fourcade *et al.*, correlated the TIM-3 and PD-1 expression and effector cytokine production in patients with melanoma and found that, TIM-3⁺PD-1⁺ tumor infiltrating CD8⁺ T cells become more dysfunctional than TIM-3⁻PD-1⁻ and TIM-3⁻PD-1⁺, producing less IL-2, IFN- γ and TNF- α .(105) Woo *et al.*, observed the co-expression of LAG-3/PD1 tumor infiltrating CD8⁺ T cells. They found that LAG3⁺PD1⁺ CD8⁺ T cells become more exhausted than single negative CD8⁺ T cells.(106) In another study, Fourcade *et al.*, observed the potential role of BTLA in combination with PD-1 and TIM-3 expression on tumor infiltrating CD8⁺ T in melanoma patients. They found that BTLA⁺PD-1⁺TIM-3⁺ CD8⁺ T cells become more dysfunctional and common blockade of BTLA, PD-1 and TIM-3 enhanced effector function and proliferation of tumor specific CD8⁺ T cells.(101) Chauvin *et al.*, observed high expression of TIGIT with co expression of PD-1 on tumor infiltrating CD8⁺ T cell from melanoma patients and their combined blockade enhanced CD8⁺ T cell effector function and proliferation.(107) However, these studies revealed that exhausted CD8⁺ T cells posses a unique molecular signature that is make them distinct from the

naïve and effector population. Exhausted tumor infiltrating CD8⁺ T cell population is defined with the decreased proliferation, cytolytic activity and effector cytokine production. Restoring their anti-tumor effector function by blocking exhaustion receptors represents an effective strategy to treat melanoma.

4.7. Tumor infiltrating CD8⁺ T cells in melanoma

Tumor infiltrating immune cells population is a heterogeneous group of CD8⁺ T cells, regulatory T cells (T_{reg}), DCs, NKs, MDSCs and macrophages.(108) As effector cells play important role to kill the malignant cells, CD8⁺ T cells comprise the major population of TILs and it is highly associated with the better prognosis in several types of cancer.(109-111) During melanoma progression, tumor cells overlaying epidermis and surrounding stroma are infiltrated with tumor infiltrating lymphocytes, mostly cytotoxic CD8⁺ T cells (CTLs). Tumor infiltrating CTLs recognize and kill the targeted melanoma cells.(112) CD8⁺ T cells after maturation in the thymus, enter in the secondary lymphoid organs, such as spleen and lymph nodes, where they undergo division and multiplication. In the lymph node CD8⁺ T cells encounter with the foreign antigen presented by APCs through TCR and become activated.(113,114) The mature activated CD8⁺ T cells (CTLs) exit the lymph node and reach at the target tissue by passing through circulatory system to perform effector function.(115,116) CD8⁺ T cell trafficking is mediated by the interaction of CXC-chemokine receptor 3 (CXCR3) expressed by CD8⁺ T cells and C-X-C motif chemokine 10 (CXCL-10) produced by DCs.(117) Furthermore, DCs express CD70 ligand and CD80-CD86 to bind with their respective CD27 and CD28 receptors on CD8⁺ T cells. These interactions are considered to be the first step in CD8⁺ T cell priming and trafficking.(118) First time, in the mid-1980s, Rosenberg *et al.*, isolated highly cytotoxic lymphocytes from the tumor patients and that restrict tumor growth following adoptive transfer in patients with lung, liver and skin cancer.(119,120) Shankaran *et al.*, observed that both IFN- γ and tumor infiltrating lymphocytes are critical for antitumor immunity and suggested that tumor infiltrating CD8⁺ T cells are essential for tumor regression.(121) Shortly after, Dudley *et al.*, evaluated that clonal expansion of tumor infiltrating CD8⁺ T cells is responsible tumor regression patients with metastatic melanoma.(122) Before infiltration and accumulation at a tumor peripheral site, effector CD8⁺ T cells initially encounter with antigen present in tumor draining lymph nodes. Poor infiltration is correlated with poor immunogenicity of tumor due to the lack of antigens. In

this regards, Peske *et al.*, reported that murine B16 melanoma expressing ovalbumin (B16.OVA) as a neoantigen infiltrated more effector CD8⁺ T cells than the parental B16 line.(123) Dendritic cells (DCs) maturation and trafficking also affect the CD8⁺ T cell infiltration into the tumor. Poor antigen presentation by DCs limits effector CD8⁺ T cell priming and infiltration. Wang *et al.*, reported that unregulated STAT-3 signaling in melanoma inhibits DCs maturation and results into limited CD8⁺ T cell activation.(124) In addition to the importance of tumor antigenicity, DCs maturation in the vasculature of tumor also restricts the activation and accumulation of effector CD8⁺ T cells in the tumor. Mullin *et al.*, reported that subcutaneous (SC) and intravenous (IV) administration of bone marrow derived DCs robustly induce CD8⁺ T cell activation, but differentially infiltrate effector CD8⁺ T cells in SC sites or lungs.(125) However, these studies highlighted the potential role tumor infiltrating CD8⁺ in antitumor immune responses and supporting the application of tumor-specific CD8⁺ T cells in adaptive immunotherapy. Melanoma cells express melanoma antigens such as MART-1, gp100, gp75 and tyrosinase, which increase their immunogenicity. The expression level of these antigens affect CTLs activation their effector function. CTLs detect gp100, MART-1 (melanoma antigen recognized by T cells-1) but not tyrosinase by MHC-I restricted pathways.(126) Thus, CTLs are considered to be a useful target for melanoma specific immune therapy.

4.8. Regulatory T cell (T_{reg}):

CD4⁺ regulatory T cells (T_{reg}) are a highly immune suppressive subtype of CD4⁺ T cells expressing transcription factor FOXP3.(127,128) T_{reg} are originally identified as CD4⁺ CD25⁺ T cells and are proven to play an essential role in maintaining self-tolerance.(129-132) Deficiency of FOXP3⁺ T_{reg} results in allergy and fatal autoimmune disorder in both humans and mice.(131-133) T_{reg} are classified into natural/thymus (nT_{reg}) and peripherally/induced (iT_{reg}) regulatory T cell populations based on the site where they are generated.(131-133) Natural regulatory T_{reg} are generated in the thymus and their development is initiated by TCR signaling that is activated in CD4⁺ T cells upon recognition of self-peptides presented by APCs on their surface via MHC-II molecules. TCR signaling is followed by activation of IL-2 receptor subunit as, also known as CD25. Furthermore IL-2 signaling and expression of forkhead box protein P3 (FOXP3) orchestrate the differentiation of T_{reg} cells.(134) T_{reg} cells exert their suppressive mechanism through various humoral and cellular mechanisms, such as CTLA-4 mediated suppressive

function of APCs, which inhibits the priming and activation of effector CD8⁺ T cells, competition in IL-2 consumption, thereby reduces the amount of IL-2 for effector CD8⁺ T cells, conversion of ATP into adenosine, an immunomodulatory metabolite that can prevent optimal T cell activation and production of immunosuppressive cytokines (such as TGFβ, IL-10 and IL-35 and).(134) nT_{reg} cells express high level of Neuropilin-1(a type-1 transmembrane protein) and Helios (a member of the Ikaros transcription factor family) while iT_{reg} cells often lack or express very low level of these molecules. Helios and Neuropilin-1 overexpression in nT_{reg} cells is associated with their immunosuppressive activity by enhancing antigen recognition by nT_{reg} cells.(135,136)

The role of T_{reg} cells in antitumor immunity was first time reported in 1999, demonstrating that depletion of CD4⁺ CD25⁺ T_{reg} cells in mice results in increased tumor regression.(137,138) Tumor infiltrating lymphocytes comprise 10-50% of CD4⁺ T cells compared with the 2-5% in the peripheral blood of healthy individual.(139-141) Relatively high abundance of T_{reg} population in the tumor microenvironment results in poor prognosis in patients with various type of cancer including melanoma.(131,139).

4.9. Infectious bacteria as vaccine modality:

An efficient immunity against cancer needs a specific recognition and removal of tumor antigen-expressing malignant cells. The better understanding of pathophysiology and host-pathogen interaction, combined with advancement in cellular/molecular immunology, has helped to design and use of live/attenuated bacteria as conventional vaccine directed to infectious diseases. Importantly, practical usage of live/attenuated vaccines relies on getting a proper balance between the low virulence toxicity and high immunogenicity of the vaccine.(142) Therapeutic approaches to generate antigen-specific immune responses against tumors have included both passive and active immunization using purified tumor antigens, DNA or dendritic cells expressing tumor antigens.(143) Cancer vaccination by using bacterial and viral vector for site directed delivery of tumor associated antigen (TAA) is a promising approach to enhance tumor immunogenicity. Although the conventional therapeutic approaches comprising of surgical resection, chemo and radiotherapy are effective against some type of cancer, an enormous amount of patients do not benefit from such therapies. Novel experimental and medical therapies are claimed to treat cancer by improving and/or replacing conventional

methods. These may include gene therapy, telomerase therapy, insulin potentiating therapy, human alpha-lactalbumin made lethal to tumor cells (HAMLET) and bacterial-related therapies.(144) In this regard, it has been observed that infectious bacteria such as *Salmonella* (145) and *Listeria* (146-149), exhibit preferential accumulation in the tumor microenvironment thus offering a great potential for cancer therapy. Moreover, they possess certain advantageous features such as motility, capacity to simultaneously carry and express multiple therapeutic proteins and elimination by antibiotics, thus making bacterial-related therapies a promising new class of strategy in cancer treatment.(150) Recombinant bacteria and virus expressing tumor-associated antigen as a vector system has been considered as an attractive tool to induces a powerful humoral and cell mediated immune response against cancer. Bacterial and viral pathogenicity helps to induce strong antigen specific CD8⁺ T response to overcome the weak tumor immunogenicity. To achieve a long lasting protection against intracellular pathogen and to induce strong antigen specific CD8⁺ T cell response, CD8⁺ T cells must be activated through innate immune system for their replication and effector CTLs differentiation. Bacterial phagocytosis stimulates macrophages to secrete a variety of chemokines that recruit new cells to the site of infection. Inflammatory cytokines increase vascular permeability and lymphokines promote the expression of MHC molecules, co-stimulatory molecules and proteins associated with antigen processing. This entire set of modification promotes an early Th1 response and cell-mediated immunity. IL-12, produced by macrophages and dendritic cells in response to bacterial PAMPs (pathogen-associated molecular patterns), is the key lymphokine in this process. IL-12 acts on NK cells to release IFN- γ that further activates macrophages and promotes the destruction of the intracellular bacteria in the phagosome. The production of IFN- γ by NK cells, promoted by IL-12, has been shown to be a crucial factor in early host defense mechanisms against intracellular bacteria such as *Salmonella* and *Listeria*.(151)

4.10. *Listeria monocytogenes*:

Listeria monocytogenes is a food born gram-positive facultative intracellular bacterium that is associated with Listeriosis and gastrointestinal infections.(152,153) *Listeria* uses its virulence factor known as Internalin A (InIA) to interact E-cadherin on the surface polarized epithelium to invade into the cell.(154,155) Inside the cells, *Listeria* uses another virulence factor known as positive regulatory factor A (PrfA) for its intracellular survival and propagation.(156)

PrfA further activates phospholipases and pore-forming toxin Listeriolysin O (LLO) to escape from infected cells.(157) *Listeria* induces strong innate and adaptive immune response. The innate immune response is generated by the surface recognition of *Listeria* by toll-like receptors (TLRs) which downstream activate myeloid differentiation primary response 88 (MyD88) adaptor protein and induce proinflammatory cytokines production.(158,159) Once phagocytized by APCs *Listeria* is processed and their peptides are presented on the MHC-II to induce *Listeria*-specific CD4⁺ T cell response. Alternatively, on escape from the phagosome to cytosol nuclear oligomerization domain-like receptors (NLRs) and AIM2 sense *Listeria* and induce the activation of inflammatory cascades.(160,161) In the cytosol, *Listeria* secretes its proteins that are processed and presented by MHC-I molecules to CD8⁺ T cells the induction of *Listeria* specific CTL response.(162) Thus, the generation of innate and adaptive immune response makes *Listeria* a powerful vaccine vector.

4.11. Immunogenic cell death

Immunogenic cell death (ICD) is defined as a unique type of regulated cell death that is capable to elicit antigen-specific adaptive immune response through the release of spatiotemporally danger signals or damage associated molecular patterns (DAMPs).(163) These danger signals or DAMPs are endogenous cellular molecules that perform conventional cells functions, but on their extracellular exposure, they gain the immunogenic competence. After extracellular release, these molecules encounter with innate immune cells such as macrophages, monocytes and DCs via their cognate pattern recognition receptors (PRRs). This leads to the activation and maturation and migration of innate immune cells to tumor draining lymph nodes. Further these cells encounter with tumor specific-antigens in the tumor draining lymph nodes and presents them to CD4⁺ and CD8⁺ T cells, which initiate anti-tumor adaptive immune response.(164)

The immune system regularly encounters dead cells during normal cell turnover, injury and infection. Discrimination between different forms of cell death mechanisms is required to eliminate pathogens and proper healing by avoiding responses to self, which may result in autoimmunity. Tumor cells are often unable to induce strong activation of antitumor immune response due to their low antigenicity and poor adjuvant capacity. One putative way to increase the generation of effective antitumor immune response is to trigger ICD (Figure 3).(163)

Consequently, a major issue in immunology is to understand how the immune system recognizes whether cell death is immunogenic, tolerogenic and/or silent.(163) ICD is characterized by secretion of damage associated molecular patterns (DAMPs), including Calreticulin (CRT), ATP and HMGB1 (high-mobility group box 1) and their binding with CD91, P2RX7 and TLR-4, respectively. DAMPs facilitate the recruitment of immature DCs into tumor microenvironment and engulf tumor-antigens (CRT and HMGB1) to initiate antigen presentation to CTLs. The whole process results in the release of IL-1 β from mature DCs and IL-17 from $\gamma\delta$ T cells and finally leads to the tumor cell eradication by CTL response.(165)

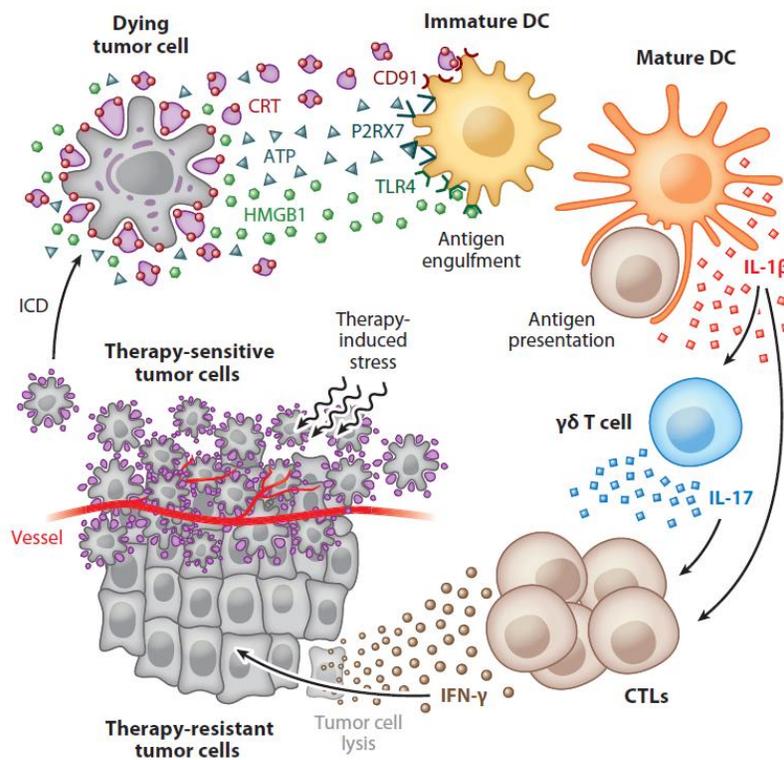


Figure 3: Immunogenic cell death (ICD). As a result of endoplasmic reticulum stress and autophagy, cancer cells respond to immunogenic cell death (ICD) inducers expose calreticulin (CRT) on the outer leaflet of their plasma membrane at a preapoptotic stage and secrete ATP during apoptosis. In addition, cells undergoing ICD release the nuclear protein high-mobility group box 1 (HMGB1) as their membranes become permeabilized during secondary necrosis. CRT, ATP, and HMGB1 bind to CD91, P2RX7, and TLR4, respectively, which facilitates the recruitment of DCs into the tumor bed (stimulated by ATP), the engulfment of tumor antigens by DCs (stimulated by CRT), and optimal antigen presentation to T cells (stimulated by HMGB1). Altogether, these processes result in a potent IL-1 β - and IL-17-dependent, IFN- γ -mediated immune response involving both $\gamma\delta$ T cells and CTLs, which eventually lead to the eradication of chemotherapy-resistant tumor cells. Adapted from Kroemer *et al.*, (2013) *Annual Review of Immunology* (163)

4.12. Necroptosis:

Necroptosis is a programmed inflammatory cell death pathway that is characterized by cell swelling, rupturing of plasma membrane and release of cellular organelles into the extracellular space.(166) Unlike apoptosis, necroptosis is caspases-independent programmed cell death which signals through receptor-interacting serine/threonine-protein kinase 1 (RIPK1), RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL). Necroptosis is highly immunogenic and mediates the release damage associated molecular patterns (DAMPs), high-mobility group box 1 (HMGB1), DNA, uric acid, IL-1 α and ATP which induce a robust immune response and inflammation.(167-169)

Necroptosis is mainly governed by stimuli that also initiate apoptosis extrinsic pathway such FAS ligand (FASL; CD95), TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor (TNF) family of cytokines.(170) One of the best-characterized signaling pathways inducing necroptosis is initiated by the binding of TNF with tumor necrosis factor receptor 1 (TNFR1). The binding of TNF with TNFR1 recruits cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2), tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), and TNF receptor associated factor 2 (TRAF2) to form membrane bound complex I and ubiquitylate RIPK1. Ubiquitination of RIPK1 leads to the activation of pro-survival mitogen-activated protein kinases (MAPK) and nuclear factor- κ B (NF- κ B) pathways.(171) Ubiquitination of RIPK1 prevents the formation of RIPK1 necroptotic and apoptotic complex. Subsequent degradation of cellular inhibitor of apoptosis proteins (cIAPs) by second mitochondria-derived activator of caspases (SMAC) facilitate deubiquitination of RIPK1 by deubiquitinases, including A20 and cylindromatosis (CYLD).(172-174) After deubiquitination, RIPK1 dissociates from the plasma membrane form a cytosolic complex II so called “riposome” containing RIPK1, caspase-8, and FAS-associated death domain protein (FADD).(175,176) In complex II, caspase-8 cleaves and/or inactivates RIPK3 to block its interaction with RIPK3 to induce necroptosis. In the ablation of caspase-8 activity, RIPK1 binds with RIPK3 to form necrosome, where RIPK3 phosphorylates MLKL. On activation MLKL oligomerizes and translocates to the plasma membrane to trigger necroptosis (Figure 4).(177,178) In addition to death-receptors ligands, RIPK3 dependent necroptosis can be triggered by other stimuli such as T-cell receptor (TCR) ligation, engagement of Toll-like receptor (TLR) 3 and TLR-4, DNA damage and viral infection.(179) Depending on the signals,

RIPK3 can be activated through RIPK1 dependent and or independent mechanism. For example, TLRs can directly activate RIPK3 without the activation of RIPK1 via another RHIM-containing protein known as TRIF, (180,181) where as during viral infection, DNA-dependent activator of interferon regulatory factors (DAI), another RHIM-containing cellular protein can activate RIPK3 through RHIM-RHIM interaction.(182)

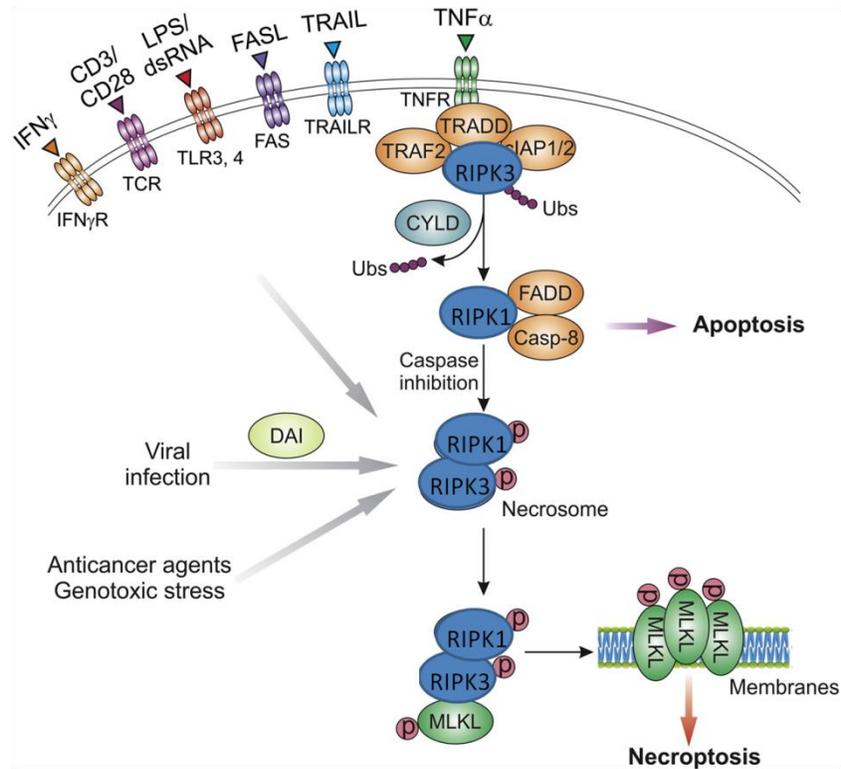


Figure 4: Necroptosis regulators and pathways. Binding of TNF- α with TNFR interacts with RIPK1 to recruit cIAP1 and cIAP2 to form a membrane bound complex I which results in the polyubiquitination of RIPK1. cIAPs inhibition leads deubiquitination of RIPK. RIPK1 binding with FADD and caspase-8 leads to apoptosis. Ablation of caspase-8 activity results in RIPK1 binding RIPK3 to form necrosome and subsequent phosphorylation of MLKL by RIPK3. This results in oligomerization and translocation of MLKL, towards plasma membrane to execute necroptosis. Other necroptotic stimuli, include, TRAIL, FASL, LPS, interferon γ (IFN γ) and dsRNA stimulate their respective receptors to activate RIPK1 and/or RIPK3 to promote necroptosis. Viral infection directly activates RIPK3 through DAI. Adapted from Chen *et al.*, (2016) *Biochimica et Biophysica Acta* (183)

Cancerous cells going through the process of necroptosis produce release DAMPs and release cytokines in a RIPK1 dependent manner to attract tumor infiltrating DCs. Tumor infiltrating DCs uptake the tumor antigen in tumor draining lymph nodes and presented them to

naïve CD8⁺ T cell by MHC-I molecules in a process called as cross priming. Upon antigen interaction, CD8⁺ T cells are differentiated into effector CD8⁺ T cells (CTLs) that infiltrate into the tumor site and kill the malignant cells. In parallel, RIPK3 can activate NKTs by upregulated the cytokines productions.(184) Previously, it has been reported that immunization with necroptotic cells induces CD8⁺ T cells priming and provide a strong antitumor immune response by exhibiting *in vivo* cytolytic activity.(185) Another study, reported that RIPK3 is necessary for the function of NKT cells by regulating the cytokine production via its potential effect on the activation of mitochondrial phosphatase phosphoglycerate mutase 5 (PGAM5). Furthermore, RIPK3-mediated activation of PGAM5 enhances cytokine production by nuclear translocation of nuclear factor of activated T-cells (NFAT) and dephosphorylation of dynamin-related protein 1 (Drp1) which are essential for mitochondrial homeostasis.(186)

Necroptosis is involved in the clearance of viral infection.(187) Number of studies highlights the role of receptor-interacting serine/threonine-protein kinases to control viral(188-193) and bacterial(194-200) infection. Most recently, it has been observed that, *Listeria monocytogenes* directly activates RIPK3, which further phosphorylates MLKL to restrict intracellular replication of *Listeria* without inducing necroptosis. Furthermore the phosphorylation of MLKL does not result into its own oligomerization and translocation to the plasma membrane (Figure 5).(201,202) However, these studies highlights the role of necroptosis or necroptotic cell death mediators such as RIPK1, RIPK3 and MLKL in regulating cellular inflammation, adaptive immunity and pathogen infection.

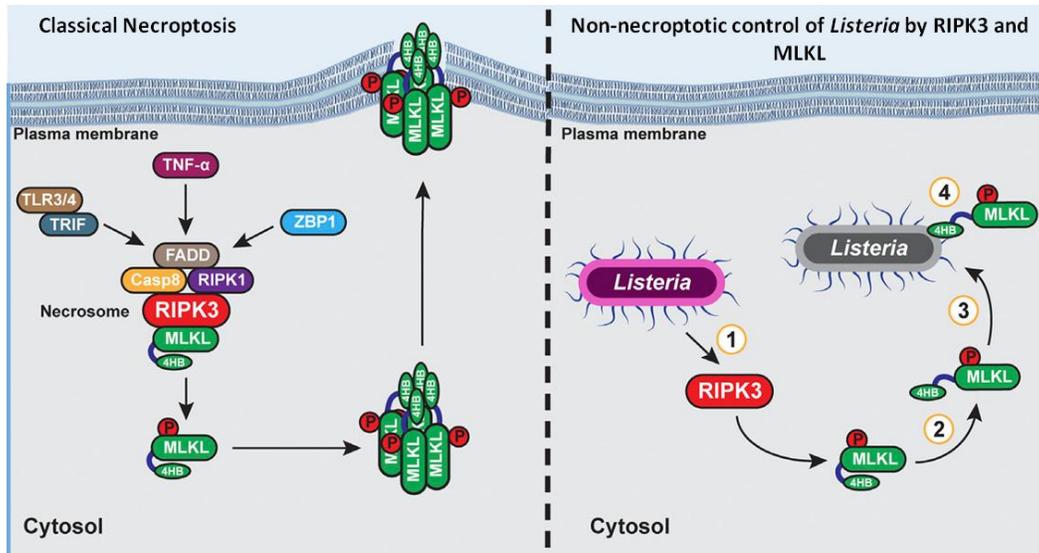


Figure 5: RIPK3 and MLKL clear *Listeria* from epithelial cells. In classical necroptosis signaling (left), activation of RIPK3 leads to the phosphorylation of MLKL. Activated MLKL subsequently oligomerizes and expose its phospholipid binding 4HB domain. Active MLKL translocates to the plasma membrane and induce necroptosis. In contrast, during *Listeria* infection of epithelial cells (right), *Listeria* directly activate RIPK3 which phosphorylates MLKL, but MLKL does not oligomerize and translocate to the plasma membrane. Instead, phosphorylated MLKL directly binds with cytosolic *Listeria* and inhibits its replication. Adapted from Zhang and Balachandran (2019), *Journal of Cell Biology* (202)

4.13. Pyroptosis:

Pyroptosis is another type of programmed cell death mechanism which is characterized by the cell swelling, rupturing of plasma membrane release of cellular contents into the extracellular environment and induction of strong inflammatory response.(203) First time, Mathan and Mathen in 1991, observed that *Shigella* enters in the colonic mucosa of human host and infects the phagocytic cells in lamina propria to induce extensive macrophages cells death and abscess formation.(204) In 1992, Zychlinsky *et al.*, observed that *Shigella* induces programmed cell death in infected macrophages and considered it to be apoptosis.(205) Further studies revealed that this cell death mechanism is a new mode of programmed cell death which is independent of caspase-3 activity and highly depends of caspase-1.(206-208) In 1999, Miao *et al.*, confirmed the role of caspase-1 in the induction of this programmed cell death mechanism in macrophages after infection with *Salmonella*. Furthermore, they observed the release of pro-inflammatory cytokines without the involvement of caspase-3, caspase-6 and caspase-7.(209) Subsequently, different studies were also confirmed the role of caspase-1 in the induction of this

novel programmed cells death mechanism distinct from apoptosis, during various pathogen infection such as *Listeria monocytogenes*, *Yersinia pseudotuberculosis*, *Legionella pneumophila* and *Pseudomonas aeruginosa*.(210) In 2000, first time the term pyroptosis was used to describe this programmed cell death pathway.(211) Within the past three years, Gasdermin D (GSDMD) was discovered and indentified as a downstream substrate of caspase-1/11 which is cleaved by caspase-1/11. After cleavage GSDMD releases N-terminal pore-forming domain (PFD) to exert pore formation in the plasma membrane and the release of cellular contents in the extracellular environment.(212-214)

Pyroptosis is initiated by the activation of either caspase 1 or caspase 11 (Figure 6). Caspase-1 is activated by one of several inflammasomes such as NLR family, pyrin domain-containing 3 (NLRP3), interferon- γ (IFN γ)-inducible protein 16 (IFI16), AIM2, pyrin, NLR family, CARD-containing 4 (NLRC4) and NLRP1b.(215) NLRP3 recognizes viral double-stranded RNA, bacterial toxins, ROS and adenosine triphosphoric acid.(216) AIM2 mainly recognize cytosolic DNA(217) during viral and bacterial infection, whereas IFI16 detects viral DNA.(218) Pyrin recognizes Rho family GTPases by bacterial toxins.(219) Bacterial flagellin, type III secretion system (T3SS) rod or needle proteins activate NLRC4.(220) NLRs recognition of their respective stimuli leads to the recruitment of inflammasome adaptor apoptosis-associated speck-like protein containing a CARD (ASC) to activate caspase-1 that coincides the release of IL-1 β and IL-18.(215) By contrast, caspase-11 is activated via direct binding of bacterial cytosolic lipopolysaccharide (LPS).(221,222) Furthermore, caspase 1 or caspase 11 independently cleaves gasdermin D that ruptures cell membrane and release of cellular contents in the extracellular environment to amplifies the inflammatory process.(212,223-226)

Listeria monocytogenes (LM) is recognized by toll like receptors (TLRs) on the surface of cells and cytoplasmic pattern recognition receptors NOD to induce innate immune response. TLR2 and TLR5 mainly recognized the extracellular LM, whereas NOD1 and NOD2 recognize cytoplasmic LM.(227) In addition to NODs cytoplasmic LM can also be recognized by inflammasomes such as NLRP3, NLRC4 and AIM2.(228) Once these inflammasomes are activated, they recruit the procaspase-1 and activate caspase-1 to eventually induce pyroptosis.(209) Substantial information reported that LM infection activates NLRP3 and caspase-1 to release proinflammatory cytokines and induces proinflammatory cell death to eliminate intracellular infection of LM.(229) Wu *et al.*, observed that LM infection activates

NLRP3 and ASC to induce caspase-1 dependent proinflammatory cell death.(230) William *et al.*, reported that NLRC4 attenuates CD8⁺ T cell response on infection with LM.(231) Thus these studies indicates that during LM infection, pyroptosis can serve a body defense mechanism which regulates the innate and adaptive immune response and clear intracellular pathogen infection.

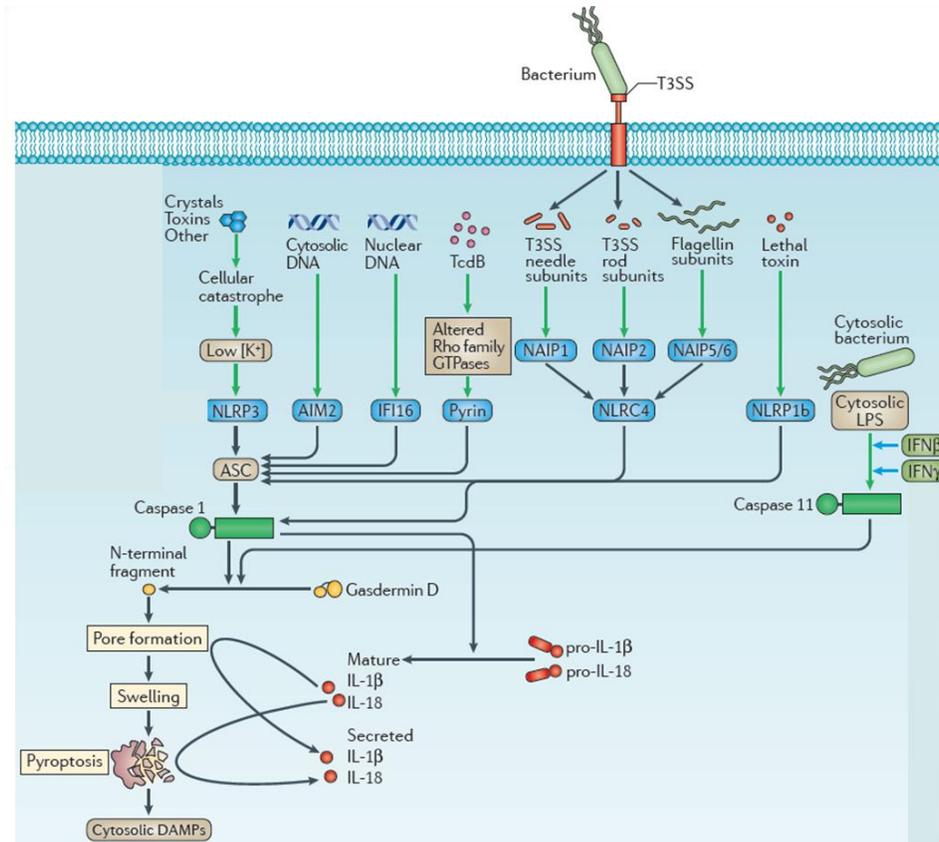


Figure 6: Pyroptosis in response to infection. Caspase-1 is activated downstream of inflammasomes activation by their respective stimuli. By contrast, caspase 11 senses cytosolic lipopolysaccharide (LPS). Either caspase 1 or caspase 11 independently cleaves gasdermin D to form the pyroptotic pores. The cell then ruptures the membrane and executes pyroptosis. Additionally, caspase 1 cleaves pro-interleukin-1β (pro-IL-1β) and pro-IL-18 to their mature forms (caspase 11 cannot do this directly). Adapted from Jorgensen and Miao, (2015) (232)

Considering the ability of intracellular bacteria (*Listeria monocytogenes*) such as, preferential accumulation into the tumor, induction of strong adaptive immune response and the potential role of inflammatory cells death mechanisms in the control of intracellular pathogen infection, we hypothesized this study as follow:

5. Hypothesis:

Hypothesis of this work is that the type of cell death follows the vaccination with recombinant bacteria, is important for subsequent immune response.

The hypothesis of this work is further divided into two aspects. The first aspect is that the infection of wild-type C57Bl/6 mice with OVA-expressing *Listeria monocytogenes* (LM-OVA) will induce resistance to subsequent inoculation with OVA-expressing syngeneic melanoma cell lines (preventive action) as well as tumor regression when inoculated in mice already bearing OVA-expressing tumor cells (therapeutic action). A second aspect of our hypothesis is that, this protective effect is dependent of the type of cell death inflicted by LM-OVA in infected host cells. Therefore, RIPK3^{-/-} (necroptosis), caspase1/11^{-/-} (pyroptosis) and caspase1/11^{-/-} RIPK3^{-/-} double deficient mice will display an incomplete/deficient protection.

6. Objectives:

The main goal of this project is to study the efficiency of cancer immunotherapy triggered by LM-OVA against syngeneic melanoma cell line expressing ovalbumin, in wild-type, RIPK3^{-/-} (necroptosis), caspase-1/11^{-/-} (pyroptosis) and caspase-1/11^{-/-} RIPK3^{-/-} double deficient mice.

6.1. Specific goals:

- 1) To compare anti-OVA cellular immune responses in wild type, RIPK3^{-/-} (necroptosis), caspase-1/11^{-/-} (pyroptosis) and Caspase1/11^{-/-} RIPK3^{-/-} double deficient mice C57Bl/6 mice infected with LM-OVA.
- 2) To investigate the effect of individual infection with LM-OVA on the growth of melanoma cell lines expressing ovalbumin, in wild-type, RIPK3^{-/-} and caspase-1/11^{-/-} and caspase-1/11^{-/-} RIPK3^{-/-} double deficient mice C57Bl/6 mice.

7. MATERIALS AND METHODS:

7.1. Antibodies and other reagents:

Recombinant mouse IL-2 (Sigma-Aldrich, I0523), purified Rat Anti-Mouse IFN-gamma (BD Biosciences, 551216), Biotin Rat Anti-Mouse IFN- γ (BD Biosciences, 554410), Anti-Mouse TNF monoclonal antibody (BD Biosciences, 51-26732E) Biotinylated Anti-Mouse TNF monoclonal antibody (BD Biosciences, 51-26731E) were used for ELISPOT assay. H2-K^b-SIINFEKL Dextramer (Immudex, Copenhagen, Denmark, JD2163), Anti-mouse CD8 (BD Biosciences, 551162), Anti-mouse CD8 (BioLegend, 100707) Anti-mouse CD8 (eBioscience, 48-0081-82) Anti-mouse CD45.1 (eBioscience, 12-0453-82), Anti-mouse IFN- γ (BD Biosciences, 563376), Anti-mouse TNF- α (BD Biosciences, 554418), Anti-mouse CD107a (LAMP1) (BioLegend, 121609), Anti-mouse CD127 (eBioscience, 17-1278-42), Anti-mouse KLRG1 (eBioscience, 11-5893-80), Anti-mouse CD4 (BD Biosciences, 563106) and Anti-mouse CD25 (eBiosciences, 17-0251-82), Anti-mouse FOXP3 (BD Biosciences, 560082), Carboxyfluorescein succinimidyl Ester (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, C34554) and Cell tracer Violet (CellTrace™ Violet Cell Proliferation Kit, Invitrogen, C34557) were used for flow cytometric analysis.

7.2. Cell lines:

B16-F0 and B16.OVA (B16 expressing-ovalbumin) cell lines were kindly provided by Prof. Subash Sad (University of Ottawa, Canada). B16.OVA cell line was previously generated Brown *et al.* (233) Both cell lines were cultured/maintained in RPMI-1640 (RPMI) medium supplemented with 8% FBS, 10% L-Glut and 10% Pen-Strep in 5% CO₂ at 37°C. *In vitro* growth curve for B16 and B16.OVA was drawn by counting of cells after subsequent intervals of time. The use of cells for experiments was conditioned to a minimum of 95% viability checked by the exclusion of 0.2% Trypan Blue.

7.3. Bacteria:

Recombinant *Listeria monocytogenes* strain (10403S) expressing-ovalbumin (LM-OVA) was kindly provided Prof. Subash (University of Ottawa, Canada). LM-OVA was previously was generated by Dudani *et al.* (234) From the frozen aliquot LM-OVA was grown on brain heart infusion (BHI)-streptomycin agar plates. A single colony was picked to grow in BHI medium (Sigma-Aldrich) supplemented with 50 μ g/ml streptomycin at 37°C under constant shaking. At

mid log phase ($OD_{600}=0.4$) bacteria was harvested, frozen in 20% glycerol and stored at -80°C . Colony-forming unit (CFU) was determined by performing serial dilution in 0.9% NaCl, which were spread on BHI-streptomycin agar plates.

7.4. Viral strain

Recombinant human adenovirus expressing-ovalbumin (rhAd5.OVA) was kindly provided by Prof. José Ronnie C. Vasconcelos (Universidade Federal de São Paulo, Brazil). WT and knockout (KO) mice were immunized or not with 2×10^6 plaque-forming unit (PFU) of rhAd5.OVA.

7.5. Mice:

C57BL/6 $\text{RIPK3}^{-/-}$ and $\text{Casp-1/11}^{-/-}$ mice were generously provided by Vishva Dixit (Genentech, Inc, USA) and Richard Flavell (Yale University, USA), respectively. $\text{RIPK3}^{-/-}$ and $\text{Casp-1/11}^{-/-}$ mice were crossed to generate $\text{Casp-1/11}^{-/-}/\text{RIPK3}^{-/-}$ double-deficient mice. 6-8 weeks-old WT (Wild type), $\text{RIPK3}^{-/-}$, $\text{Casp-1/11}^{-/-}$ and $\text{Casp-1/11}^{-/-}/\text{RIPK3}^{-/-}$ double-deficient mice were used as experimental controls or infected groups. OT-I mice $\text{CD45.1}^+\text{45.2}^+$ were generated by mating OT-I males ($\text{CD45.1}^-\text{45.2}^+$) with B6.SJL ($\text{CD45.1}^+\text{45.2}^-$) females. All mice experiments were performed in the animal facilities of Institute of Biomedical Sciences, University of São Paulo, and of University of Ottawa under the guidelines of Ethics Committee on Animal Use, University of São Paulo and Canadian Council on Animal Care (CCAC), respectively.

7.6. Mice Infection/Immunization:

C57BL/6 WT and KO mice were generated in-house at animal facility of Institute of Biomedical Sciences, University of São Paulo and maintained under the guidelines of Ethics Committee on Animal Use, University of São Paulo. For infection, frozen stock of LM-OVA was thawed and serially diluted in 0.9% NaCl. 6-8 weeks-old WT and KO mice were divided into three experimental control and infected groups. All the experimental infected groups were infected with 10^3 CFU of LM-OVA in $100\mu\text{l}$ of 0.9% NaCl via lateral tail vein (intravenously). While control groups were inoculated by $100\mu\text{l}$ of PBS.

WT and KO mice were immunized or non-immunized intramuscularly with 2×10^6 PFU of rhAd5.OVA in a total volume of $100\mu\text{l}$ of PBS ($50\mu\text{l}$ injected into the left and $50\mu\text{l}$ into the right Tibialis anterior muscle). All contaminated materials were disposed of as bio-hazardous waste by following proper standard operating procedure (SOPs).

7.7. Bacterial burden assay:

Spleens from all the infected mice at three and seven days post-infection were harvested independently and kept in RPMI medium (Life Technologies, Burlington, Ontario, Canada). Single cell suspension was prepared by tweezing each spleen separately between the frosted ends of two sterile glass slides in RPMI medium. CFU/spleen was determined by plating 10-fold serial dilutions of single cell suspension from each spleen separately on BHI-Streptomycin plates.

7.8. Assessment of antigen-specific CD8⁺ T cell population:

WT and KO mice were infected or not with LM-OVA for seven days. At seven days post-infection, spleens were harvested, processed and stained individually with anti-mouse CD8 antibody (BD Biosciences, 563898) and H2-K^b-SIINFEKL Dextramer (Immudex, Copenhagen, Denmark, JD2163) as per manufacture's instruction. Samples were read by FACS using BD FACSCelesta™ (BD, Mountain View, CA). Each sample was analyzed by using the following gate strategy. Finally, by using FlowJo v10 workspace, the frequency of CD8⁺ H2-K^b-SIINFEKL⁺ population of each sample was evaluated.

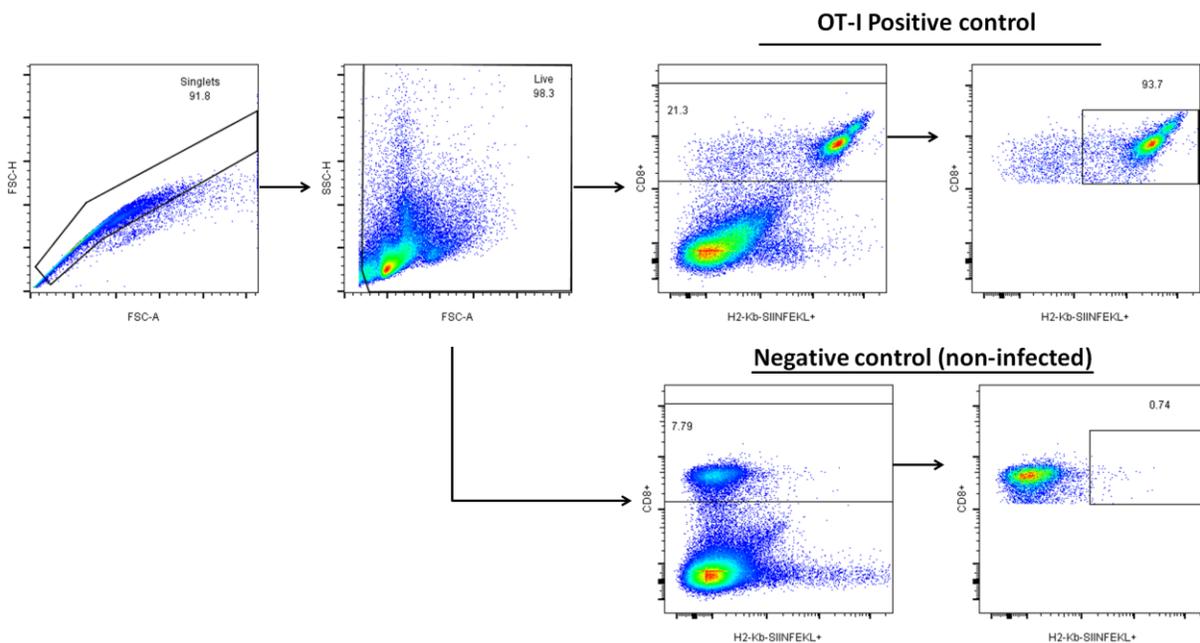


Figure 7: Gate strategy to access CD8⁺ H2-K^b-SIINFEKL⁺ population. FSC-A x FSC-H (Forward Scattered-Area x Forward Scattered-Height) to exclude doublets, then FSC-A x SSC-H (Forward Scattered-Area x Side

Scattered-Height) to exclude debris and finally H2-K^b-SIINFEKL⁺ x CD8⁺ to separate CD8⁺ H2-K^b-SIINFEKL⁺ population.

7.9. *In vivo* proliferation of antigen-specific OT-I CD8⁺ T cells:

In vivo proliferation of OT-I CD8⁺ T cells (CD45.1⁺ and CD45.2⁺) was performed to evaluate the differences in the priming and proliferation pattern of the OT-I CD8⁺ T cells in WT, and KO mice. OT-I splenocytes were labeled with 5uM of Cell tracer Violet (CTV) (CellTrace™ Violet Cell Proliferation Kit, Invitrogen, C34557) and 10⁷ cells in 100μl of un-supplemented RPMI were adoptively transferred by retro-orbital sinus in each mouse. After one hour, mice were infected with LM-OVA, while control groups remained uninfected. Four days later, the spleens of recipient mice were collected and processed independently to make single cell suspension. Splenocytes were labeled with anti-CD8 (BioLegend, 100707) for 30 minutes in PBS containing 1% bovine serum albumin (BSA). The reduction of CTV staining in dividing adoptively transferred OT-I cells was analyzed by FACS using BD FACSCelesta™ (BD, Mountain View, CA). The following gate strategy was used evaluate *in vivo* proliferation of OT-I cells. Finally, FlowJo v10 workspace, was used to evaluate the frequency of dividing adoptively transferred OT-I cells.

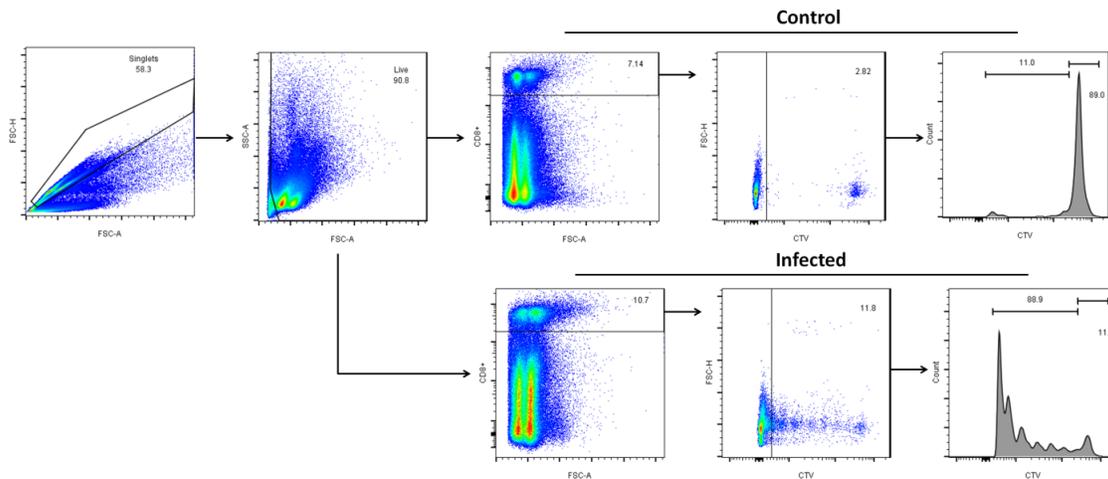


Figure 8: Gate strategy to access *in vivo* proliferation of OT-I cells. FSC-A x FSC-H to exclude doublets, FSC-A x SSC-A (Forward Scattered-Area x Side Scattered-Area) to exclude debris, FSC-A x CD8⁺ to separate CD8⁺ T cell population, FSC-H x CTV to separate adoptively transferred OT-I CD8⁺ cell population and finally CTV x Count to observe proliferation/division of OT-I CD8⁺ T cell population.

7.10. *In vivo* cytotoxic assay:

In vivo cytotoxicity was performed as previously described with slight modifications and optimization.(235) Briefly, at seven days post-infection, spleens from WT donor mice were harvested and processed for single cell suspension by tweezing each spleen between the frosted ends of two sterile glass slides. Cells were counted and divided equally into four populations and marked separately with two different concentrations of both Carboxyfluoresceine succinimidyl Ester (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, USA, Cat. No. C34554) and CTV; CFSE^{High} (10μM), CFSE^{Low} (1μM), CTV^{High} (10μM) and CTV^{Low} (1μM). CFSE^{High} cells were pulsed with 10nM of OVA₂₅₇₋₂₆₄ (SIINFEKL) peptide (InvivoGen, vac-sin) while the control CFSE^{Low} remained un-pulsed. CTV^{High} and CTV^{Low} cells were pulsed with 0.1nM and 0.001nM of OVA₂₅₇₋₂₆₄ peptide, respectively. All four populations of cells were washed and mixed in 1:1:1:1 ratio. Total of 4x10⁷ cells in 100μl of un-supplemented RPMI were injected in each infected and control experimental mouse by retro-orbital sinus. After 16-20 hours spleen from each mouse was excised, processed to obtain single cell suspension and analyzed by FACS using BD LSRFortessa™ (BD, Mountain View, CA). The percentage of target cells lysis was determined using the following formula.

$$1 - \frac{\% \text{CFSE}_{\text{high}} \text{ Or CTV}_{\text{high}} \text{ Infected} / \% \text{CFSE}_{\text{low}} \text{ Or CTV}_{\text{low}} \text{ Infected}}{\% \text{CFSE}_{\text{high}} \text{ Or CTV}_{\text{high}} \text{ Naïve} / \% \text{CFSE}_{\text{high}} \text{ Or CTV}_{\text{high}} \text{ Naïve}} \times 100$$

The following gate strategy was used to evaluate *in vivo* target cell killing by CTLs.

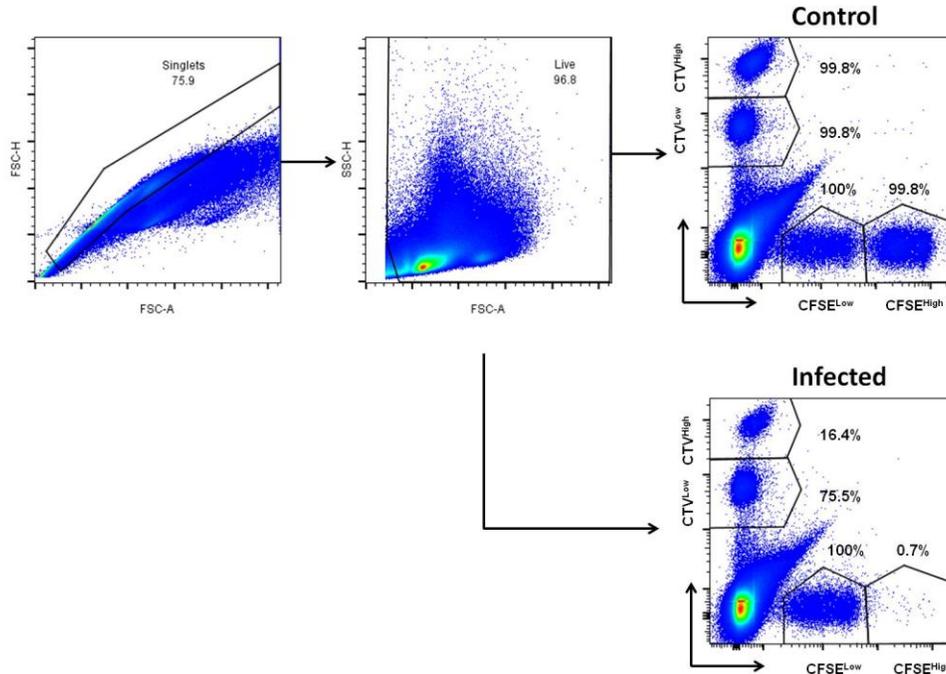


Figure 9: Gate strategy to access in vivo target cell killing by CTLs. FSC-A x FSC-H to exclude doublets, FSC-A x SSC-H to exclude debris and finally CFSE^{High}, CFSE^{Low}, CTV^{High} and CTV^{Low} populations showing percentage of target cell killing by CTLs.

7.11. ELISPOT Assay:

ELISPOT assay was performed to enumerate the frequency of IFN- γ -producing cells by using a protocol previously established by Boscardin *et al.*, (236), with slight optimization and modifications. Initially, 96-well nitrocellulose plate (Multiscreen HA Millipore) was coated with 60 μ l per well of sterile 1 X PBS containing 10ng/ml of mouse anti-IFN γ capture antibody (BD Bioscience, 551216). A separate 96-well nitrocellulose plate was coated with 100 μ l per well of sterile 1 X PBS containing 1:100 dilution of mouse anti-TNF- α (BD Biosciences, 51-26732E). Both plates were incubated for overnight at room temperature. After incubation plates were washed 3 times with 100 μ l RPMI under sterile conditions. Subsequently, plates were blocked by adding 100 μ l per well of 10% RPMI medium for 2 hours at 37°C. 10⁶ responder cells from each experimental mouse were separately added in anti-IFN- γ and anti-TNF- α Ab-coated ELISPOT plates, with 3x10⁶ feeder cells (from non-infected WT mice) and pulsed with 10 μ M OVA₂₅₇₋₂₆₄ peptide. The culture was established in RPMI medium supplemented with 1% NEAs (non-

essential amino acids) (Gibco), 1% L-Glut (L-Glutamine) (Gibco), 0.1% β -mercapto ethanol (Gibco), 1% sodium pyruvate (Gibco), 1% Pen-Strep (Penicillin-Streptomycin) (Gibco), 1% vitamins (MEM vitamin solution) (Gibco), 10% FBS (Fetal bovine serum) (Gibco) and recombinant mouse IL-2 (5ng/ml) (ThermoFisher, 701080) for 24h and 36h (IFN- γ and TNF- α , respectively) at 37°C with 5% CO₂. After incubation plates were washed three times with PBS containing 0.05% of Tween-20 (Fisher BioReagents) (PBS-T). IFN- γ specific plate was incubated with 100ul per well of Biotin Rat Anti-Mouse IFN- γ (BD Biosciences, 554410) at a final concentration of 20ng/ml in 0.05% PBS Tween-20 at 4°C for overnight. TNF- α specific plate was incubated with 100ul per well of biotinylated anti-mouse TNF monoclonal antibody (BD Biosciences, 51-26731E) at a 1:100 PBS-T at 4°C for overnight. Next day, the plates were washed 5 times with PBS-T and 3 times with 1 X PBS. Subsequently, 100 μ L per well of PBS-T containing streptavidin-HRP complex (BD Bioscience, 554066) was added at a 1:800 dilution and incubated at room temperature for 2 hours. Plates were washed 3 times with PBS-T and 5 times with 1 X PBS, respectively. Spots were visualized by using 3-Amino-9-ethylcarbazole (AEC) substrate (BD Biosciences, 551951) as per manufacture's instruction and washed with distilled water. The plate was dried at room temperature and the spots were quantified by ELISPOT reader (AID ELR06).

7.12. Intracellular cytokines staining:

For intracellular staining splenocytes from infected and non-infected mice were obtained independently after 7 days of infection. Total of 4 million splenocytes from each mouse were cultured separately in RPMI medium supplemented with 1% NEAs, 1% L-Glut, 0.1% β -mercapto ethanol, 1% sodium pyruvate, 1% Pen-Strep, 1% vitamins (MEM vitamin solution), 10% FBS, recombinant mouse IL-2 (5ng/ml), purified Na/LE Hamster Anti-mouse CD28 (BD, PharmingenTM, 553294) 2 μ g/ml, 1% brefeldin A (Biolegend, 420601) and monensin (Biolegend, 420701). Cells were pulsed with 10 μ g of OVA₂₅₇₋₂₆₄ peptide and incubated for 8 hours at 37°C with 5% CO₂. To stain the surface marker CD107a (LAMP1), anti CD107a (BioLegend, 121609) was added in the complete medium. After 8 hours of incubation cells surface markers were stained with H2-K^b-SIINFEKL Dextramer (Immudex, Copenhagen, Denmark, JD2163) and anti-CD8 (BD Biosciences, 551162). Intracellular staining of IFN- γ and TNF- α was done by using

BD Cytfix/Cytoprem kit (BD Biosciences, 554714), as per manufacture’s instruction. The following gate strategy was used to evaluate the CD8⁺ LAMP1 (CD107a) population.

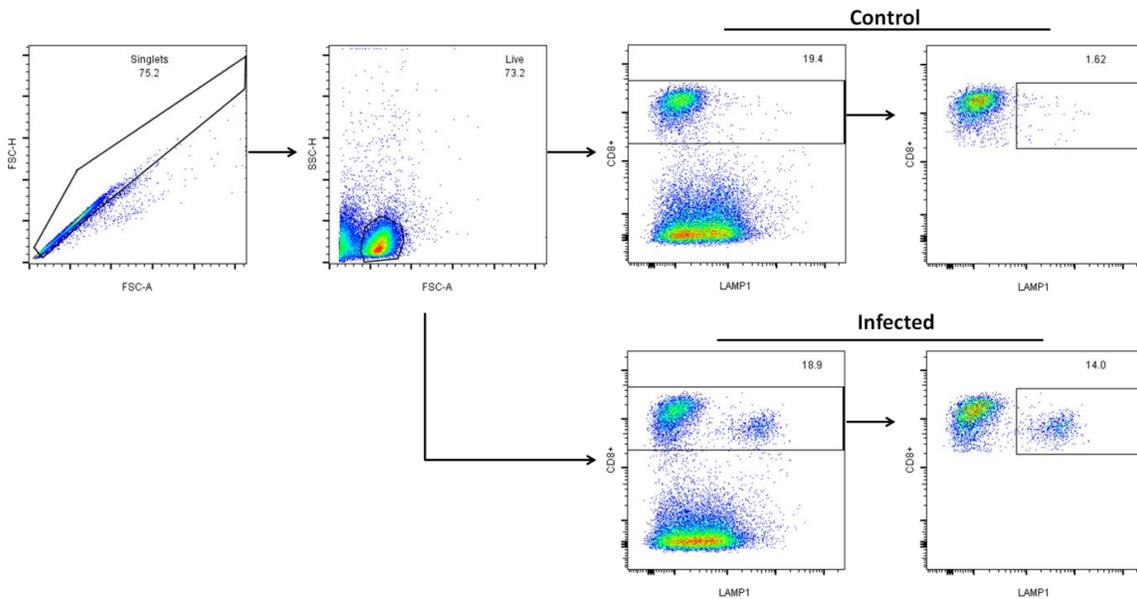


Figure 10: Gate strategy to observe CD8⁺ LAMP1⁺ population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris and finally LAMP1 x CD8⁺ to separate CD8⁺ LAMP1⁺ population.

The following gate strategy was used to analyzed CD8⁺ H2-K^b-SIINFEKL⁺ cells expressing IFN- γ and TNF- α populations.

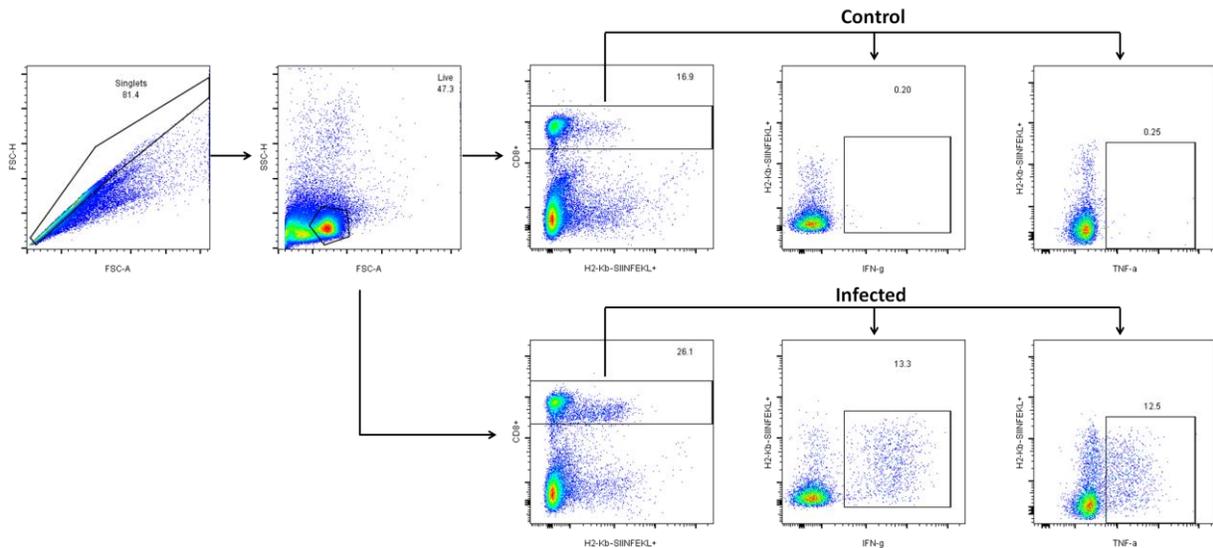


Figure 11: Gate strategy to observe IFN- γ and TNF- α expressing CD8⁺ H2-K^b-SIINFEKL⁺ population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris, H2-K^b-SIINFEKL⁺ x CD8⁺ to separate

H2-K^b-SIINFEKL⁺ x CD8⁺ population, IFN- γ x H2-K^b-SIINFEKL⁺ to separate IFN- γ ⁺ population and TNF- α x H2-K^b-SIINFEKL⁺ to separate TNF- α ⁺ population.

To correlate the LM-OVA burden and expansion of regulatory T cell (T_{reg}) response, we infected the mice with LM-OVA for 3 days. For intracellular staining splenocytes from infected and non-infected mice were obtained after 3 days of infection with LM-OVA. Total of 4 million cells from the spleen of each mouse were separated and used for surface staining with anti-mouse CD4 (BD Biosciences, 563106) and anti-mouse CD25 (eBiosciences, 17-0251-82) antibodies. Intracellular staining of fork head box P3 (FOXP3) was done by using anti-mouse FOXP3 (BD Biosciences, 560082) Fix/Perm Buffer Set (Catalog No. 421403), as per manufacture's instruction. The following gate strategy was used to evaluate CD4⁺ CD25⁺ FOXP3⁺ T_{reg} population.

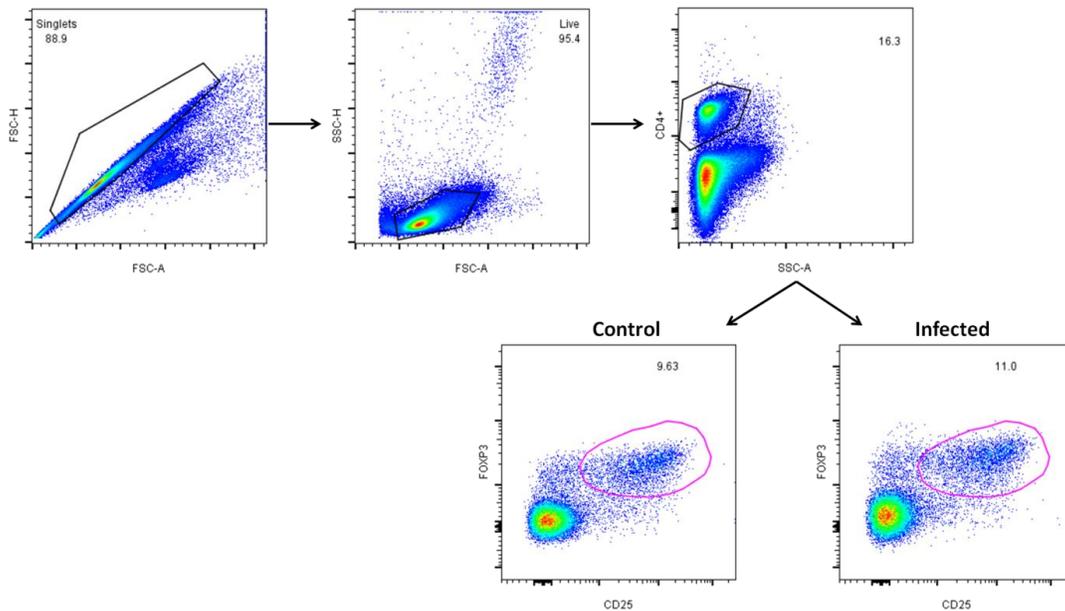


Figure 12: Gate strategy to evaluate CD4⁺ CD25⁺ FOXP3⁺ T_{reg} population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris, SSC-A x CD4⁺ to separate CD4⁺ T cell population and finally CD25 x FOXP3 to separate CD25⁺ FOXP3⁺ T_{reg} cell population.

7.13. Adoptive transfer:

For adoptive transfer, splenocytes from OT-1 (CD45.1⁺ and CD45.2⁺) mice were obtained, processed and inoculated (10⁷ cells/mouse) in each experimental mouse by retro-orbital

sinus. After one hours mice were infected or not with LM-OVA. At day 7 of post inoculation and infection, splenocytes from infected or non-infected mice were obtained, processed and stained independently with anti-CD8, anti-CD45.1, anti-CD127 and KLRG1. Cells were harvested from the spleen of mice and analyzed by FACS using BD LSRFortessa™ (BD, Mountain View, CA). The following gate strategy was used to evaluate the CD127 and KLRG1 positive CD8⁺ OT-1 (CD45.1⁺ and CD45.2⁺) population.

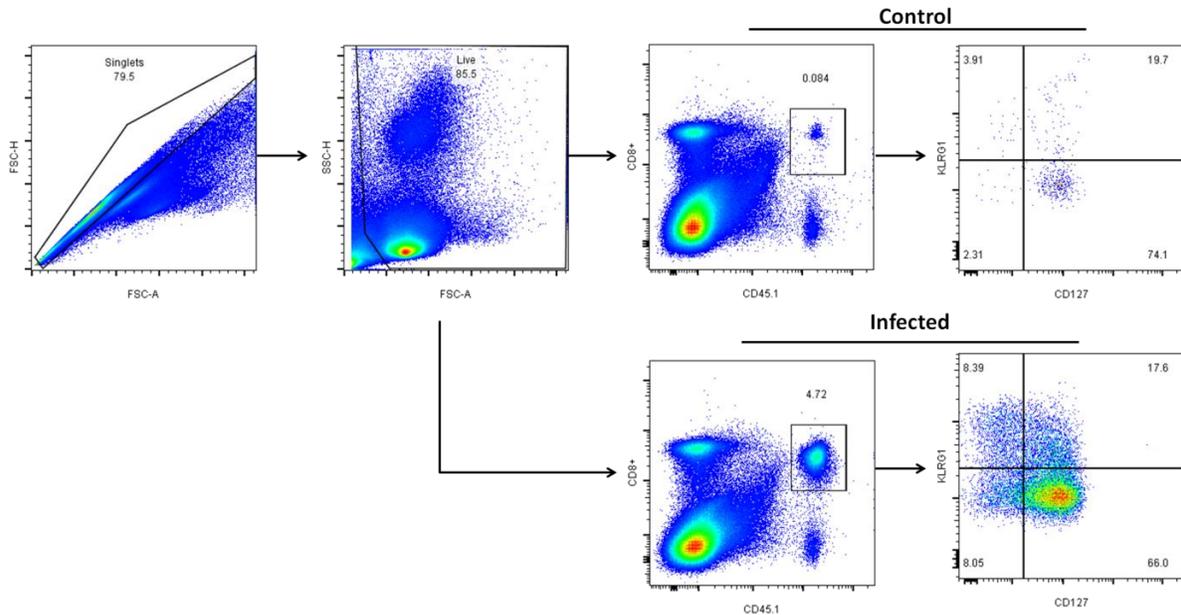


Figure 13: Gate strategy to evaluate KLRG1⁺ CD127⁺ OT-I CD8⁺ (CD45.1 CD45.2⁺) population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris, CD45.1 x CD8⁺ to separate CD45.1⁺ CD45.2⁺ adoptively transferred CD8⁺ OT-1 cells and finally CD127 x KLRG1 to observe the frequency of KLRG1 and CD127⁺ adoptively transferred CD8⁺ OT-1 cells.

7.14. Quantitative PCR:

B16 and B16.OVA cells were harvested from culture and counted (5×10^6 Cells) for total RNA extraction. Total RNA extraction was done by Trizol[®] reagent (Life Technologies, USA) according to the manufacturer's instruction. RNA was resuspended in 20 μ l DNase/RNase free water and stored at -70°C. Total RNA was quantified by using Nanodrop-2000 spectrophotometer (Thermo Scientific USA). cDNA was prepared by using 2 μ g of RNA for total of 10 μ l reaction. Reverse transcription was performed using ImProm-II™ Reverse Transcription System (Promega USA) according to manufacturer's instructions with Mastercycler[®] Pro-

thermo cycler (Eppendorf USA). Quantitative Real-Time PCR (polymerase chain reaction) was performed by using reaction mixture having: Syber® Green PCR Master Mix (Applied Biosystem UK), cDNA 80ng, 0.6µM primers (for GAPDH) and 50µM (for Ovalbumin), and RNase free water in QuantStudio™ 12 Flex Real-Time PCR System (Applied Biosystem USA). The following primers sets used were used to amplify ovalbumin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH):

OVA Forward: TCAAGCACATCGCAACCAAC

OVA Reverse: AGGGGAAACACATCTGCCAA

GAPDH Forward: GAGCAACTCCCCTCTTCC

GAPDH Reverse: CCATGTAGGCCATGAGGTTC

The reaction conditions for qPCR were used as: a preheat temperature of 94°C for 10 min, 40 cycles of 94°C for 15 sec, 60°C for 1 min and a final elongation at 72°C for 10 min. For gene expression analysis, the relative quantification approach was used, in which we used the CT (threshold cycle) values of each of the samples obtained at the exponential phase of reaction. The values were used for $2^{-\Delta\Delta CT}$ calculation and each value was normalized by housekeeping GAPDH.

7.15. Protein electrophoresis and Western Blot analysis:

Total of 1×10^6 B16 and B16.OVA cells were two times washed with 1X PBS and centrifuged at 5000 x g for five minutes. Thereafter, 1X protein sample buffer was added to the pallet and incubated for 5 minutes at 95°C. Cell lyses were stored -20°C. The ovalbumin (OVA) protein expression was analyzed by sodium dodecyl sulphate-containing polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blot. 40µl of each sample containing 40µg of protein were applied with BenchMark Ladder (Invitrogen) to 10% running gel. After electrophoresis, the gel proteins were transferred through a semi-dry system (Trans-Blot Tuber Transfer system (BIO-RAD) to a 0.22mm polyvinylidene difluoride (PVDF) membrane for 9 minutes at 25V. Thereafter, the membrane was left in the blocking solution containing skimmed milk in TBS-Tween (150mM NaCl, 50mM Tris-HCL, 0.05% Tween-20) plus 0.1g azide for 2 hours at room temperature. Then the membrane was incubated overnight at 4°C with the primary antibody. After incubation the membrane was washed three times in TBS-Tween and incubated for 1 hour at room temperature with the appropriate secondary antibody conjugated to

peroxidase. After this period the membrane was washed again three consecutive times and the immune-complexes were detected by the chemiluminescence method (ECL) (enhanced chemiluminescence) prepared in our laboratory [Solution A: 9 ml H₂O, 1ml Tris-HCL 1M pH 8.5, 22µl p-coumaric acid 90mM, 50µl Luminol 250µm and Solution B: 450µl H₂O and 50µl H₂O₂ 30%]. The bands were revealed on membrane by Gel Doc XR + System - BIO-RAD).

Primary antibodies: Anti-OVA, (SIGMA, ABS818) and β-Actin, (SIGMA, A1978).
Secondary Antibody: Anti-mouse α-IgG (BIO-RAD, 1721034).

7.16. *In vivo* tumor growth:

2.5x10⁵ B16 and B16.OVA cell were inoculated subcutaneously on the right flank of Rag1^{-/-} mice to draw the *in vivo* growth curve of tumors. WT and KO mice were subcutaneously challenged with 10⁶ B16 and B16.OVA cells. To observe preventive action of LM-OVA vaccination (as prophylactic vaccination), WT and KO mice were vaccinated with 10³ CFU of LM-OVA. After 7 days of vaccination mice were subcutaneously challenged with 10⁶ B16 and B16.OVA cells. To observe the therapeutic action LM-OVA vaccination (as therapeutic vaccination), WT and KO mice were challenged with 10⁶ B16 and B16.OVA cells. After 3 days, mice were vaccinated intravenously with 10³ CFU of LM-OVA. Tumors were excised when the diameter reached to 1cm³. The diameter of tumor was measured after subsequent interval of 2 days by using the following formula.

$$V = 0.5 a \times b^2 \text{ (a= long diameter of the tumor, b= short diameter of the tumor)}$$

7.17. Statistical analysis:

Statistical analysis was performed by using Graphpad Prism version 5 (Graphpad Software Company Incorporation). Statistical significance was determined by using two-way ANOVA followed by *Bonferroni* posttests. Statistical differences were considered significant when the *P* value was <0.05.

8. RESULTS:

8.1. RIPK3 and Casp-1/11 deficiencies differentially impair host ability to control *Listeria* infection

First, we evaluated the impact of individual or combined RIPK3 and Casp-1/11 deficiencies on the ability of C57Bl/6 mice to handle recombinant LM-OVA infection. We observed no difference in the size of the spleens from WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO (double knock-out) mice at day 3 post-infection (peak of infection in the spleen) with LM-OVA (Figure 14a). However, at this time point, bacterial burden in spleen was significantly higher in RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} double deficient in comparison to WT mice, suggesting that both RIPK3 and Casp-1/11 are important to control the early phase of *Listeria* infection (Figure 14b). Interestingly, at day 7 post-infection (time of resolution) the spleen size was augmented in all RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice in comparison to WT (Figure 14c). Most importantly, at this time point, bacterial burden was only detected in Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice, suggesting that Casp-1/11 but not RIPK3 is essential to clear LM-OVA infection (Figure 14d).

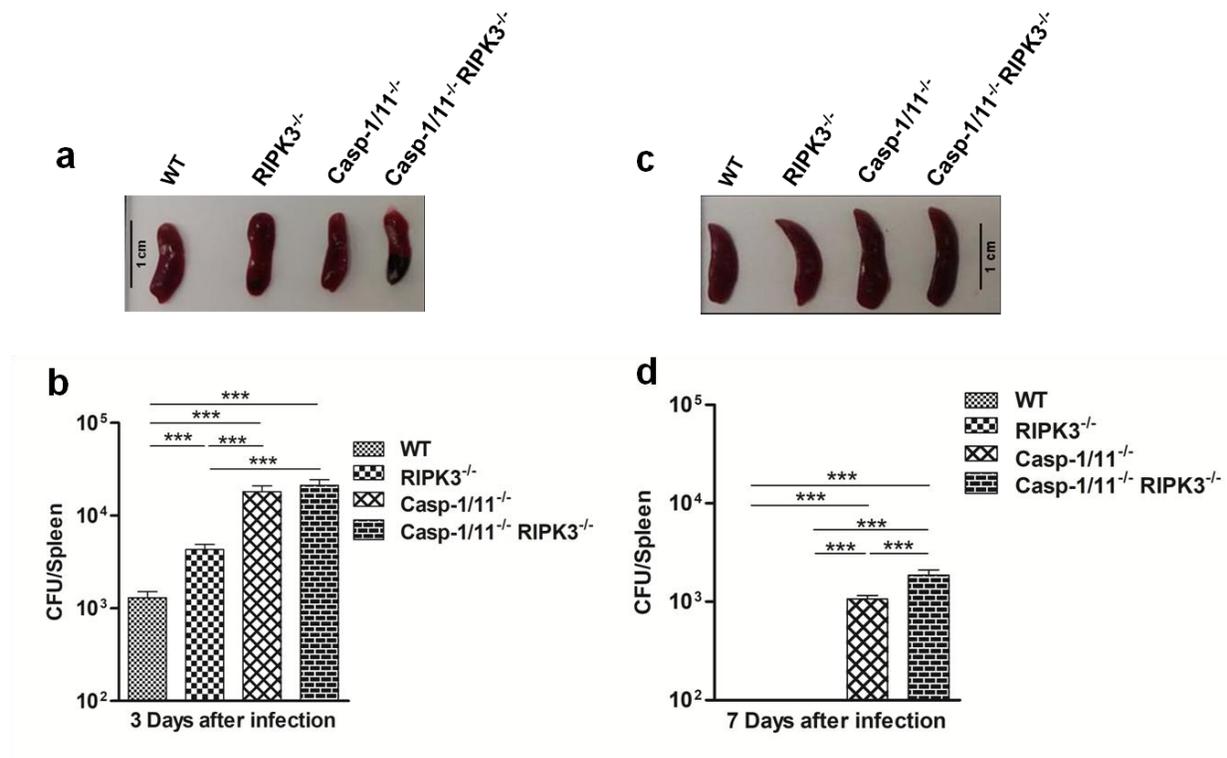


Figure 14: Casp-1/11 but not RIPK3 is necessary for the host ability to clear LM-OVA infection. Size of the spleens (a, c) and bacterial burden (b, d) were measured in infected mice at 3 (a, b) and 7 (c, d) days after LM-OVA vaccination. The data is expressed as means of five individual mice per group and is representative of three independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***($p < 0.001$)

8.2. Casp-1/11 deficiency interferes with antigen-specific CD8⁺ T cell expansion in response to LM-OVA

To evaluate whether the ability to clear LM-OVA would influence the level of OVA-specific CD8⁺ T cell response, we used a MHC class I multimer technology to measure the population of OVA₂₅₇₋₂₆₄ (SIINFEKL)-specific CD8⁺ T cells generated in response to LM-OVA vaccination. In comparison to WT mice, we observed significantly lower frequencies and numbers of OVA₂₅₇₋₂₆₄ (SIINFEKL)-specific CD8⁺ T cells in Casp-1/11^{-/-} and Casp-1/11^{-/-} RIPK3^{-/-} DKO but not in RIPK3^{-/-} mice (Figure 15a-c), suggesting a positive correlation between the capacity of mice to clear LM-infection and the amplitude of succeeding OVA-specific CD8⁺ T cell response.

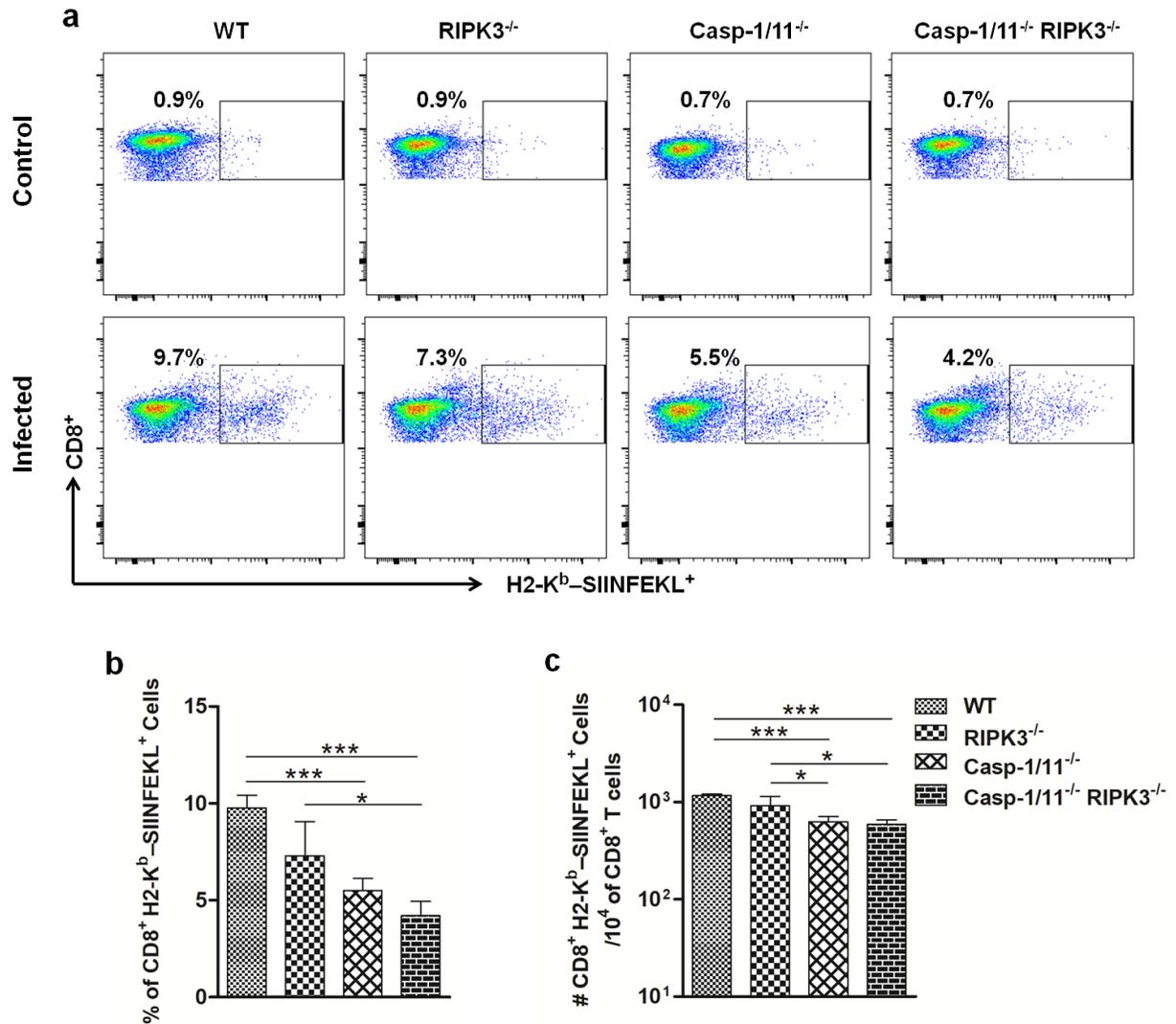


Figure 15: Casp-1/11 deficiency impairs *in vivo* antigen-specific CD8⁺ T cell expansion in response to LM-OVA. (a,b) Frequency and (c) total number of OVA₂₅₇₋₂₆₄ (SIINFEKL)-specific CD8⁺ T cells at 7 days of infection in the spleens of infected mice, revealed by staining with anti-CD8 antibody and H2-K^b-SIINFEKL dextramer. The data is expressed as means of three to five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***($p < 0.001$) *($p < 0.05$)

8.3. RIPK3 and/or Casp-1/11 deficiencies limit antigen-specific CD8⁺ T cells priming and proliferation

The reduced LM-OVA-triggered, OVA-specific CD8⁺ T cell numbers observed in the absence of Casp-1/11 (but not of RIPK3) could be the result of impaired proliferation of these

cells. To approach this question, we examined the *in vivo* proliferation of OT-I CD8⁺ T cells adaptively transferred to WT, RIPK3^{-/-}, Casp-1/11^{-/-} or Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice at 96 hours after vaccination with LM-OVA. As expected, we retrieved roughly the same frequency of OT-I CD8⁺ T cells in all non-infected mice strains, which means that the RIPK3 and/or Casp-1/11 deficiency does not affect the homeostatic proliferation or the survival of donor OT-I CD8⁺ T cells (Figure 16a, b). In contrast, compared with WT mice, we observed a significantly lower proliferation of OT-I CD8⁺ T cells in all RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice in response to LM-OVA vaccination (Figure 16a, c). Since the bacterial burden was the higher in the RIPK3 and Casp-1/11 KO mice, this suggests that the impaired proliferation of CD8⁺ T cells in these mice is not due to poor antigenic levels. Accordingly, the frequency of non-dividing (>1 division) OT-I CD8⁺ T cells population remained significantly higher in RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice (Figure 16a, c). Our results suggest that RIPK3 and Casp-1/11 are important for optimal priming and proliferation of antigen-specific CD8⁺ T cells in response to LM-OVA vaccination.

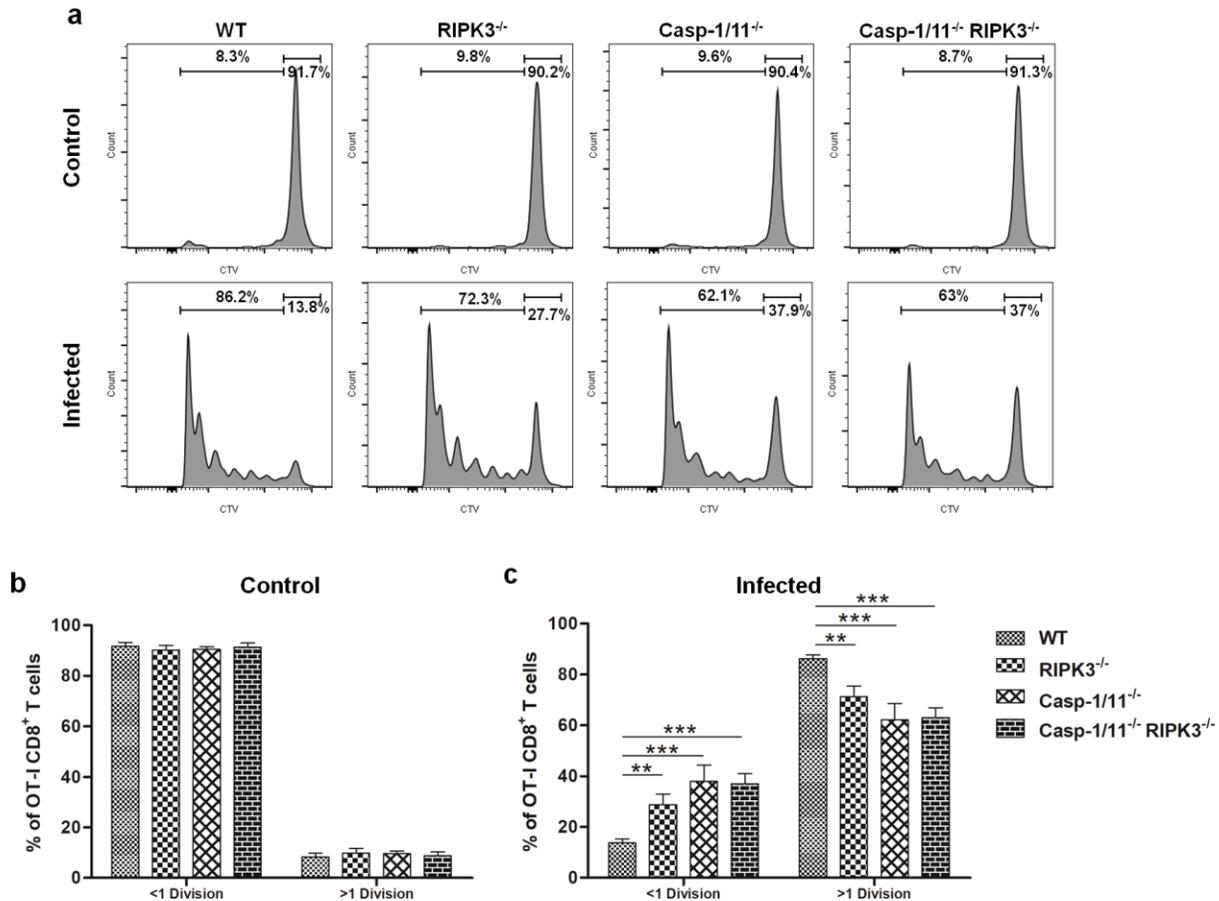


Figure 16: RIPK3 and/or Casp-1/11 deficiencies negatively impact on OVA-specific CD8⁺ T cells priming and proliferation in response to LM-OVA. (a) *In vivo* proliferation of OT-I CD8⁺ T cells in non-infected and LM-OVA-infected WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice. Frequencies of <1 division and >1 division OT-I CD8⁺ T cell populations in (b) non-infected controls and (c) LM-OVA-infected mice. The data is expressed as means of five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***($p < 0.001$) *($p < 0.01$)

8.4. RIPK3 and/or Casp-1/11 deficiencies impair OVA-specific CD8⁺ T cell cytolytic activity and cytokine production in response to LM-OVA vaccination

Initially, we established a dose dependent LM-OVA response to generate antigen-specific CD8⁺ T cells response by infecting the WT mice with 10^2 , 10^3 and 10^4 CFU of LM-OVA for 7 days. We performed *in vivo* cytotoxicity to evaluate the CTL killing of targeted cells pulsed with three different concentrations of OVA₂₅₇₋₂₆₄ peptide, namely high (10nM), intermediate (0.1nM)

and low (0.001nM) concentrations. We observed that 10^3 and 10^4 LM-OVA induce strong OVA-specific $CD8^+$ T cells response and kill more than 99% target cells pulsed with high concentration (10nM) of OVA peptide (Figure 17a, b).

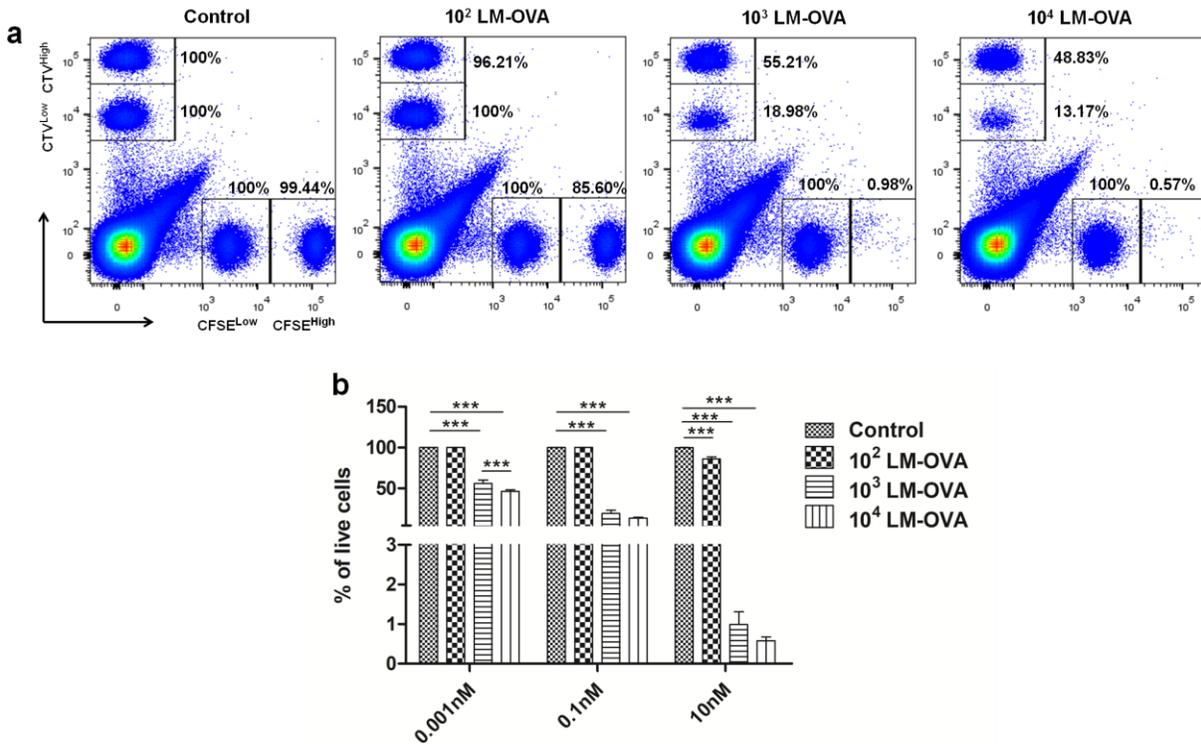


Figure 17: LM-OVA induce strong OVA-specific CTL response. (a-b) WT C57Bl/6 mice were infected or not with 10^2 , 10^3 and 10^4 CFU of LM-OVA. *In vivo* cytotoxic activity was estimated by injecting syngeneic target cells labeled with CFSE^{High}, CFSE^{Low}, CTV^{High} and CTV^{Low} and pulsed 0.001nM (CTV^{High}), 0.1nM (CTV^{Low}), 10nM (CFSE^{High}) or not (CFSE^{Low}) with OVA₂₅₇₋₂₆₄ peptide. Percentage of CFSE and CTV shows the frequency of viable cells after CTL-mediated target cell elimination. The data is expressed as means of three individual mice per group. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***($p < 0.001$)

We also enumerated the frequency OVA-specific IFN- γ secreting $CD8^+$ T cells and found that 10^2 CFU of LM-OVA is not enough generate detectable IFN- γ response after 7 days of infection. A high frequency of OVA-specific IFN- γ secreting $CD8^+$ T cells was observed in mice infected with 10^3 and 10^4 CFU of LM-OVA (Figure 18a, b). Taken together our results show that antigen-specific $CD8^+$ T cell response induced by LM-OVA is highly dose-dependent.

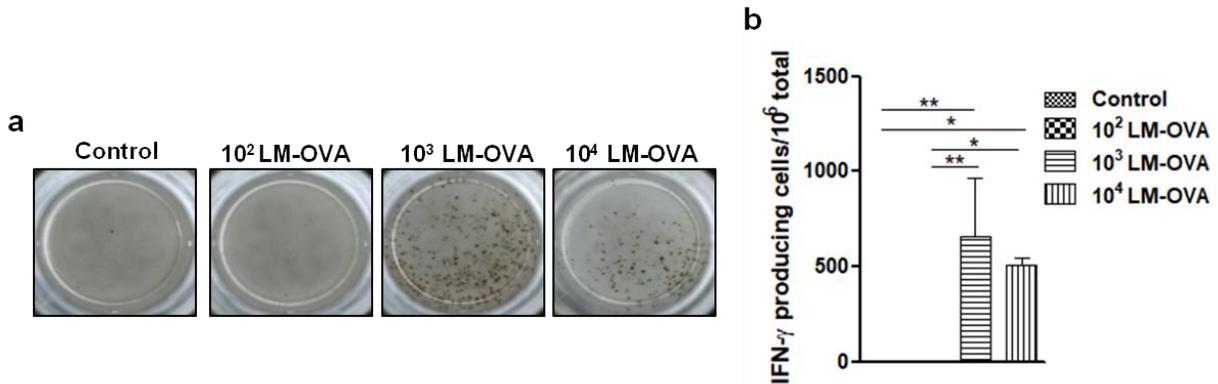


Figure 18: LM-OVA induces OVA-specific IFN- γ production by CD8⁺ T cells. (a, b) Splenocytes from non-infected and infected mice (10^2 , 10^3 and 10^4 CFU of LM-OVA) were pulsed with $10\mu\text{M}$ of OVA₂₅₇₋₂₆₄ peptide. Frequency of IFN- γ producing OVA-specific CD8⁺ T cells was determined by ELISPOT assay. The data is expressed as means of three individual mice per group. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

Next, we analyzed the importance of RIP3K and Casp-1/11 for the functional profile of CD8⁺ T cells. First, we evaluated the cytolytic activity of OVA-specific CD8⁺ T cells at day 7 post-infection with LM-OVA. As expected, we retrieved more than 99% of all target populations in control, non-infected mice (Figure 19a, b). A significant weakening in elimination of target cells pulsed with 0.1nM of OVA₂₅₇₋₂₆₄ peptide was observed in RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice (Figure 19a, c), suggesting that at least for this particular concentration of cognate peptide (therefore avidity of CTL/target interaction may be critical) both RIPK3 and Casp-1/11 are important for optimal *in vivo* CTL effector response. Interestingly, we also observed a small, yet significantly reduced target elimination at the lower (0.001nM) peptide concentration in Casp-1/11^{-/-}/RIPK3^{-/-} DKO mice (Figure 19a, c), suggesting that these proteins may act in concert to optimize the protective effect of LM-OVA vaccination. Further, we assessed the ability of OVA-specific CD8⁺ T cells to produce IFN- γ or TNF- α by ELISPOT and intracellular staining. A significantly lower frequency of IFN- γ - (Figure 20a, b) and TNF- α - (Figure 20c, d) producing CD8⁺ T cells was observed in RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} mice. To determine the prerequisite of cytolytic activity of OVA-specific CD8⁺ T cells, we assessed the degranulation of OVA-specific CD8⁺ T cells by measuring the surface expression of CD107a, a lysosomal associated membrane protein (LAMP-1). We observed that RIPK3^{-/-} and Casp1-11^{-/-} deficiencies result in a significant reduction in lysosomal-

associated membrane protein 1 (LAMP-1) expression (Figure 21a, b). Similarly, we found a significantly reduced expression of IFN- γ (Figure 22a, b) and TNF- α (Figure 23a, b) in OVA-specific CD8⁺ T cells by intracellular staining in RIPK3^{-/-}, Casp-1/11^{-/-} and Casp-1/11^{-/-}/RIPK3^{-/-} mice. Taken together, our results indicate that antigen-specific CD8⁺ T cells depend on RIPK3 and Casp-1/11 for cytolytic activity and cytokine production.

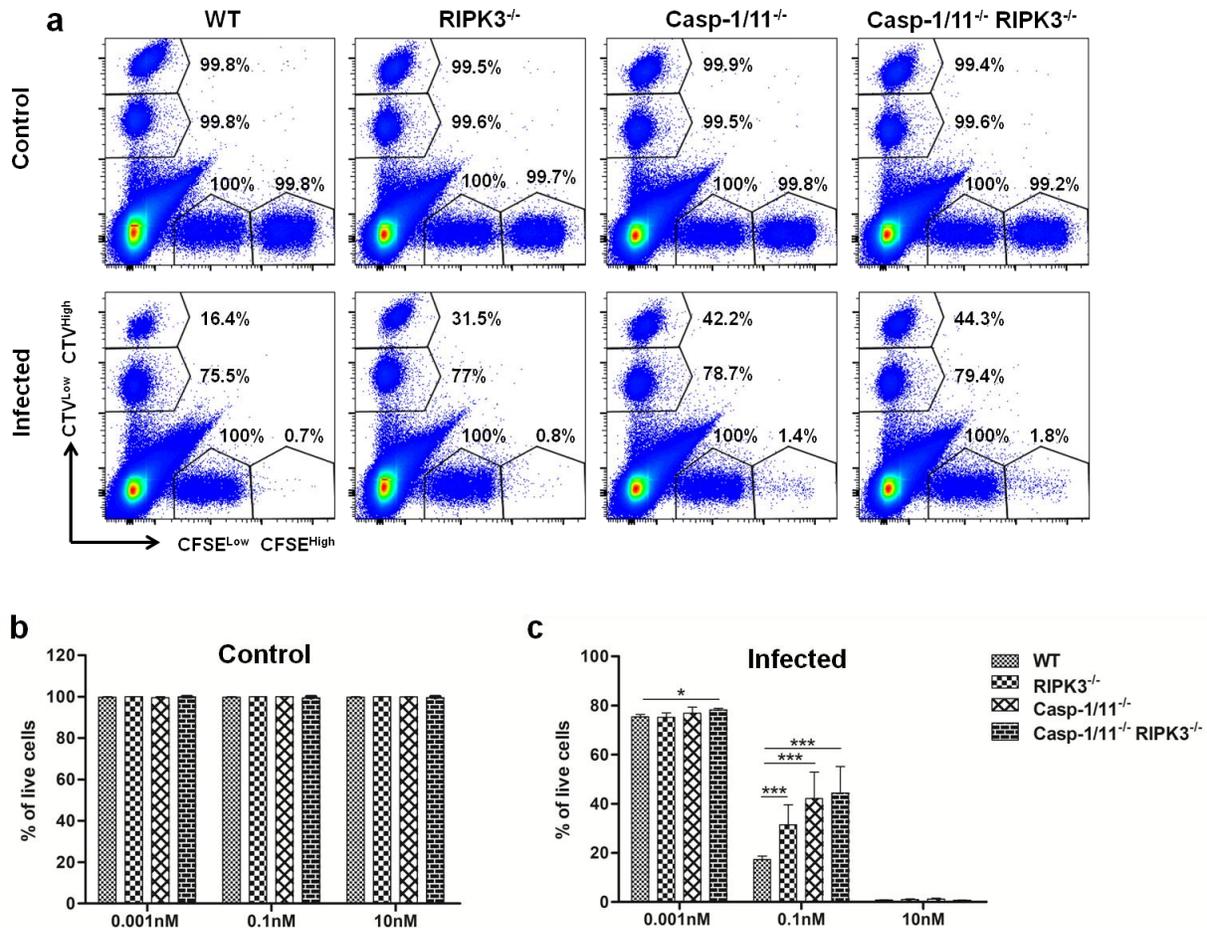


Figure 19: RIPK3 and/or Casp-1/11 deficiencies negatively impact on *in vivo* antigen-specific CTL activity. *In vivo* elimination of target cells pulsed with high (10nM), intermediate (0.1nM) and low (0.001nM) concentration of OVA₂₅₇₋₂₆₄ peptide at day 7 post-infection with LM-OVA. (a-c) Percentage of CFSE^{High}, CFSE^{Low}, CTV^{High} and CTV^{Low} shows the frequency of remaining cells after CTL-mediated target cell elimination. Percentage of live target cells in (b) non-infected and (c) infected mice. The data is expressed as means of five individual mice per group and is representative of three independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***(p<0.001) *(p<0.05)

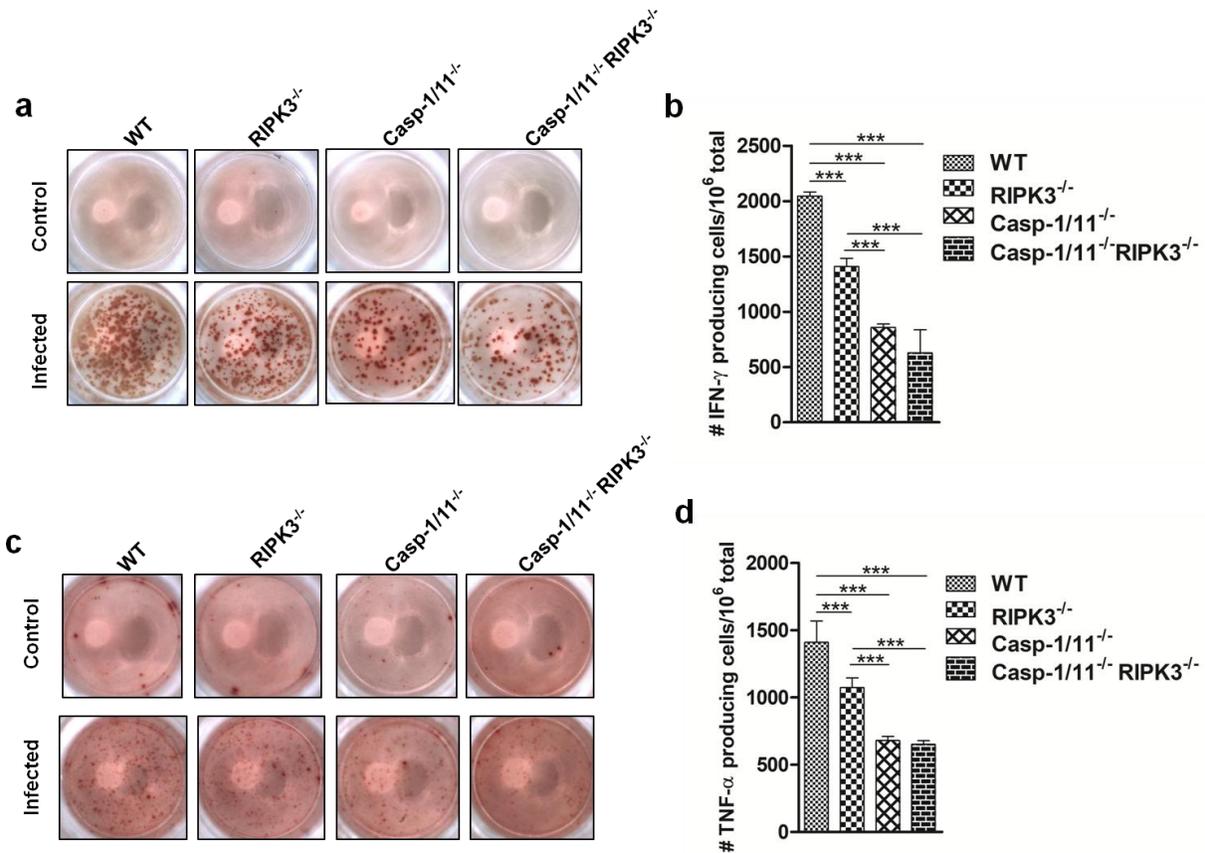


Figure 20: RIPK3 and/or Casp-1/11 deficiencies limit cytokine production by OVA-specific CD8⁺ T cells in response to LM-OVA. ELISPOT assay was performed to determine the frequency of OVA₂₅₇₋₂₆₄ peptide specific (a, b) IFN- γ - or (c, d) TNF- α -producing CD8⁺ T cells at day 7 post-infection with LM-OVA. Spots represent the frequency of OVA specific IFN- γ producing CD8⁺ T cells. The data is expressed as means of five individual mice per group and is representative of three independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***(p<0.001)

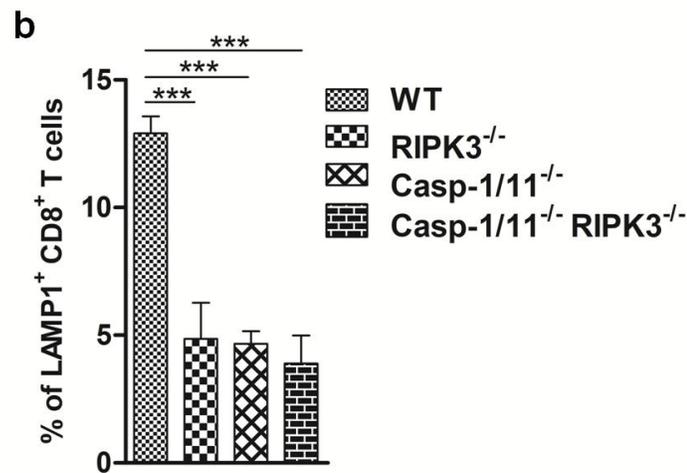
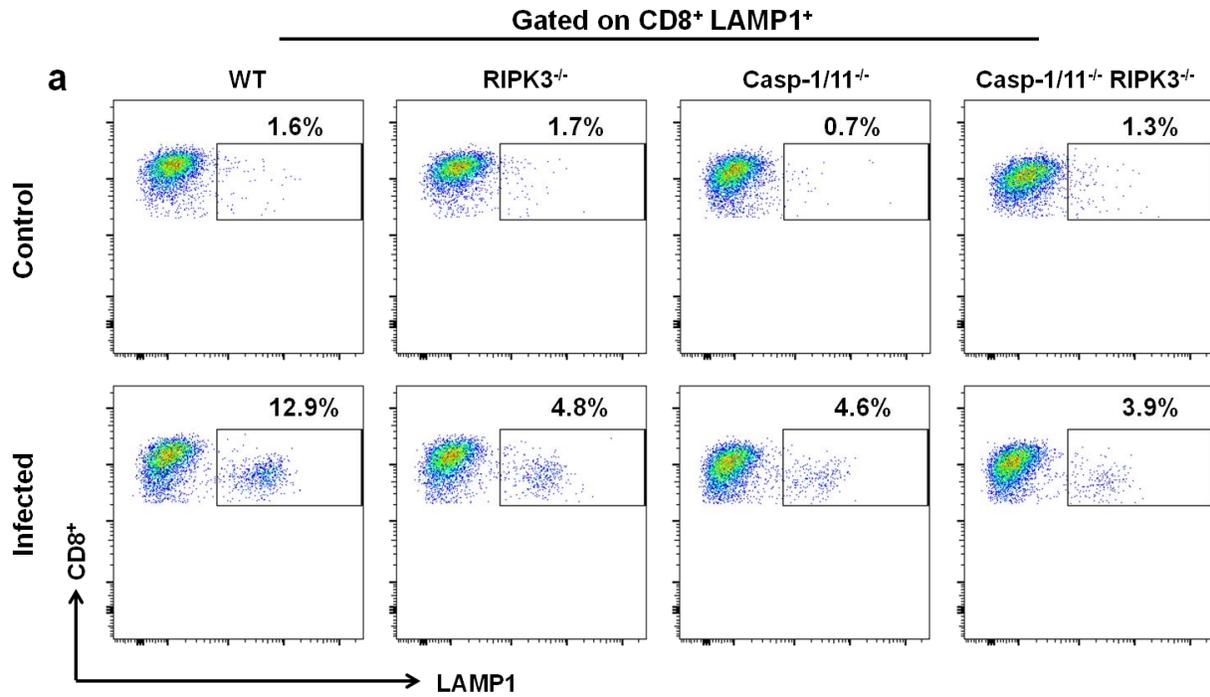


Figure 21: RIPK3 and/or Casp-1/11 deficiencies reduce antigen-specific CD8⁺ T cells degranulation. (a) Surface expression of LAMP-1 on OVA-specific CD8⁺ T cells (b) Frequency of LAMP-1⁺-CD8⁺ T cells at day 7 post-infection with LM-OVA. The data is expressed as means of five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***($p < 0.001$)

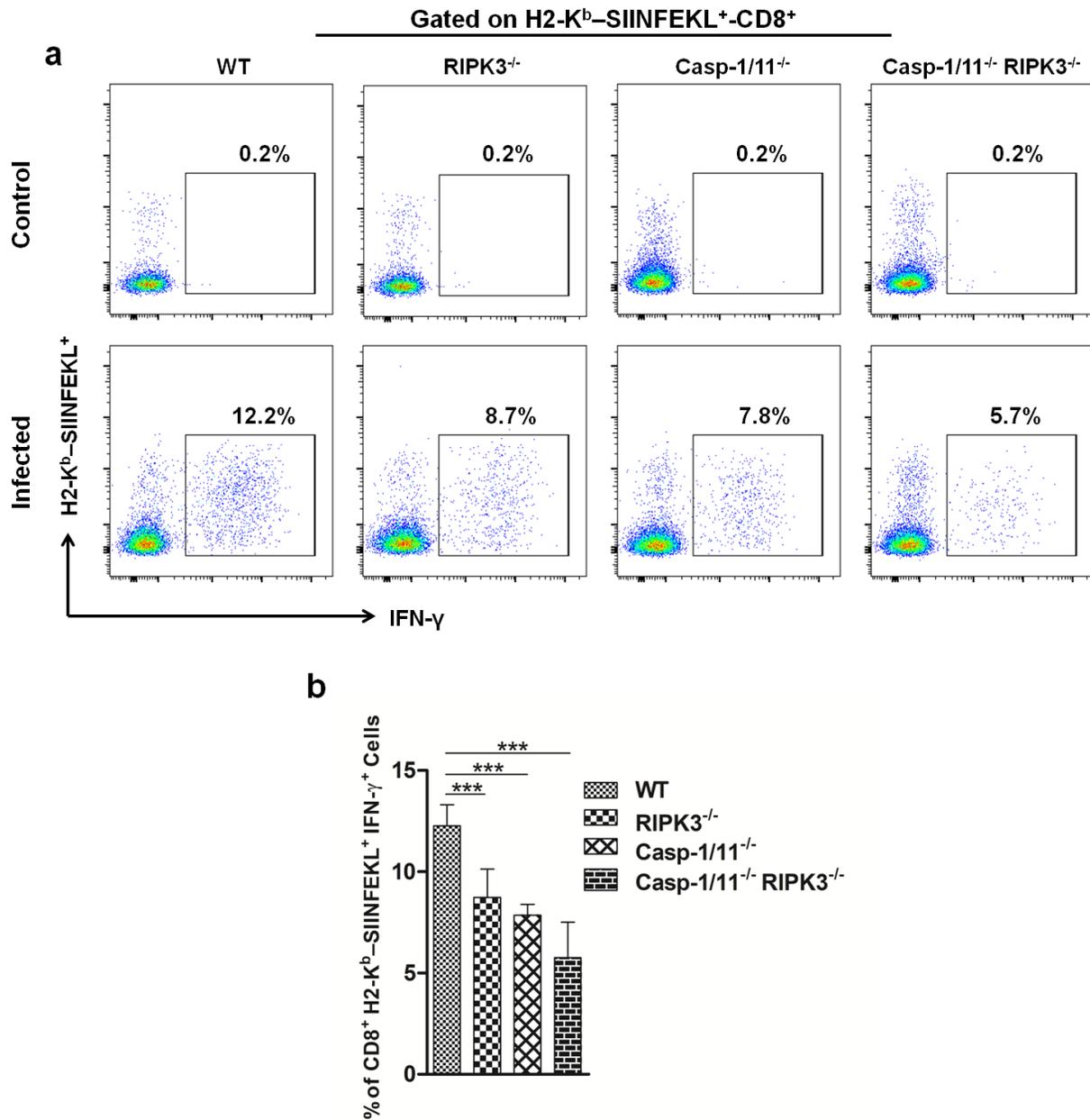


Figure 22: RIPK3 and/or Casp-1/11 deficiencies reduce antigen-specific CD8⁺ T cells intracellular IFN- γ production in response to LM-OVA. (a) Intracellular H2-K^b-SIINFEKL-specific IFN- γ producing CD8⁺ T cell population (b) Frequency of H2-K^b-SIINFEKL-specific IFN- γ -producing CD8⁺ T cells at day 7 post-infection with LM-OVA. The data is expressed as means of five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***(p<0.001)

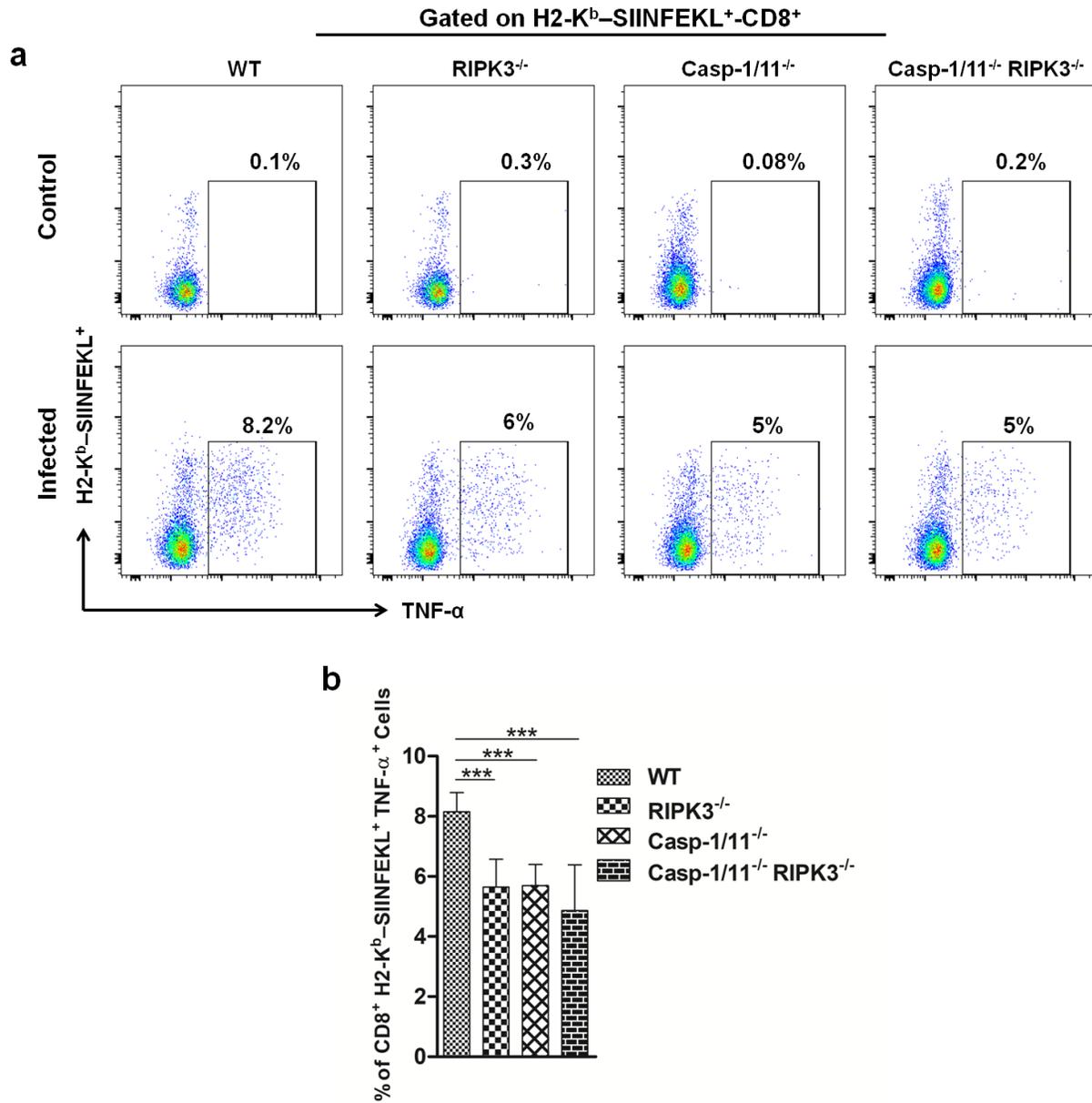


Figure 23: RIPK3 and/or Casp-1/11 deficiencies reduce antigen-specific CD8⁺ T cells intracellular TNF- α production in response to LM-OVA. (a) Intracellular H2-K^b-SIINFEKL-specific TNF- α -producing CD8⁺ T cell population. (b) Frequency of H2-K^b-SIINFEKL-specific TNF- α producing CD8⁺ T cells after 7 days of infection with LM-OVA. The data is expressed as means of five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***($p < 0.001$)

8.5. Antigen-specific CD8⁺ T response depends on antigen-delivery vector

To compare the OVA-specific CD8⁺ T cell response generated by LM-OVA with other antigen-delivery system, we used human recombinant adenovirus encoding the ovalbumin protein (rhAd5.OVA) to immunize WT, RIPK3 and Casp-1/11 KO mice. After 7 days post-immunization with rhAd5.OVA, we accessed the cytolytic activity and IFN- γ production of OVA-specific CD8⁺ T cells. Interestingly, OVA-specific CD8⁺ T cells generated by rhAd5.OVA did not show any significant difference in elimination of target cells pulsed with either three different concentrations of OVA₂₅₇₋₂₆₄ peptide, namely high (10nM), intermediate (0.1nM) and low (0.001nM) concentrations (Figure 24a-c). Moreover, similar IFN- γ production was observed in WT and RIPK3^{-/-}, Casp-1/11^{-/-} mice after 7 days post-immunization with rhAd5.OVA (Figure 25a, b). Our results show that RIPK3 and Casp-1/11 are not important to induce antigen-specific effector CD8⁺ T cell response after rhAd5.OVA. Therefore, it seems to be relatively specific for the generation of optimal CD8⁺ T cell responses after vaccination with recombinant LM-OVA.

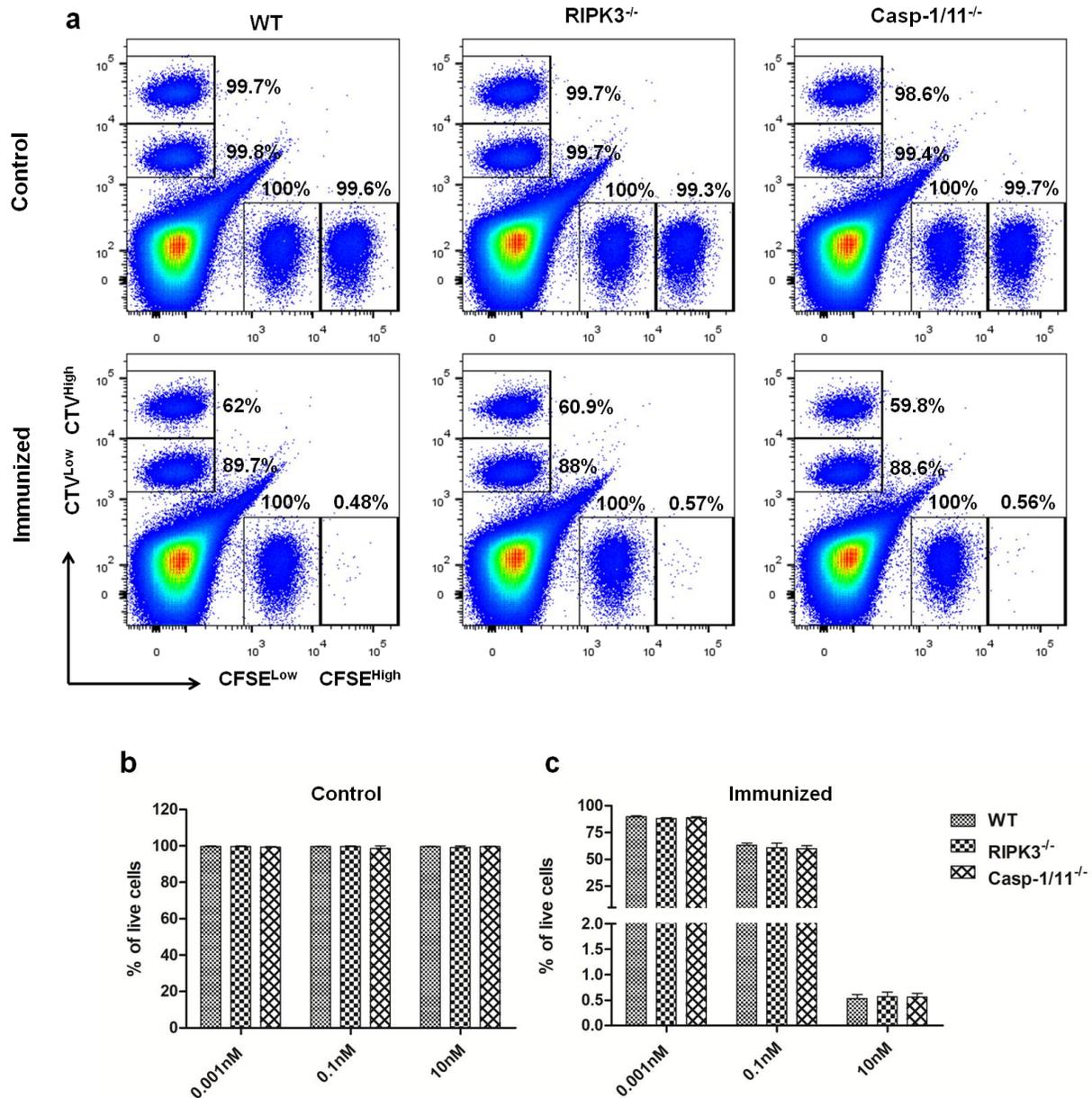


Figure 24: Antigen-specific CD8⁺ T cell response depends on antigen-delivery vector (*In vivo* cytotoxicity). (a-c) *In vivo* elimination of target cells pulsed with high (10nM), intermediate (0.1nM) and low (0.001nM) concentration of OVA₂₅₇₋₂₆₄ peptide at day 7 post-immunization with rhAd5-OVA. (a) Percentage of CFSE^{High}, CFSE^{Low}, CTV^{High} and CTV^{Low} shows the frequency of remaining cells after CTL-mediated target cell elimination. Percentage of live target cells in (b) non-immunized and (c) immunized mice. The data is expressed as means of five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

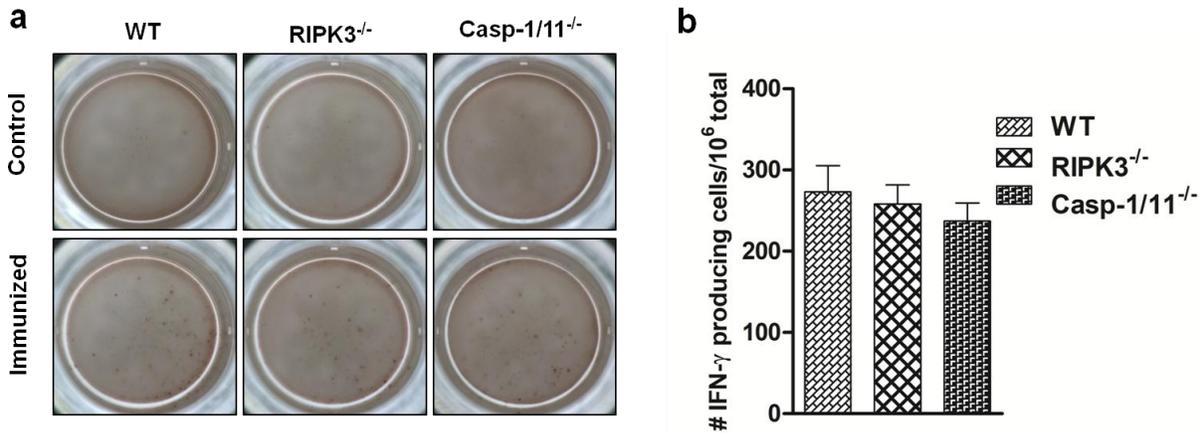


Figure 25: Antigen-specific CD8⁺ T cell response depends on antigen-delivery vector (ELISPOT assay). (a-b) Frequency of IFN- γ producing cells: Splenocytes from WT, RIPK3^{-/-} and Casp-1/11^{-/-} immunized or non-immunized mice pulsed with 10 μ M of OVA₂₅₇₋₂₆₄ peptide. Spots represent the frequency of OVA specific IFN- γ producing CD8⁺ T cells. The data is expressed as means of five individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

8.6. RIPK3 deficiency limits regulatory T cells (CD4⁺ CD25⁺ FOXP3⁺) expansion at the peak of LM-OVA infection

To correlate the LM-OVA burden and expansion of regulatory T cell population, we infected the mice with LM-OVA for 24 and 72 hours. We observed that RIPK3 and Casp-1/11 deficiencies do not affect the expansion of regulatory T cells after 24 hours of infection (Figure 26a-c). Further, we assessed the regulatory T cell expansion after 72 hours of LM-OVA infection (at peak of LM-OVA burden). RIPK3 and Casp-1/11 deficiencies do not enhance the regulatory T cell expansion even after 72 hours of infection. Interestingly, we found a small but significant contraction of regulatory T cell population in RIPK3 deficient mice in comparison to WT and Casp-1/11 (Figure 27a-c). Taken together, our results show that RIPK3 is necessary for regulatory T cell expansion at the peak of LM-OVA infection.

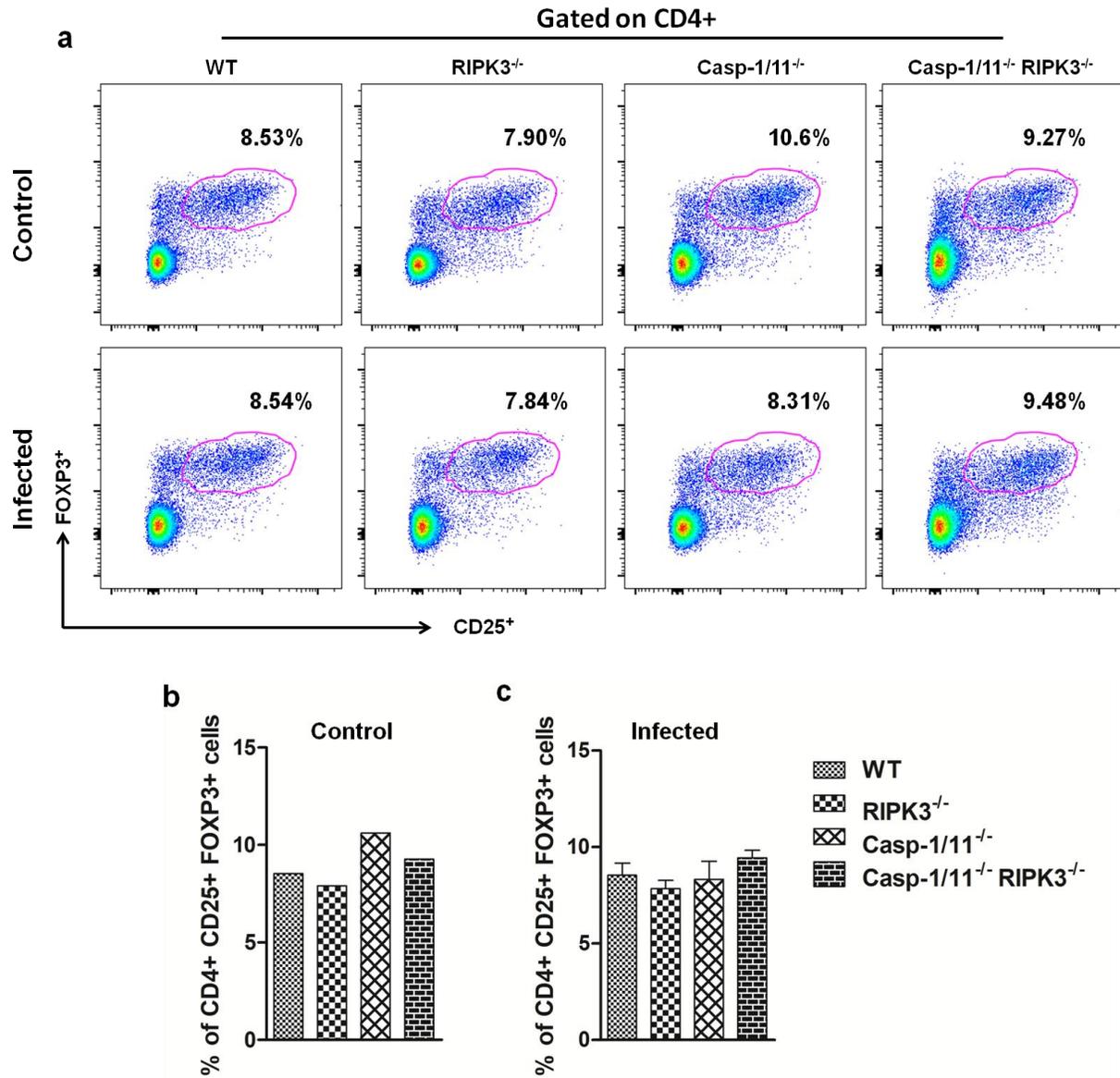


Figure 26: Regulatory T cell (CD4⁺ CD25⁺ FOXP3⁺) response is independent of RIPK3 and Casp-1/11 after 24 hours of LM-OVA infection. (a) Population of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in non-infected and infected mice. (b) Percentage of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in non infected mice. (b) Percentage of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells after 24 hours of infection with LM-OVA. The data is expressed as means of five individual mice per group. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

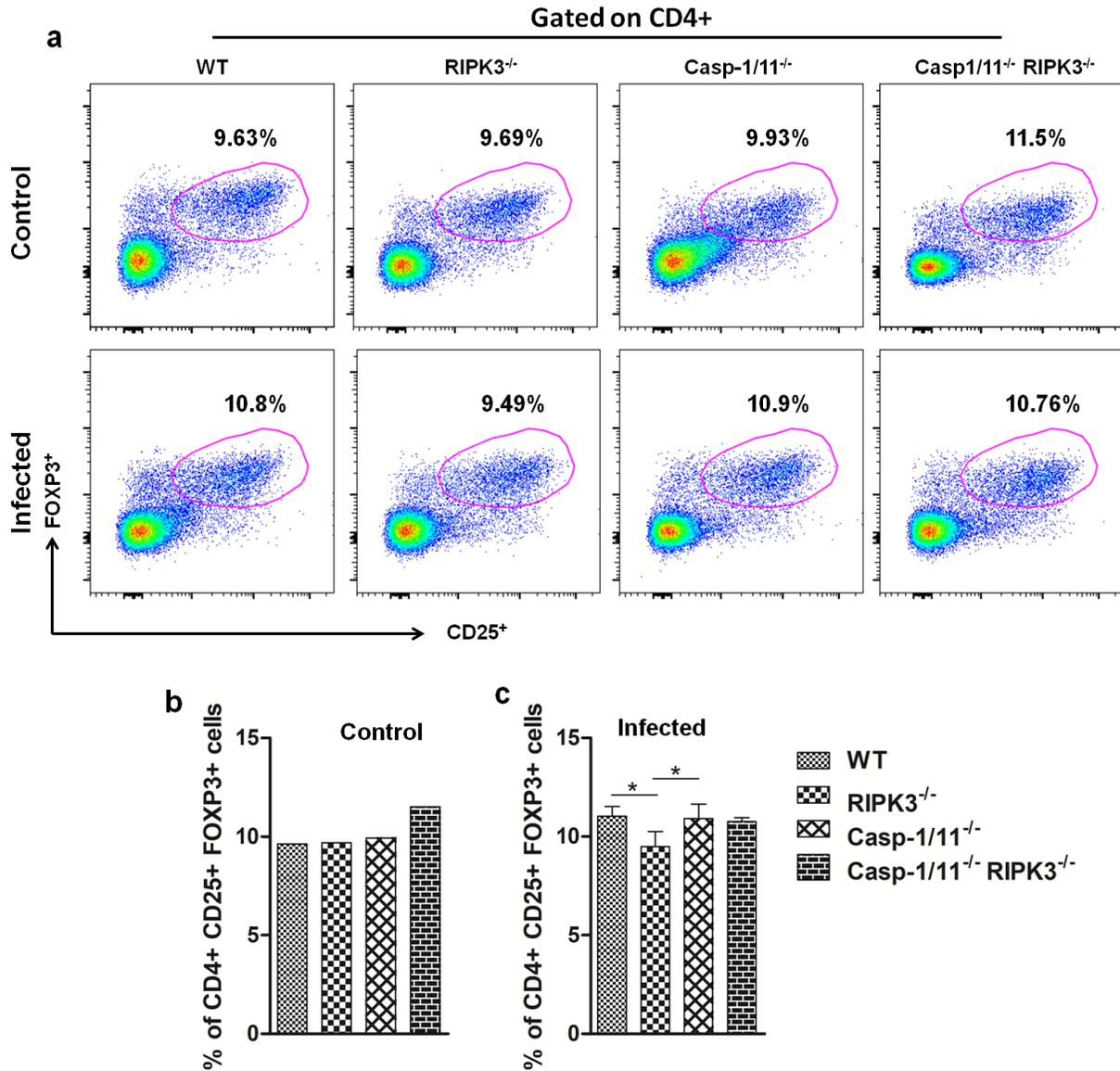


Figure 27: RIPK3 deficiency limits regulatory T cell (CD4⁺ CD25⁺ FOXP3⁺) expansion at peak of LM-OVA burden (after 72 hours of infection). (a) Population of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in non-infected and infected mice. (b) Percentage of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells in non infected mice. (c) Percentage of CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells after 72 hours of infection with LM-OVA. The data is expressed as means of five individual mice per group. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. *(p<0.05)

8.7. Casp-1/11 and Casp-1/11/RIPK3 combined deficiencies restrict OVA-specific CD8⁺ T memory cell differentiation

To correlate the downgrading of OVA-specific CD8⁺ T cytolytic activity and cytokines production with early initiation of memory response cells, we evaluated the surface expression of memory precursor molecules (CD127^{high} KLRG1^{low}) on adoptively transferred OT-I CD8⁺ T cells after 7 days of infection. Casp-1/11 but not RIP3K deficiency significantly reduced the expression of CD127 and increased KLRG1 expression on OT-I CD8⁺ T cells after 7 days of infection (Figure 28a-d). Thus, our results indicate that Casp-1/11 differentially modulate the early differentiation of memory precursor OVA specific CD8⁺ T cells.

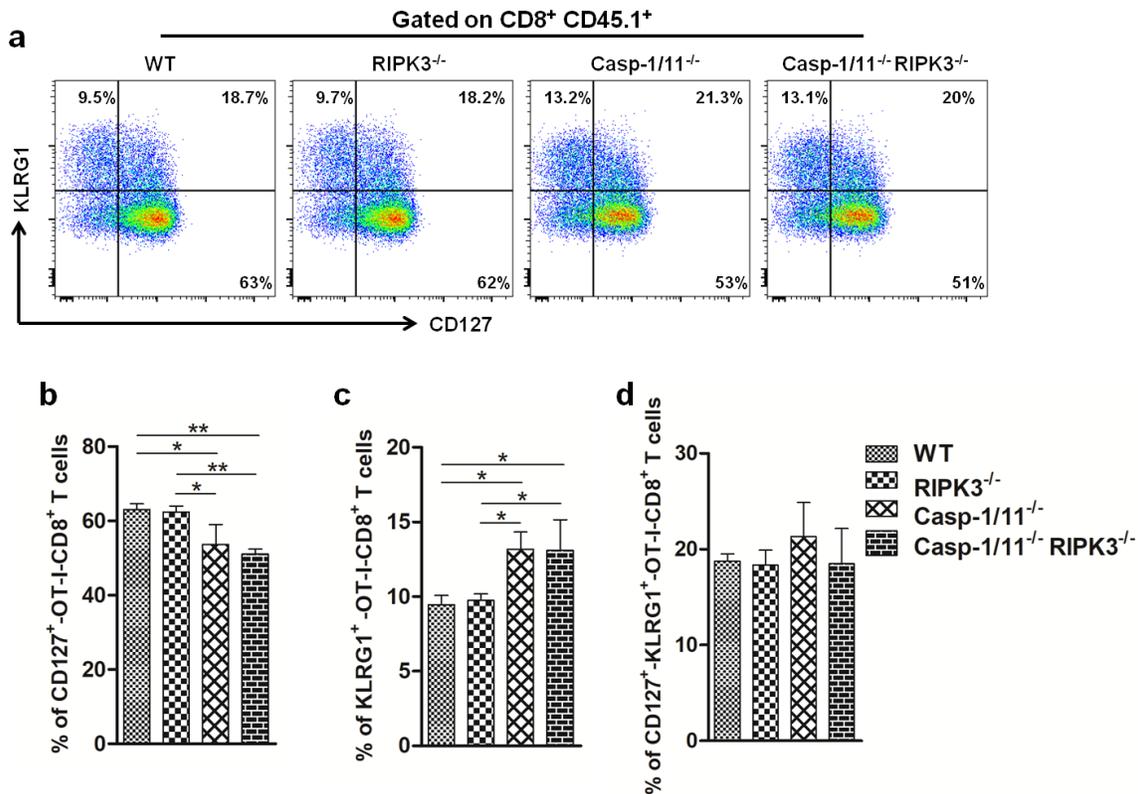


Figure 28: Casp-1/11 and Casp-1/11/RIPK-3 combined deficiencies restrict early OVA-specific CD8⁺ T memory cells response. (a) Surface expression of CD127 and KLRG1 on adoptively transferred CD8⁺ T cells after 7 days of infection. (b) Frequency of CD127 (c) KLRG1 (d) and CD127, KLRG1 positive OT-I CD8⁺ T cells. Results are expressed as means of five individual mice per group and are representative of three independent experiments. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. **($p < 0.01$) *($p < 0.05$)

8.8. RIPK3 and Casp-1/11 are essential for optimal CD8⁺ T cell response to control tumor growth

Initially, we confirmed the relative mRNA and protein expression of ovalbumin in B16 and B16.OVA of melanoma cells lines (Figure 29a, b). Further, *in vitro* (Figure 29c) and *in vivo* (in Rag1^{-/-} mice) (Figure 29d, e) growth curves were drawn to compare the growth rate of B16 and B16.OVA. We did not find any significant difference in both *in vitro* and *in vivo* growth rate of B16 and B16.OVA. Similar *in vitro* and *in vivo* growth rate of B16 and B16.OVA suggests that ovalbumin expression does not affect the growth rate of B16.

To find the impact of RIPK3 and Casp-1/11 deficiencies on the *in vivo* tumor growth, we challenged the B16 and B16.OVA in WT, RIPK3^{-/-} and Casp-1/11^{-/-} mice without LM-OVA vaccination (Figure 30a). We did not observe any significant *in vivo* growth difference of B16 and B16.OVA in WT, RIPK3^{-/-} and Casp-1/11^{-/-} mice without LM-OVA vaccination (Figure 30b-e).

As, we found that RIPK3 and Casp-1/11 are essential for optimal CD8⁺ T cell response generated by LM-OVA, we further evaluated the impact this reduced OVA-specific CD8⁺ T cell response on *in vivo* growth of B16 and B16.OVA with LM-OVA vaccination. To observe the preventive action of LM-OVA vaccination, all the experimental mice were initially vaccinated with LM-OVA. After 7 days of vaccination mice were challenged independently with B16 and B16.OVA (Figure 31a). We observed that OVA-specific CD8⁺ T cell response generated by LM-OVA was strong enough to eradicate all the B16.OVA cells in the WT, RIPK^{-/-} and Casp-1/11^{-/-} mice, while the B16 cells were grown normally (Figure 31b-d). To observe the therapeutic action of LM-OVA vaccination, all the experimental mice were challenged separately with B16 and B16.OVA. At day 3 post-tumor challenge, mice were vaccinated or not with LM-OVA (Figure 32a). We observed that RIPK3 and Casp-1/11 deficiencies do not affect the *in vivo* growth (Figure 32b, d) (Figure 33a) and weight (Figure 33c) of B16 by OVA-specific CD8⁺ T cell response generated by LM-OVA. In comparison to B16, we found a significantly high growth (Figure 32c, e) (Figure 33b) and weight (Figure 33d) of B16.OVA in Casp-1/11 and Casp-1/11/RIPK3 double deficient mice (Figure 33e). Taken together, our results show that prophylactic LM-OVA vaccination is independent of RIPK3 and Casp-1/11 to control B16.OVA growth. Whereas therapeutic LM-OVA vaccination depends on Casp-1/11 and Casp-1/11/RIPK3 to control B16.OVA growth under our experimental conditions.

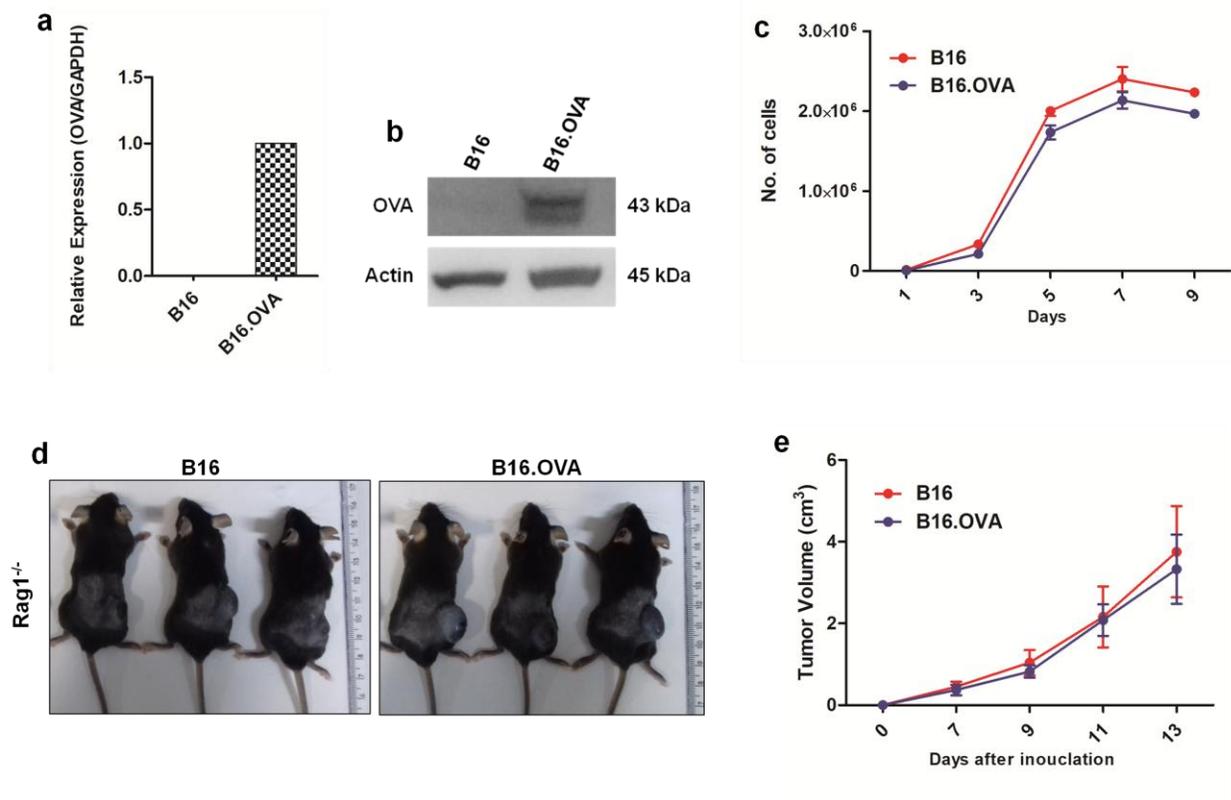


Figure 29: Ovalbumin expression does not affect B16 growth. Relative mRNA and protein expression of ovalbumin in B16.OVA: B16 was used as an experimental control (a) Relative mRNA expression of ovalbumin in B16.OVA was detected by quantitative-PCR. Statistical analysis was performed by using one way ANOVA followed by Tukey's Multiple Comparison Test. The data is representative of two independent experiments (b) Relative protein expression of ovalbumin in B16.OVA was detected by Western blot analysis. (c) *In vitro* growth of B16 and B16.OVA. (d-e) *In vivo* growth of B16 and B16.OVA in Rag1^{-/-} mice. The data is expressed as means of three individual mice per group and is representative of two independent experiments with similar results. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

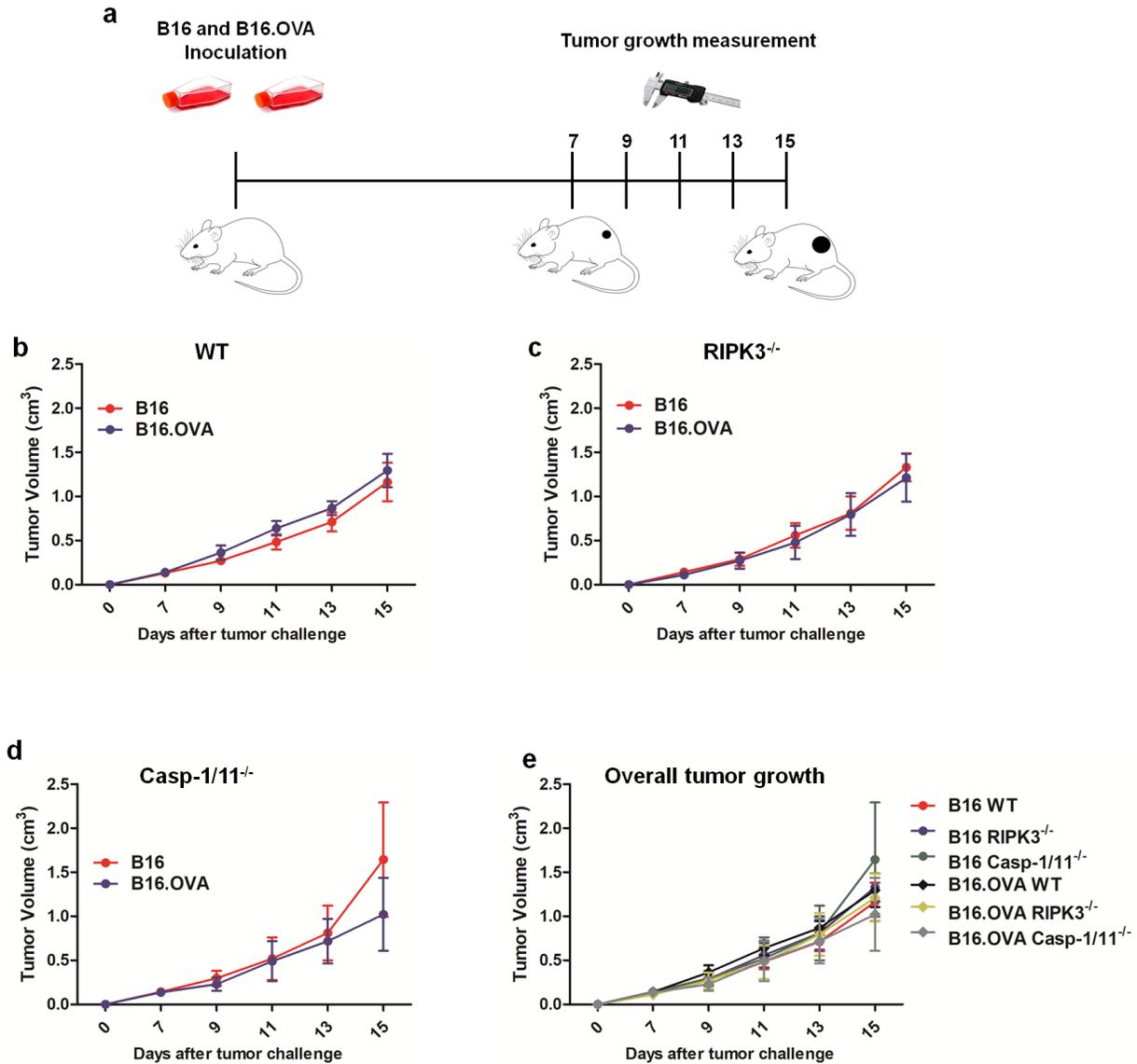


Figure 30: RIPK3 and Casp-1/11 do not regulate *in vivo* growth of B16 and B16.OVA melanoma cell lines without LM-OVA vaccination. WT, RIPK3^{-/-} and Casp-1/11^{-/-} mice were challenged subcutaneously with B16 and B16.OVA in the right and left (respectively) flank of each mouse. Tumor growth was measured after subsequent interval of 2 days. (a) Experimental design, (b) *In vivo* growth of B16 and B16.OVA in WT (c) RIPK3^{-/-} (d) Casp-1/11^{-/-} mice. (e) *In vivo* growth comparison of B16 and B16.OVA in all experimental groups. The data is expressed as means of six individual mice per group and is representative of two independent experiments. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

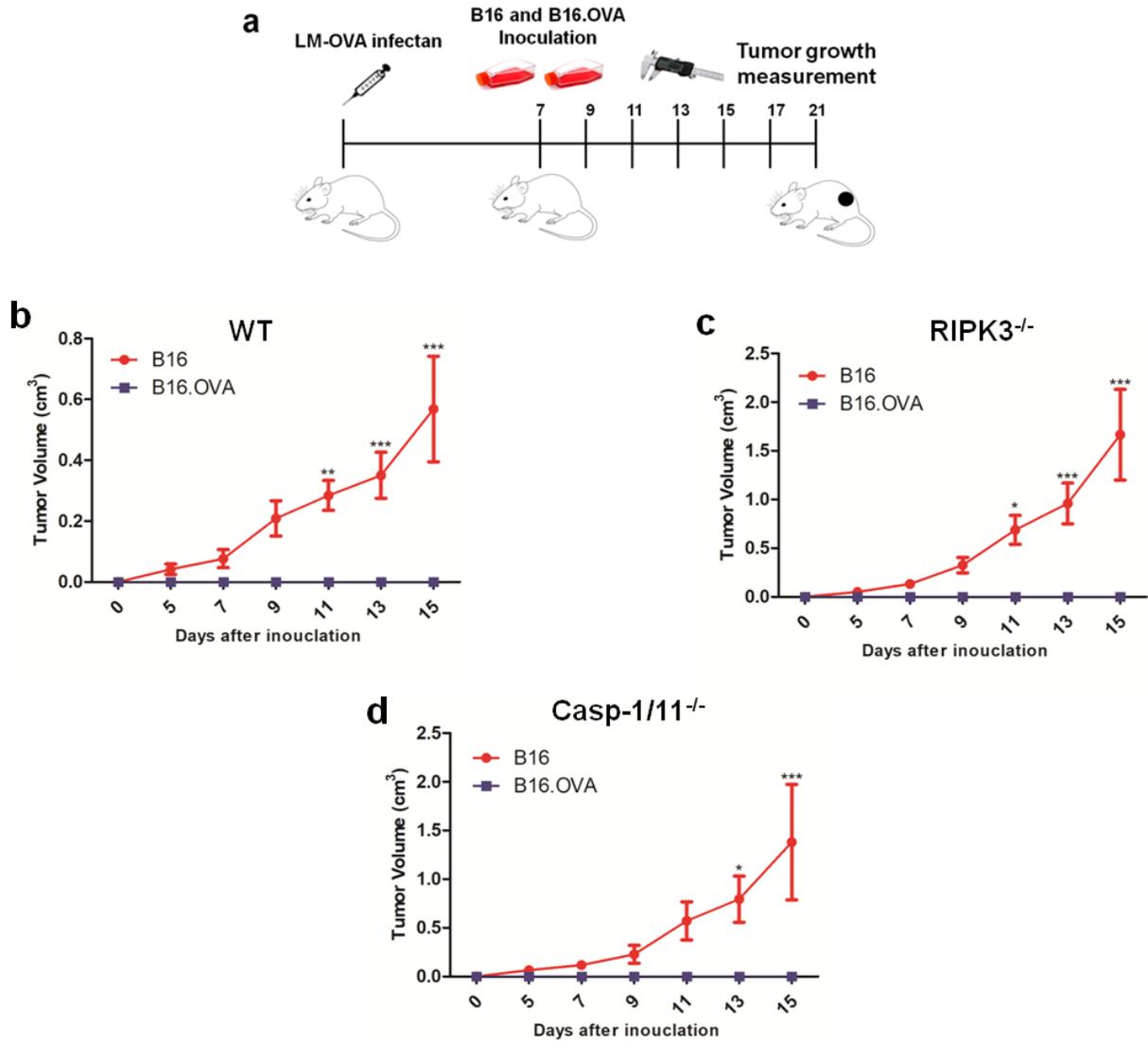


Figure 31: Prophylactic LM-OVA vaccination is independent of RIPK3 and Casp-1/11 to control *in vivo* growth of B16.OVA cells (Preventive action). WT, RIPK3^{-/-} and Casp-1/11^{-/-} mice were vaccinated with 10³ LM-OVA. At day 7 of post-vaccination, all the experimental mice were independently challenged subcutaneously with B16 and B16.OVA. Tumor growth was measured after subsequent interval of 2 days. (a) Experimental design, (b) *In vivo* growth of B16 and B16.OVA in WT (c) RIPK3^{-/-} (d) Casp-1/11^{-/-} mice. The data is expressed as means of six individual mice per group and is representative of two independent experiments. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests.

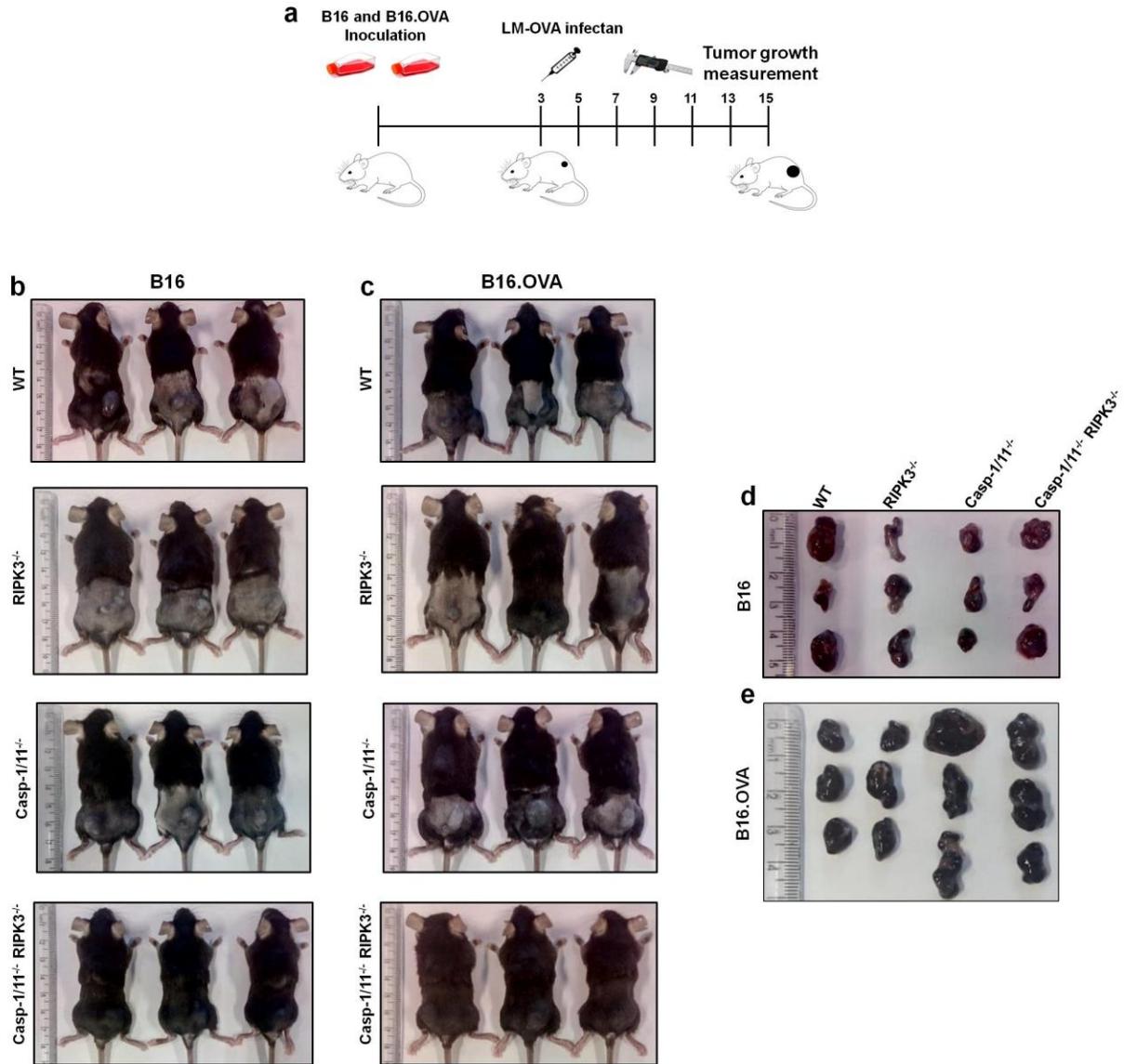


Figure 32: OVA-specific CD8⁺ T cell response generated by LM-OVA is insufficient to control tumor growth in Casp-1/11 and Casp-1/11/RIPK3 deficient mice (Therapeutic Action). (a) Experimental design (b, d) *In vivo* growth of B16 in WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp1/11^{-/-}/RIPK3^{-/-} mice. (c, e) *In vivo* growth of B16.OVA in WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp1/11^{-/-}/RIPK3^{-/-} mice. All experimental mice were vaccinated with LM-OVA at day 3 post-tumor challenge. Six mice in each group are used for tumor challenge and LM-OVA vaccination. Three mice from each group are used to represent the tumor *in vivo* growth.

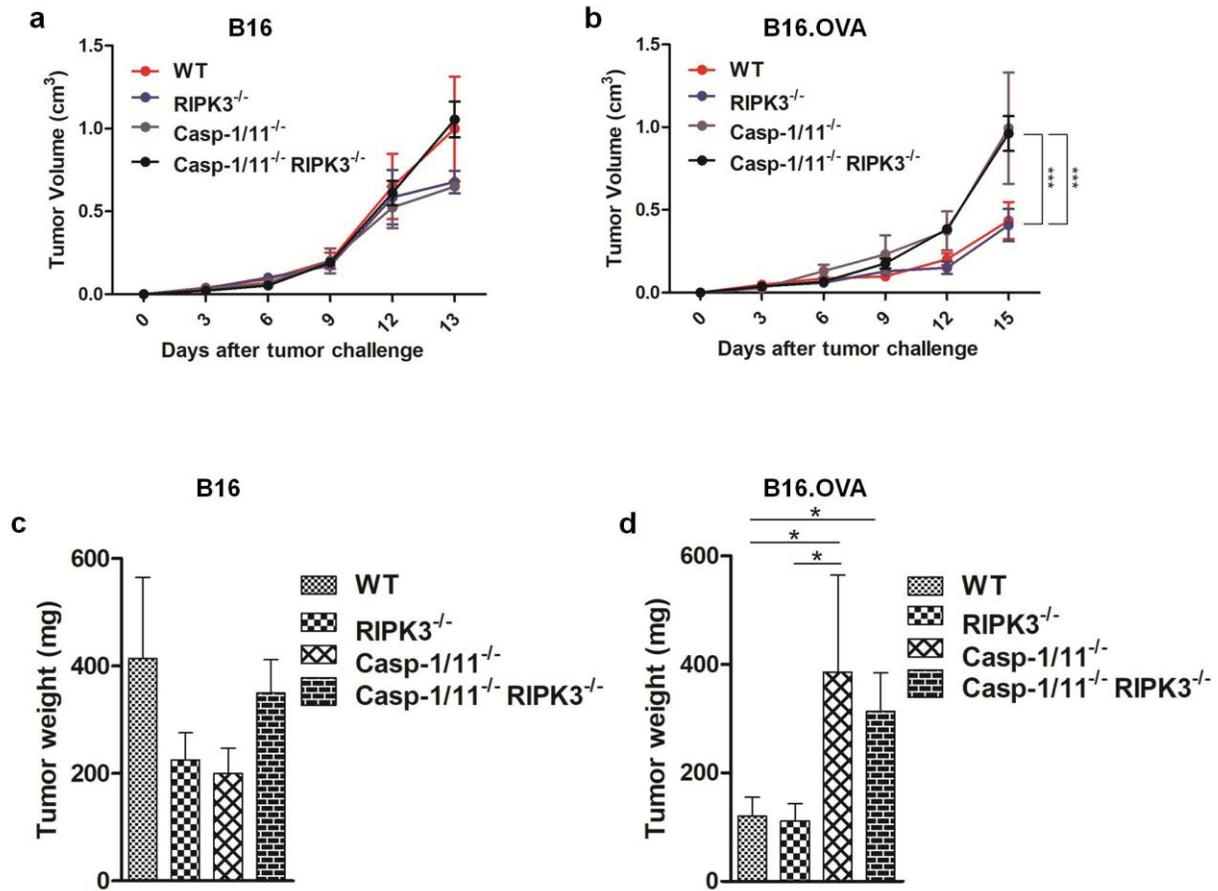


Figure 33: OVA-specific CD8⁺ T cell response generated by LM-OVA is insufficient to control tumor growth in Casp-1/11 and Casp-1/11/RIPK3 deficient mice (Therapeutic Action). (a) *In vivo* growth of B16 in WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp1/11^{-/-}/RIPK3^{-/-} mice. (b) *In vivo* growth of B16.OVA in WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp1/11^{-/-}/RIPK3^{-/-} mice. (c) Weight of B16 tumors excised from WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp1/11^{-/-}/RIPK3^{-/-} mice. (d) Weight of B16.OVA tumors excised from WT, RIPK3^{-/-}, Casp-1/11^{-/-} and Casp1/11^{-/-}/RIPK3^{-/-} mice. Results are expressed as means of six individual mice per group and are representative of one independent experiment. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttests. ***(p<0.001) *(p<0.05)

9. DISCUSSION:

An efficient immunity against cancer and pathogens involves specific recognition and removal of malignant or infected cells. Strategies aimed to elicit optimized effector CD8⁺ T cells response, in a way that single antigen-specific clones may present multiple effector functions and differentiate to long-lived memory T cells, have been proposed.(237) Among such strategies, the use of intracellular infectious bacteria carrying unique single or multiple therapeutic proteins holds promise.(238-240) *Listeria* not only infects antigen-presenting cells (APCs), but also tumor infiltrating myeloid-derived suppressor cells (MDSCs) to accumulate in the tumor microenvironment and avoid immune clearance.(146) Also, *Listeria* persists within tumors for longer time even after its clearance from spleen and liver.(147-149) In order to maintain potential benefits of *Listeria*-induced long-term antitumor immunity, recombinant *Listeria* expressing tumor-associated antigens (TAAs) have been developed for activating tumor-specific CTL response.(241)

In fact, it has been previously reported that recombinant *L. monocytogenes* carrying the ovalbumin gene (LM-OVA) induces strong OVA-specific CD8⁺ T cell response and protect mice against B16-OVA melanoma cell line.(242) Interestingly, the role of RIPK3 and Casp-1/11 proteins known to be involved in the control of *L. monocytogenes* infection, on OVA-specific CD8⁺ T cell response after LM-OVA vaccination remained unexplored.

Host defends itself against intracellular pathogen infection such as *Listeria* by evolving multi-layered defense system. Macrophages recognizes pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) and induce inflammatory response to clear pathogen infection.(243-245) In classical necroptosis signaling, RIPK3 phosphorylates mixed lineage kinase domain like pseudokinase (MLKL), which subsequently oligomerizes and binds to plasma membrane of cell for its disruption that eventually induces necrotic cell death.(202) *Listeria* induces rapid necroptotic cell death in macrophages (246,247), but the direct interaction of RIPK3-MLKL with *Listeria* to clear the infection remained unclear. Recently, it has been reported that activation of RIPK3 during LM infection contributes to restrict intracellular replication of the parasite.(201) Interestingly enough, LM-induced activation of RIPK3 results in phosphorylation of MLKL without inducing necroptosis (201), suggesting a necroptosis-independent role of RIPK3 in LM restriction. Here, we observed that RIPK3 deficiency negatively impact the control of LM-OVA burden only at early stage (day 3) of infection (Figure

14b). In contrast, at the peak of antigen-specific CD8⁺ T cell response (day 7) both WT and RIPK3-deficient mice were able to completely eliminate LM-OVA infection (Figure 14d). We were not able to determine whether the effect of RIPK3 deficiency on the early control of LM-OVA was necroptosis-dependent or -independent. Regardless, although the impact of the absence of RIPK3 on the control of LM-OVA burden was mild and temporary, it significantly reduced the OVA-specific CD8⁺ T cell proliferation, cytolytic activity (Figure 19) and cytokine production (Figures 20-23), suggesting a role of RIPK3 in controlling optimal adaptive immune responses following recombinant LM vaccination.

Similarly to RIPK3, it has been reported that deficiency of Casp-1/11 renders mice more susceptible to LM infection (248,249). Tsuji *et al*, reported that, Casp-1 activation clear *Listeria* during infection by innate immune recognition of microorganism-associated molecular pattern and enhance IL-18 and IFN- γ production.(249) In agreement, we observed that Casp-1/11 deficiency also facilitate LM-OVA infection, both at day 3 (Figure 14b) and day 7 of infection (Figure 14d).

The extent of CD8⁺ T cell proliferation is depends on the amount of antigen available *in vivo* and the strength of T cell receptors (TCR) signals. The magnitude of CTL response is directly proportional to the antigen and or epitope abundance.(250) *Listeria* induce strong antigen-specific CD8⁺ T cell response during first day of infection and the primary CD8⁺ T cell expansion is independent on antigen persistence.(251) In response to *Listeria* infection CD8⁺ T cell primary expansion peaks between 7-10 days.(252,253) Here, we established a dose-dependent response for LM-OVA and found out that 10² CFU is insufficient to induce a significant CD8⁺ T cell response against OVA after 7 days post-infection (Figure 17 and 18). On the other hand, we observed maximum elimination of OVA-expressing targets in mice infected with both 10³ and 10⁴ LM-OVA. Similarly, OVA-specific, IFN- γ -secreting CD8⁺ T cells were observed only with 10³ and 10⁴ LM-OVA infection. Our results indicate that increasing bacterial burden results in additional *in vivo* antigen-specific priming. Furthermore, we evaluated single and combined deficiency of RIPK3 and Casp-1/11 on the functional profile of OVA-specific CD8⁺ T cell response. Our results show that RIPK3 and Casp-1/11 are essential for cytolytic activity (Figure 20), cytokine production (Figure 22 and 23) and degranulation of OVA-specific CD8⁺ T cells (Figure 21).

Interestingly, the absence of Casp-1/11 had an even more profound negative effect on OVA-specific CD8⁺ T cell responses compared to the lack of RIPK3. Importantly, the combined RIPK3 and Casp-1/11 deficiencies did not result in synergistic or additive effects. Actually, Casp-1/11, RIPK3 double-deficient mice behaved similarly to Casp-1/11-deficient mice in every aspect investigated in our work. Our data suggest that RIPK3 and Casp-1/11 operates in the same functional pathway (see below) and that Casp-1/11 seems to be dominant over RIPK3.

The observed deficiency of OVA-specific CD8⁺ T cell response does not seem to be related to intrinsic flaw of CD8⁺ T cells from RIPK3^{-/-} or Casp-1/11^{-/-} mice. In fact, *in vivo* proliferation of RIPK3/Casp-1/11-sufficient OT-I CD8⁺ T cells occurred normally in LM-OVA-infected WT but not on RIPK3-, Casp-1/11- or Casp-1/11/RIPK3-deficient mice (Figure 16), suggesting that the deficiency is set at the level of antigen-presentation. In this regard, efficient activation and optimal expansion of effector CD8⁺ T cell response depend on LM intracellular burden and the level of infection was shown to impact the priming ability of infected antigen-presenting cells (APCs).(253) Importantly, deficient antigen-presentation in response to higher levels of LM seems to be at least in part due to significant LM-induced death of APCs.(254) We did not formally address as whether LM-OVA differentially kills RIPK3- or Casp-1/11-deficient cells *in vivo*. However, our results clearly show that by day 7 post-infection, only Casp-1/11-deficient mice harbor significant bacteria in the spleen. This difference in LM burden could account for the worst OVA-specific CD8⁺ T cell responses observed in Casp-1/11-deficient mice compared to RIPK3 mice. If this is true, LM-induced APC death would be the functional pathway interfered by both RIPK3 and Casp-1/11 deficiencies, as mentioned before.

We also compared the role of RIPK3 and Casp-1/11 deficiencies on the induction of early memory antigen-specific CD8⁺ T cells during LM-OVA infection. Interestingly, our data shows that Casp-1/11 and Casp-1/11/RIPK3 double deficiency restricts initiation of early memory precursor antigen-specific CD8⁺ T cell response, as observed by the CD127^{high} KLRG1^{low} phenotype, while individual deficiency of RIPK3 does not affect the dynamic of memory precursor CD8⁺ T cell population. These results may suggest that Casp-1/11 but not RIPK3 are necessary to the proper generation and differentiation of memory CD8⁺ T cells in response to LM-OVA.

Interestingly, the combined deficiency of RIPK3 and Casp-1/11 allows survival of OVA-expressing *Salmonella typhimurium* (ST-OVA) in DCs and macrophages leading to antigen-

specific CD8⁺ T cells that overexpress TIM3 and PD-1.(254) Furthermore, ST-OVA infection results in higher frequency of IFN- γ -producing CD8⁺ T cells in double deficient (RIPK3^{-/-}Casp-1/11^{-/-}) mice (255). In contrast, we found significantly reduced IFN- γ - or TNF- α -producing CD8⁺ T cells after LM-OVA infection in RIPK3- and/or Casp-1/11-deficient mice (Figure 22 and 23). Moreover, we found no significant differences in antigen-specific differentiation and on the effector function of CD8⁺ T cells in RIPK3- and/or Casp-1/11-deficient mice after immunization with recombinant human adenovirus expression ovalbumin (rhAd5-OVA) (Figure 24 and 25).

Immune system provides protection against invading pathogen with minimize harm to the infected cells and tissues.(256) FOXP3⁺ T_{reg} cells maintain peripheral tolerance by suppressing self-reactive immune response. Substantial information suggests that FOXP3⁺ T_{reg} cells readily extend their population to control non-self pathogen-associated antigens.(257-259) Ertelt *et al.*, enumerated that transit ablation of FOXP3⁺ T_{reg} cells boosts the robust activation and expansion of OVA-specific CD8⁺ T cells and clear *Listeria monocytogenes* infection in an OVA-specific fashion. Furthermore, FOXP3 ablation does not impact on the OVA-specific CD8⁺ T cell priming by LM-OVA. Similarly, non-recombinant *L. monocytogenes* administration with OVA₂₅₇₋₂₆₄ peptide generates a parallel OVA-specific CD8⁺ T cell response without FOXP3 T_{reg} ablation and thus, overrides the suppression imposed by FOXP3 T_{reg}.(260) Another study by the same group revealed that *Listeria monocytogenes* infection does not essentially regulate the LM-specific proliferation, expansion and subsequent contraction of FOXP3⁺ CD4⁺ T_{reg}, but it reduces the number of LM-specific FOXP3⁻ effector CD4⁺ cells.(261) Further studies demonstrated that depletion of CD25⁺ CD4⁺ T_{reg} cells during secondary *Listeria* infection does not diminish, but enhance antigen-specific CD8⁺ T cell memory response. These results show that the T_{reg} cell regulate the antigen-specific memory CD8⁺ T cell response during repeated or chronic *Listeria* infection.(262)

Previously, Szymczak-Workman *et al.*, observed that T_{reg} cells mediated suppression of conventional T cells is independent on apoptosis and RIPK3-dependent necroptosis (263), but the direct evidence showing the expansion and/or contraction of FOXP3⁺ T_{reg} response after 24h and 72h (at peak of LM-OVA burden) remained unclear. As we found that RIPK3 and Casp-1/11 controls LM-OVA infection (Figure 14b, d), we investigated the impact of RIPK3 and Casp-1/11 on the expansion of FOXP3⁺ T_{reg} response during LM-OVA infection. We observed that RIPK3 and Casp-1/11 deficiencies do not control FOXP3⁺ T_{reg} expansion after 24 hours of LM-OVA

infection (Figure 26). Interestingly, we found a small but significant contraction of regulatory T cell population in RIPK3-deficient mice at the peak (day 3) of LM-OVA infection (Figure 27). Taken together, our results show FOXP3⁺ T_{reg} expansion depends on RIPK3 at peak of LM-OVA burden.

The ultimate goal of cancer immunotherapy is to engage tumor-specific CD8 T cells to kill tumor cells. Distinct inflammatory cell death mechanism such as pyroptosis and necroptosis can modulate antitumor immune response generated by pathogen infection. Recombinant LM-OVA has been used to induce OVA-specific cytotoxic antitumor response to kill OVA specific melanoma cells.(242) Previously, Daniels *et al.*, reported that RIPK3 restricts West Nile virus (WNV) pathogenesis during encephalitis which is independent to the induction of cell death. They observed that RIPK3^{-/-} mice were more susceptible to the WNV infection with enhanced mortality, while mice lacking MLKL and caspase-8 remained unaffected. Furthermore, they found that RIPK3 is essential to the expression of neuronal chemokines and recruitment of T lymphocytes and inflammatory myeloid cells in the central nervous system.(264) First evidence regarding *Listeria monocytogenes* (LM) intracellular clearance reported that LM activates RIPK3, which further phosphorylates MLKL, but MLKL activation does not result into plasma membrane disruption and necroptosis. Interestingly enough, phosphorylated MLKL directly binds with LM to prevent its cytosolic replication, suggesting a necroptosis-independent role of RIPK3 in LM restriction (as discussed above).(201) Although direct clearance of LM infection by RIPK3-MLKL have been observed, the role of RIPK3 deficiency in modulating the antigen-specific CD8⁺ T cell response generated by LM on tumor growth remained unclear. Most recently, Snyder *et al.*, evaluated that introduction of necroptotic cells into the tumor microenvironment promotes BATF3⁺ cDC1- and CD8⁺ T- dependent antitumor immunity. The constitutive activation of RIPK3 and delivery of a gene encoding this enzyme to tumor cells using adeno-associated viruses induces tumor cell necroptosis, which synergizes with immune checkpoint blockade (ICB) to promote durable tumor clearance.(265) Another group also delivered of mRNA-encoding MLKL intra-tumorally to promote necroptotic cell death and conferred protection in murine melanoma model.(266) Both studies defined that intra-tumoral activation of RIPK3-MLKL promotes tumor regression but how these signals interact with tumor infiltrating APCs to facilitate tumor antigen-presentation to cytotoxic CD8⁺ T cells remains still

need to be addressed. Nevertheless, these investigations support the idea that the activation of intra-tumoral necroptotic pathway can be an additional approach to promote antitumor immunity.

To obtain the desired scientific rationale for clinical immunotherapeutic achievements, *Listeria*-based vaccination is in part due to its application in mouse model where a single sublethal dose protects against lethal *Listeria* challenge.(267) Recently, Hanson *et al.*, used recombinant *L. monocytogenes*-induced intracellular death termed Lm-RIID, which commits intracellular suicide by deleting genes required for its viability. Although recombinant Lm-RIID commits suicide in the host cell by inducing Cre-recombinase and deletes essential viability genes flanked by *loxP*, it induces potent antigen-specific CD8⁺ T cell response. Furthermore, similar to live *Listeria monocytogenes* vaccination, Lm-RIID vaccination to mice protects against later virulent infection.(268) Anti-tumor CD8⁺ T cell response highly depends on CD8⁺ T cells differentiation and infiltration into the tumor microenvironment (TME).(113) Recruitment of immune suppressive cells, such as regulatory T cells (T_{reg}), M2 macrophages, immature dendritic cells (DCs) and myeloid derived suppressive cells (MDSCs) into the TME encourages tumor immune evasion.(269) Most importantly, immunosuppressive cytokines such transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) production by T_{reg} in the TME impair antitumor CTLs function.(270) Deng *et al.*, reported that recombinant *Listeria monocytogenes* (LM) infection induces tumor rejection by KLRG1⁺ PD1^{low} CD62L⁻ antigen-specific IFN-γ producing effector CD8⁺ T cells. Moreover, these cells convert tumor from an immunosuppressive to immunostimulatory state by reducing T_{reg} population and conversion of M2 macrophages to M1 phenotype.(241) Although, intratumoral activation necroptotic cell death (265,266) and the vaccination by recombinant *Listeria* (268) enhance CD8⁺ T cell response to restrict tumor growth, but the role of RIPK3 and Casp-1/11 on the antigen-specific targeted elimination of tumor cells remained unclear. In this study, we observed that both RIPK3 and Casp-1/11 are necessary for optimal antigen-specific effector and early memory CD8⁺ T cell response generated by LM-OVA. Interestingly, the reduction of OVA-specific CD8⁺ T cell response found in both RIPK3^{-/-} and Casp1,11^{-/-} mice infected with LM-OVA results in a deficiency to eliminate B16.OVA melanoma cells only in Casp1,11^{-/-} and Casp1,11^{-/-}/RIPK3^{-/-} (Figures 31 and 32). Taken together, we suggest that RIPK3 and Casp-1/11 participate differently in antigen-specific effector and memory CD8⁺ T cell response generated by each

recombinant live vector. Furthermore, our findings may help to optimize the immunotherapeutic potential of LM- or other live vector-based vaccination strategies.

Tumor-infiltrating lymphocytes (TILs) are a local histopathological reflection of immune system which have gained increasing attention in the prognosis prediction and treatment of cancer including melanoma.(271-273) Accumulating current information indicates that tumor-infiltrating CD8⁺ T cells are tissue resident memory T cells (Trm). Trm cells accumulate into non-lymphoid tissues including tumor and are defined to express CD103, CD49a integrins and C-type lectin CD69, which mostly contribute in tissue residency of CD8⁺ T cells. After initial encounter to pathogen, Trm cells also persist for longer time in non-lymphoid tissues including tumors, where they provide rapid protection against re-infecting the same pathogen.(274,275) Recently, it has been reported that CD103⁺ CD8⁺ Trm cells protect against oral and intestinal infection of *Listeria monocytogenes*(276,277) and CD49a expressing Trm are more potent to control melanoma progression.(278) Thus, Trm accumulation at the tumor site aiming to kill tumor cells and their potential role in clearing pathogen and re-infection may provide new insights with potential prognosis and immunotherapeutic applications. In our study, we did not address the composition and phenotype of innate and adaptive tumor infiltrating cells after vaccination with LM-OVA. We are still left with a number of questions to be addressed here, for instance, most importantly, the potential role of inflammatory cell death mediators such as, RIPK3 and Casp-1/11 on intratumoral CD8⁺ T cell priming, proliferation and generation of Trm cells (CD103⁺ CD8⁺ Trm cells) in TME. Furthermore, how RIPK3 and Casp-1/11 regulate intratumoral accumulation of intracellular antigen delivery vector such as *Listeria* is still need to be addressed.

10. CONCLUSION:

This study concludes that;

- RIPK3 and Casp-1/11 control *Listeria* infection, RIPK3 and Casp-1/11 deficiencies increase the susceptibility to *Listeria* infection.
- Casp-1/11 but not RIPK3 restricts antigen-specific CD8⁺ T cell expansion in response to LM-OVA.
- RIPK3 and Casp-1/11 are essential to *in vivo* priming and proliferation of antigen-specific CD8⁺ T cells.
- LM-OVA induces dose-dependent antigen-specific CD8⁺ T cell response. RIPK3 and Casp-1/11 deficiencies limit the effector function of antigen-specific CD8⁺ T cells generated in response to LM-OVA infection.
- Antigen-specific CD8⁺ T cell response depends on antigen-delivery vector. RIPK3 and Casp-1/11 mediate the induction and modulation of antigen specific CD8⁺ T cell response depending on the mode infection and/or immunization by genetically modified bacterial (LM-OVA) or viral vector (rhAd5-OVA).
- The expansion of regulatory T cells (CD4⁺ CD25⁺ FOXP3⁺) depends on RIPK3 at the peak (at day 3) of LM-OVA infection.
- Casp-1/11 and Casp-1/11/RIPK3 combined deficiencies restrict antigen-specific CD8⁺ T memory cell early differentiation.
- RIPK3 and Casp-1/11 are essential for optimal anti-tumor response to control antigen-specific tumor growth. Casp-1/11 and Casp-1/11/RIPK3 deficiencies restrict tumor regression by anti-tumor immune response generated by LM-OVA vaccination.

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