Aamir Rana

Modulação da resposta anti-tumoral por agentes infecciosos modificados geneticamente

Tese apresentada ao Programa de Pós-Graduação em Imunologia do Instituto de Ciências Biomédicas, Universidade de São Paulo, para a obtenção do titulo de Doutor em ciências

Área de concentração: Imunologia

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

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

Thesis presented to the Post-Graduate Program of 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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São Paulo
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UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS

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Titulo da Dissertação/Tese: Modulação da resposta anti-tumoral por agentes infecciosos modificados geneticamente

Orientador: João Gustavo Pessini Amarante-Mendes

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1. APPROVAL OF THE ETHICAL COMMITTEE TO WORK WITH ANIMALS

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CERTIFICADO

Certificamos que o projeto intitulado "***Modulação da resposta imune anti-tumoral por agentes infecciosos geneticamente modificados***", registrado sob o protocolo nº ***28/2016***, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de *Pesquisa Científica*, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle e Experimentação Animal (CONCEA). Ante esta conformidade, o referido projeto foi avaliado e aprovado em ***27/04/2016*** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Ciências Biomédicas da Universidade de São Paulo (CEUA-ICB/USP), outorgando esta licença de uso de animais com validade de **4 ano(s)** a partir da data de aprovação.

- Investigador Principal: **Dr.(a.)** ***João Gustavo P. Amarante-Mendes***
- Departamento: *Imunologia*
- Membros da Equipe: *Aamir Rana (Pós-graduando)*

Ao final do período outorgado por esta licença, o pesquisador responsável deverá encaminhar a esta comissão, até o último dia de validade da atual proposta, *relatório final* de acordo com a Resolução Normativa CONCEA nº 30/2016 - Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA), conforme modelo constante no endereço eletrônico *http://www3.icb.usp.br/corpoeditorial/index.php?option=com_content&view=article&id=702*. Havendo interesse na renovação do projeto, a solicitação deverá ser protocolada pela Secretaria da CEUA-ICB/USP até o último dia de validade da atual proposta. Após esta data uma nova proposta deverá ser encaminhada.

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We hereby certify that the project entitled "***Modulation of anti-tumor immune response by genetically modified infectious agents***", protocol nº ***28/2016***, which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human), for *Scientific Research Purposes*, is in accordance with the provisions of the Law nº 11.794 passed on October 8th, 2008, Decree nº 6899 passed on July 15th, 2009, and the rules issued by the National Council for Control and Animal Experimentation (CONCEA). According to this legislation, the project was evaluated and approved on ***4/27/2016*** by the ETHICS COMMITTEE ON ANIMAL USE, Institute of Biomedical Sciences, University of Sao Paulo (CEUA-ICB/USP), and the license for animal use is valid for **4 year(s)** from the date of approval.

- Principal Investigator: **Dr.(a.)** ***João Gustavo P. Amarante-Mendes***
- Team members: *Aamir Rana (Graduate Student)*

At the end of the period granted by this license, the Principal Investigator must submit a final report of the project to this committee, according to the Rule nº 30 and the Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA) issued by the CONCEA. If a renewal of the project is intended, the request must be submitted to the CEUA-ICB/USP secretary before the expiration of the current proposal. After this date, a new proposal must be prepared.

Espécie/Species	Linhagem/Strain	Sexo/Gender	Idade-Peso/ Age-Weight	Total
Mus musculus	*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

Eliane Aparecida G. M. Nascimento
Secretária 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 RIPK3 KO C57Bl/6 KO para caspase 1	Macho Macho Macho	6-12 semanas 6-12 semanas 6-12 semanas	810 270 270

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.

This is perhaps the hardest chapter that I have to write. It will be simple to name all the people that helped to get this done, but it will be tough to thank them enough. I will nonetheless try.

I feel proud to articulate some obsession about my respected research supervisor. It is difficult to overstate my gratitude to **Prof. Dr. João Gustavo Pessini Amarante-Mendes,** Molecular Cell Biology Laboratory, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo Brazil, for his skilful guidance, art of making useful suggestions and for being unquenchable in the quest for improvement. This thesis, more than most required his assistance, advice and expertise. I have to thank **Profa. Dr. Karina Ramalho Bortoluci,** Department of Biological Sciences, Federal University of São Paulo, São Paulo, Brazil, for her suggestions, encouragement and technical support to this work.

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Aamir Rana

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2. LIST OF SYMBOLS:

%	Percentage
<	Greater than
>	Less than
Δ	Delta
°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 isothyocyanate
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 (IFNγ)-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	*Listeria monocytogenes*
LM-OVA	Ovalbumin-expressing *Listeria monocytogenes*
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 efetoras 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 $Casp\text{-}1/11^{-/-}$ infectados com LM-OVA resultou na deficiência em eliminar células B16.OVA de melanoma somente em $Casp\text{-}1/11^{-/-}$ 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 heterogenicity, 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 cells inhibit the caspases activation and enhance tumor survival.(10) In contrast to the apoptotic cell death, in which the dying cells shrinks 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-fold 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 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)

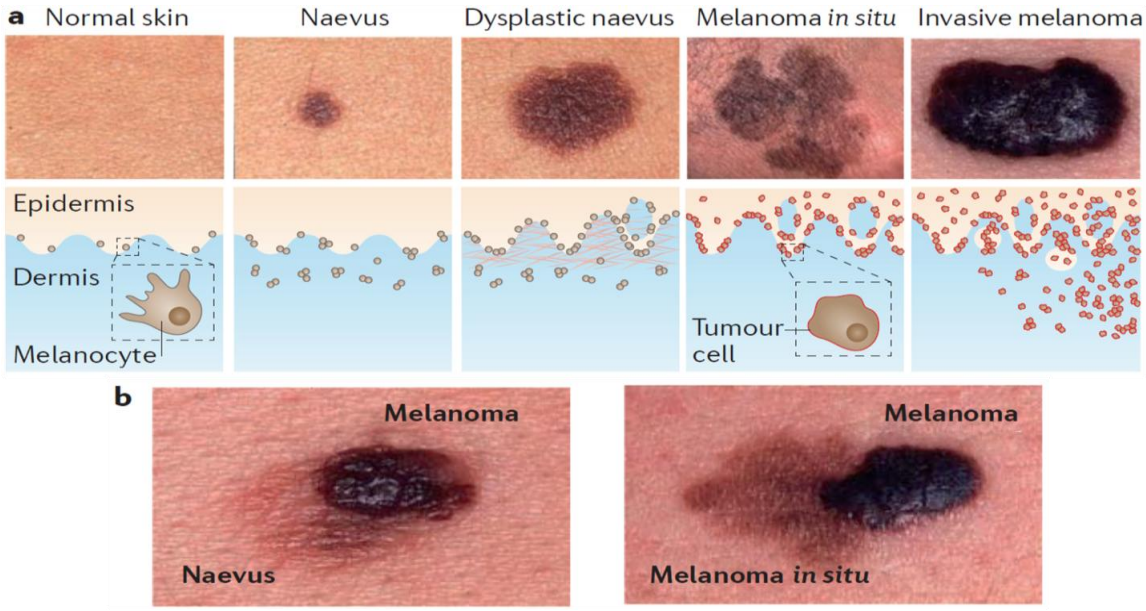

Figure 1: The morphological spectrum of melanocytic neoplasms. (a) Top row: clinical images showing a free-standing nevus, a dysplastic nevus, melanoma *in situ* and invasive melanoma. Second row: schematics illustrating the architectural features for each type of lesion. (b) Clinical images showing 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 (NARS, 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), (melanoma-associated antigen recognized by T cells) MART-1 that remain unrecognized by the immune system generate to 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 lignads 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 cykonies (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, adaptive 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 under investigations.(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, γδ 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 are integral of adaptive immunity. $CD8^+$ T cell 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 node. 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 use 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 caspase activation 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)

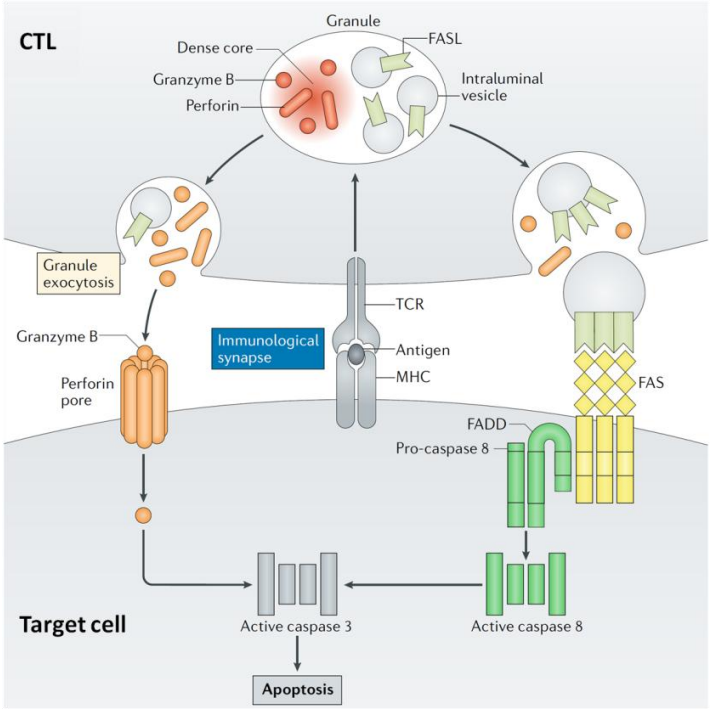

Figure 2: Mechanisms of CTL-mediated cytotoxicity. A cytotoxic T lymphocyte (CTL) recognizes an antigen-MHC complex on target cell through its T cell receptor (TCR). TCR-MHC binding triggers migration of granules containing FAS ligand (FASL), perforin and granzyme. Upon CTLs activation, granules release 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 cell 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-7Rα (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 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
Effector	$KLRG1^+$ $CD43^+$ $CD62L^-$ $CD69^+$ $CD95^+$ $CD137^+$	i) Direct cytotoxicity against malignant and pathogen infectd cells ii) Induce cytotoxicity through Fas/FasL and perforin/granzyme	81-84
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	85-90
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	85-90

4.6. $CD8^+$ T cell exhaustion

T cell exhaustion is a dysfunctional state of T cell 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.(91) Exhausted $CD8^+$ T cell population was initially defined in a chronic lymphocytic choriomeningitis virus (LCMV) infection mouse model.(92) 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.(93-95) During $CD8^+$ T cell exhaustion, exhausted cells highly express the inhibitory receptors including CTLA-4, PD-1, TIM-3, LAG-3, TIGIT and BTLA.(96-101)

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.(102) 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.(103) 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.(104) 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.(105) 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.(100) 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.(106) 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, macrophages and other immune cells.(107) 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.(108-110) 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.(111) $CD8^+$ 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.(112,113) 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.(114,115) $CD8^+$ 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.(116) 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.(117) First time, in the mid-1980s, Rosenberg *et al*., isolated highly cytotoxic lymphocytes from the tumor patients and that restrict tumor growth following adaptive transfer in patients with lung, liver and skin cancer.(118,119) Shankaran *et al*., observed that 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.(120) Shortly after, Dudley *et al*., evaluated that clonal expansion of tumor infilterating $CD8^+$ T cells is responsible tumor regression patients with metastatic melanoma.(121) 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.(122) 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.(123) 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.(124) 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.(125) 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.(126,127) T_{reg} are originally identified as $CD4^+$ $CD25^+$ T cells and are proven to play an essential role in maintaining self-tolerance.(128-131) Deficiency of $FOXP3^+$ T_{erg} results in allergy and fatal autoimmune disorder in both humans and mice.(130-132) 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.(130-132) 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.(133) 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).(133) 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.(134,135)

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.(136,137) Tumor infiltrating lymphocytes comprise 10-50% of $CD4^+$ T cells compared with the 2-5% in the peripheral blood of healthy individual.(138-140) Relatively high abundance of T_{reg} population in the tumor microenvironment results in poor prognosis in patients with various type of cancer including melanoma.(130,138).

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.(141) 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.(142) 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.(143) In this regard, it has been observed that infectious bacteria such as *Salmonella* (144) and *Listeria* (145-148), 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.(149) 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*.(150)

4.10. Listeria monocytogenes:

Listeria monocytogenes is a food born gram-positive facultative intracellular bacterium that is associated with Listeriosis and gastrointestinal infections.(151,152) *Listeria* uses its virulence factor known as Internalin A (InlA) to interact E-cadherin on the surface polarized epithelium to invade into the cell.(153,154) Inside the cells, *Listeria* used another virulence factor known as positive regulatory factor A (PrfA) for its intracellular survival and

propagation.(155) PrfA further activates phospholipases and pore-forming toxin Listeriolysin O (LLO) to escape from infected cells.(156) *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.(157,158) Once phagocytized by APCs *Listeria* is processed and their peptides are presented on the MHC-I 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.(159,160) 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.(161) 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 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).(161,162) These danger signals or DMAPs 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.(163)

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).(164)

Consequently, a major issue in immunology is to understand how the immune system understand whether cell death is immunogenic, tolerogenic and/or silent.(165) 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-1β from mature DCs and IL-17 from γδ T cells and finally leads to the tumor cell eradication by CTL response.(166)

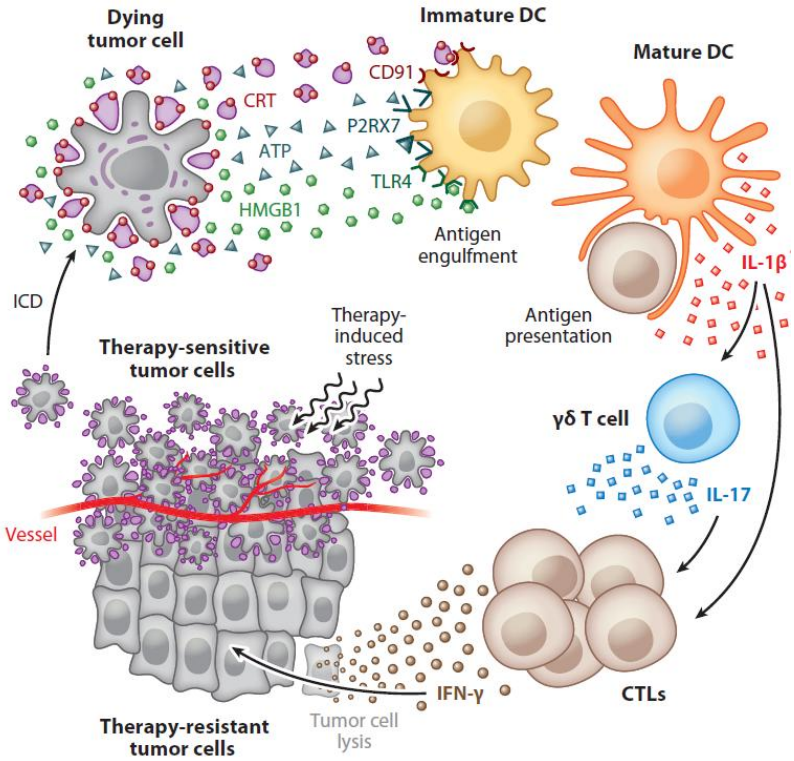

Figure 3: Immunogenic cell death (ICD). As a result of endoplasmic reticulum stress and autophagy, cancer cells respond to immunogenic cell death (ICD) inducers expose calreticulin (CRT) on the outer leaflet of their plasma membrane at a preapoptotic stage and secrete ATP during apoptosis. In addition, 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-1β- and IL-17-dependent, IFN-γ-mediated immune response involving both γδ 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.(167) 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-1α and ATP which induce a robust immune response and inflammation.(168-170)

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.(171) 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-κB (NF-κB) pathways.(172) 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).(173-175) After deubiquitination, RIPK1 dissociates form the plasma membrane form a cytosolic complex II so called “riptosome” containing RIPK1, caspase-8, and FAS-associated death domain protein (FADD).(176,177) 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).(178,179) In addition to death-receptors ligands, RIPK3 dependent necroptosis can be triggered by other stimuli such as T-cell receptor (TCR) ligantion, engagement of Toll-like receptor (TLR) 3 and TLR-4, DNA damage and viral infection.(180) 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, (181,182) 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.(183)

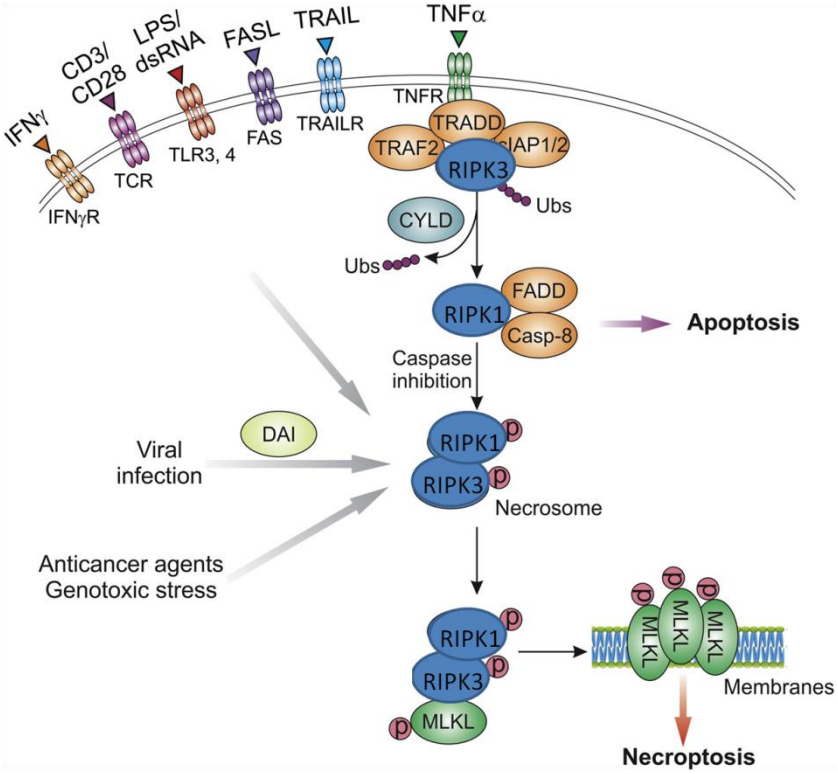

Figure 4: Necroptosis regulators and pathways. Binding of TNF- α with TNFR interacts with RIPK1 to recruit cIAP1 and cIAP2 to from a membrane bound complex I which results in the polyubiquitination of RIPK1. cIAPs inhibition leads deubiquitination of RIPK. RIPK1 binding with FADD and caspase-8 leads 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 (IFNγ) 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* (184)

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.(185) 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.(186) 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.(187)

Necroptosis is involved in the clearance of viral infection.(188) Number of studies highlights the role of receptor-interacting serine/threonine-protein kinases to control viral(189-194) and bacterial(195-201) 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 phophorylation of MLKL does not result into its own oligomerization and translalocation to the plasma membrane (Figure 5).(202,203) However, these studies highlights the role of necroptois or necrpototic cell death mediators such as RIPK1, RIPK3 and MLKL in regulating cellular inflammation, adaptive immunity and pathogen infection.

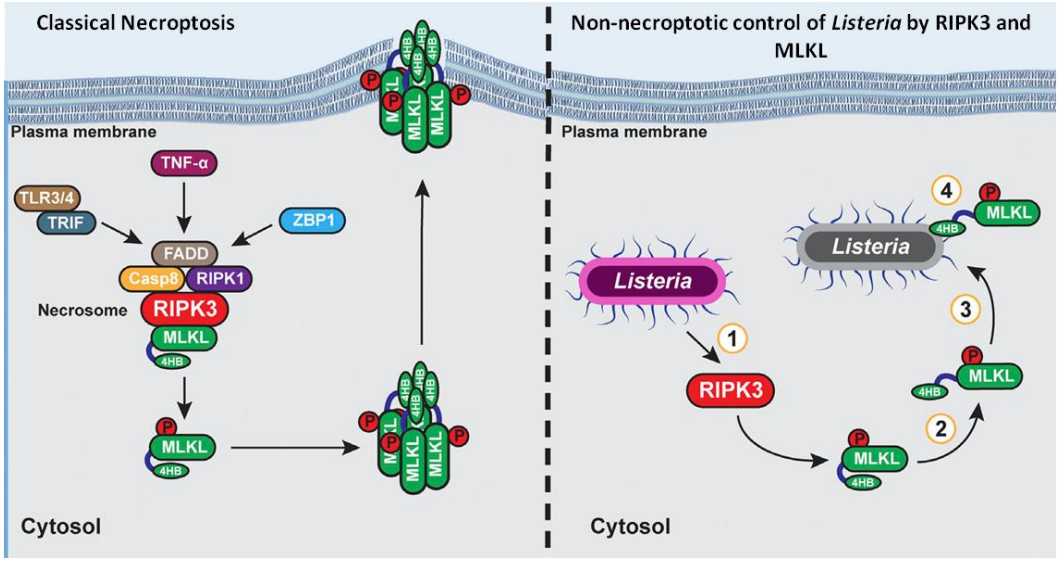

Figure 5: RIPK3 and MLKL clear *Listeria* from epithelial cells. In classical necroptosis signaling (left), activation of RIPK3 leads to the phosphorylation of MLKL. Activated MLKL subsequently oligomerizes and expose its phospholipid binding 4HB domain. Active MLKL translocates to the plasma membrane 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* (203)

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.(204) 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.(205) In 1992, Zychlinsky *et al*., observed that *Shigella* induces programmed cell death in infected macrophages and considered it to be apoptosis.(206) 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.(207-209) 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.(210) 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*.(211) In 2000, first time the term pyroptosis was used to describe this programmed cell death pathway.(212) 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 GASDMD 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.(213-215)

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 (IFNγ)-inducible protein 16 (IFI16), AIM2, pyrin, NLR family, CARD-containing 4 (NLRC4) and NLRP1b.(216) NLRP3 recognizes viral double-stranded RNA, bacterial toxins, ROS and adenosine triphosphoric acid.(217) AIM2 mainly recognize cytosolic DNA(218) during viral and bacterial infection, whereas IFI16 detects viral DNA.(219) Pyrin recognizes Rho family GTPases by bacterial toxins.(220) Bacterial flagellin, type III secretion system (T3SS) rod or needle proteins activate NLRC4.(221) 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-1β and IL-18.(216) By contrast, caspase-11 is activated via direct binding of bacterial cytosolic lipopolysaccharide (LPS).(222,223) 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**.**(213,224-227)

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 respose. TLR2 and TLR5 mainly recognized the extracellular LM, whereas NOD1 and NOD2 recognize cytolasmic LM.(228) In addition to NODs cytoplasmic LM can also be recognized by inflammasomes such as NLRP3, NLRC4 and AIM2.(229) Once these inflammasomes are activated, they recruit the procaspase-1 and activate caspase-1 to eventually induce pyroptosis.(210) 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.(230) Wu *et al*., observed that LM infection activates

NLRP3 and ASC to induce caspase-1 dependent proinflammatory cell death.(231) William *et al*., reported that NLRC4 attenuates $CD8^+$ T cell response on infection with LM.(232) 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.

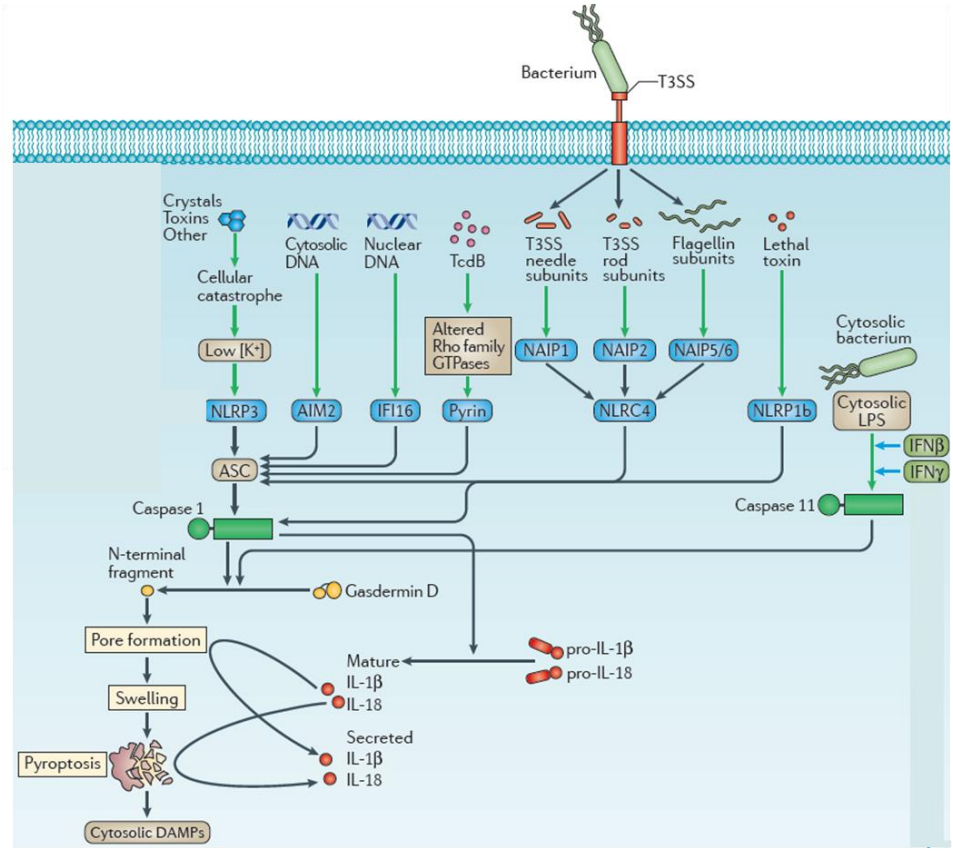

Figure 6: Pyroptosis in response to infection. Caspase-1 is activated downstream of inflammasomes activation by their respective stimuli. By contrast, caspase 11 senses cytosolic lipopolysaccharide (LPS). Either caspase 1 or caspase 11 independently cleaves gasdermin D to form the pyroptotic pores. The 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) (233)

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 it 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, 563376), 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), Carboxyflurescein 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). Both cell lines were cultured/maintained in RPMI-1640 medium supplemented with 8% FBS, 10% L-Glut and 10% Pen-Strep in 5% CO_2 at 37ºC. *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). 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 50µg/ml streptomycin at 37ºC under constant shaking. At mid log phase (OD_{600}=0.4) bacteria was harvested, frozen in

20% glycerol and stored at -80ºC. Colony-forming unit (CFU) was determined by performing serial dilution in 0.9% NaCl, which were spread on BHI-streptomycin ager 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 $2x10^6$ plaque-forming unit (PFU) of rhAd5.OVA.

7.5. Mice:

C57BL/6 RIPK3$^{-/-}$ and Casp-1/11$^{-/-}$ mice were generously provided by Vishva Dixit (Genentech, Inc, USA) and Richard Flavell (Yale University, USA), respectively. RIPK3$^{-/-}$ and Casp-1/11$^{-/-}$ mice were crossed to generate Casp-1/11$^{-/-}$/RIPK3$^{-/-}$ double-deficient mice. 6-8 weeks-old WT (Wild type), RIPK3$^{-/-}$, Casp-1/11$^{-/-}$ and Casp-1/11$^{-/-}$/RIPK3$^{-/-}$ double-deficient mice were used as experimental controls or infected groups. OT-I mice CD45.1^{+}45.2^{+} were generated by mating OT-I males (CD45.1^{-}45.2^{+}) with B6.SJL (CD45.1^{+}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 100µl of 0.9% NaCl via lateral tail vein (intravenously). While control groups were inoculated by 100µl of PBS.

WT and KO mice were immunized or non-immunized intramuscularly with $2x10^6$ PFU of rhAd5.OVA in a total volume of 100µl (50µl injected into the left and 50µl 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 infected mice were harvested at three and seven days of post-infection in RPMI-1640 medium (Life Technologies). Single cell suspension was prepared by tweezing the spleens between the frosted ends of two sterile glass slides in RPMI-1640 medium. CFU/spleen was determined by plating 10-fold serial dilutions of single cell suspension on BHI-Streptomycin plates.

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

WT and KO mice were infected or non-infected with LM-OVA for seven days. At seven days of post-infection, spleens were harvested, processed and stained with anti-mouse CD8 antibody (BD Biosciences, 563898) and H2-K^b-SIINFEKL Dextramer (Immudex, Copenhagen, Denmark, JD2163) as per manufacture's instruction. Frequency of CD8$^+$ H2-K^b–SIINFEKL$^+$ cells from each mouse was assessed by FACS using BD FACSCelesta™ (BD, Mountain View, CA). Samples were analyzed using the following gate strategy.

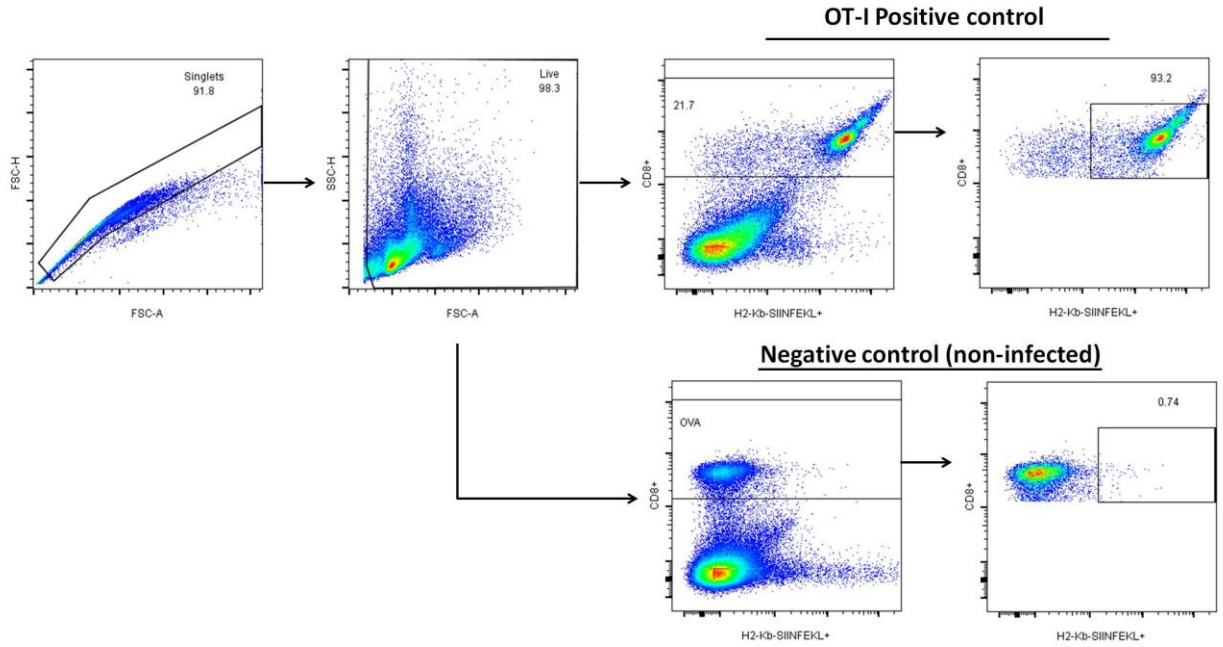

Figure 7: Gate strategy to access CD8$^+$ H2-K^b–SIINFEKL$^+$ population. FSC-A x FSC-H (Forward Scattered-Area x Forward Scattered-Height) to exclude doublets, then FSC-A x SSC-H (Forward Scattered-Area x Side Scattered-Height) to exclude debris and finally H2-K^b–SIINFEKL$^+$ x CD8$^+$ to separate 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. 10^7 OT-I splenocytes in 100µl of un-supplemented RPMI-1640 were labeled with 5uM of Cell tracer Violet (CTV) (CellTrace™ Violet Cell Proliferation Kit, Invitrogen, C34557) and 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 for single cell suspension. Splenocytes were labeled with anti-CD8 (BioLegend, 100707) for 30 minutes in 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.

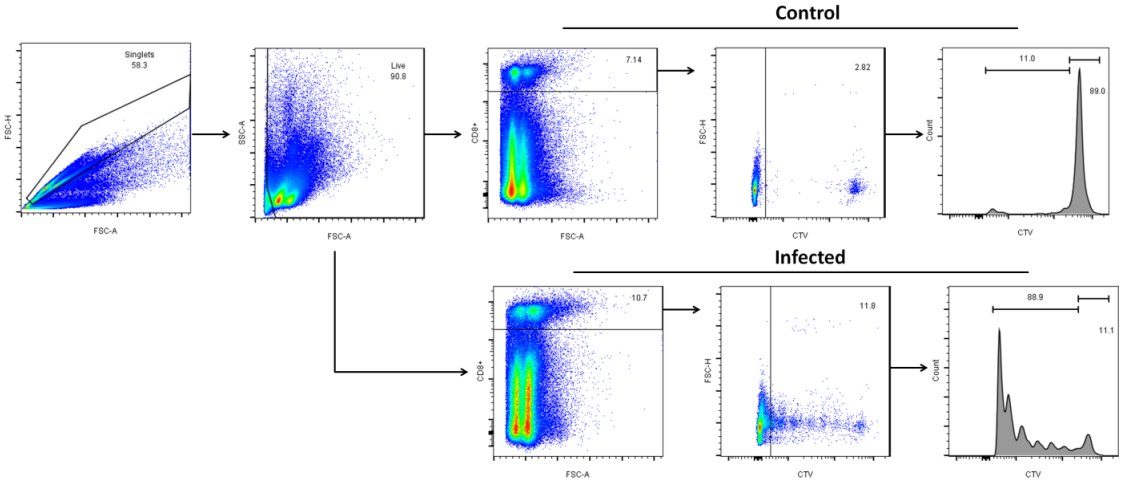

Figure 8: Gate strategy to access *in vivo* proliferation of OT-I cells. FSC-A x FSC-H to exclude doublets, FSC-A x SSC-A (Forward Scattered-Area x Side Scattered-Area) to exclude debris, FSC-A x CD8$^+$ to separate CD8$^+$ T cell population, FSC-H x CTV to separate adoptively transferred OT-I CD8$^+$ 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.(234) Briefly, at seven days of post-infection, spleens from WT donor mice were harvested and processed for single cell suspension by tweezing the spleens between the frosted ends of two sterile glass slides. Splenocytes were counted and equally divided into four

populations. Cells were separately marked with Carboxyflurescein succinimidyl ester (CFSE) (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen, C34554) $CFSE^{High}$ (10µM), $CFSE^{Low}$ (1µM), CTV^{High} (10µM) and CTV^{Low} (1µM). $CFSE^{High}$ cells were pulsed with 10nM of $OVA_{257-264}$ (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 $OVA_{257-264}$ peptide, respectively. All four populations of cells were washed and mixed in 1:1:1:1 ratio. Total of $4x10^7$ cells in 100µl of non-supplemented RPMI-1640 were inoculated into infected and control experimental mice by retro-orbital sinus. After 18 hours spleens from infected and non-infected mice were excised, processed and analyzed by FACS using BD LSRFortessa™ (BD, Mountain View, CA). The percentage of target cells lysis was determined using the following formula.

$$1- \frac{\%\ CFSE_{high}\ Or\ CTV_{high}\ Infected\ /\ \%\ CFSE_{low}\ Or\ CTV_{low}\ Infected}{\%\ CFSE_{high}\ Or\ CTV_{high}\ Naïve\ /\ \%\ CFSE_{high}\ Or\ CTV_{high}\ Naïve} \quad X\,100$$

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

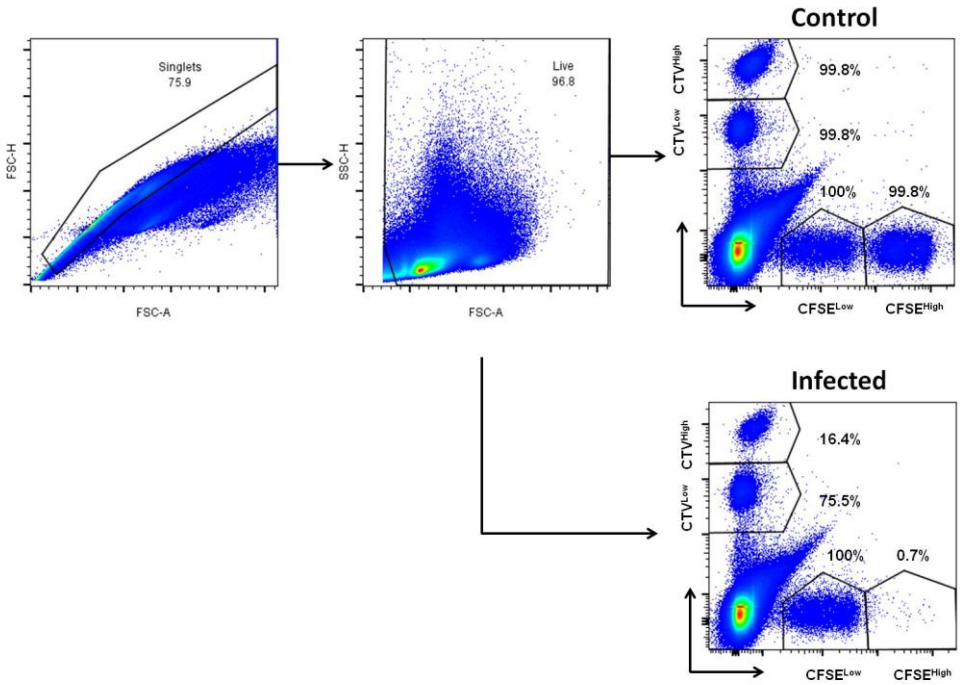

Figure 9: Gate strategy to access in vivo target cell killing by CTLs. FSC-A x FSC-H to exclude doublets, FSC-A x SSC-H to exclude debris and finally $CFSE^{High}$, $CFSE^{Low}$, CTV^{High} and CTV^{Low} populations showing percentage of target cell killing by CTLs.

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*., (235), with slight optimization and modifications. Initially, 96-well nitrocellulose plate (Multiscreen HA Millipore) was coated with 60µl per well of sterile 1 X PBS containing 10ng/ml of mouse anti-IFNγ capture antibody (BD Bioscience, 551216). A separate 96-well nitrocellulose plate was coated with 100ul 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 overnight incubation plates were washed 3 times with 100µl RPMI under sterile conditions. Subsequently, plates were blocked by adding 100µl per well of 10% RPMI medium for 2 hours at 37°C. 10^6 responder cells from each experimental mouse were separately added in anti-IFN-γ and anti-TNF-α Ab-coated ELISPOT plates, with $3x10^6$ feeder cells (from non-infected WT mice) and pulsed with 10µM $OVA_{257-264}$ peptide. The culture was established in RPMI-1640 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% CO_2. After incubation plates were washed three times with 0.05% PBS-T (Fisher BioReagents). 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 4ºC 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 dilution in 0.05% PBS Tween-20 at 4ºC for overnight. The next day, the plates were washed 5 times with PBS Tween-20 (0.05%) and 3 times with 1 X PBS. Subsequently, 100µL per well of PBS Tween-20 (0.05%) 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 Tween-20 (0.05%) and 5 times with 1 X PBS, respectively. The reaction was stopped with 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 after 7 days of infection. Total of 4 million cells from each mouse were cultured in complete medium of RPMI-1640 (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, Pharmingen^{TM}, 553294) 2µg/ml, 1% brefeldin A (Biolegend, 420601) and monensin (Biolegend, 420701). Cells were pulsed with 10µg of $OVA_{257-264}$ peptide and incubated for 8 hours at 37°C with 5% CO_2. 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 Cytofix/Cytoprem kit (BD Biosciences, 554714), as per manufacture's instruction. The following gate strategy was used to evaluate the $CD8^+$ LAMP1 (CD107a) population.

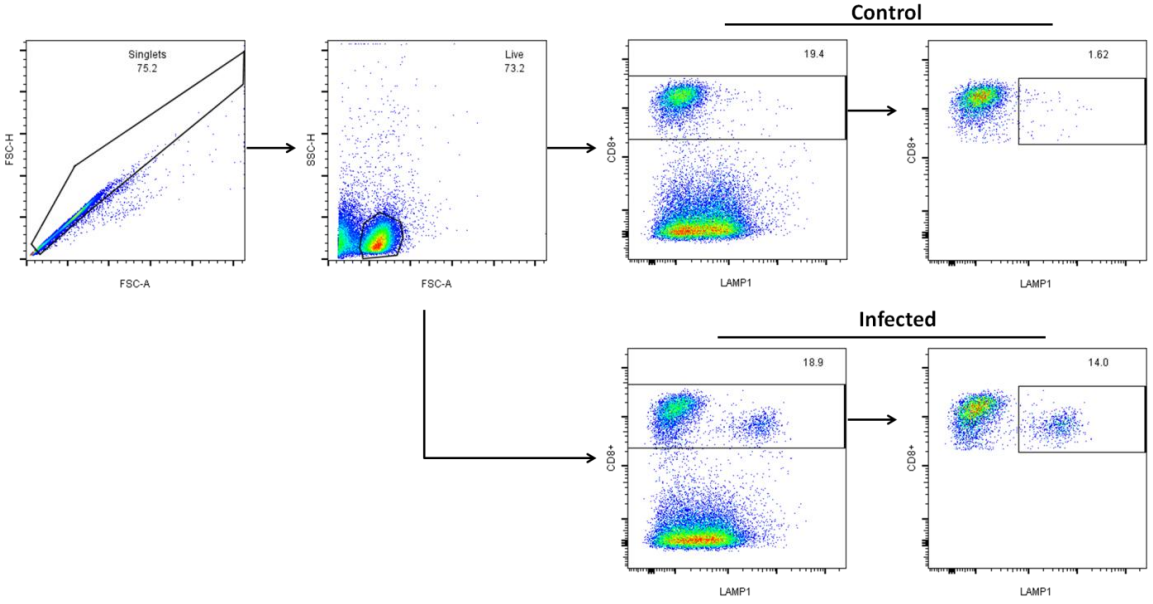

Figure 10: Gate strategy to observe $CD8^+$ $LAMP1^+$ population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris and finally LAMP1 x $CD8^+$ to separate $CD8^+$ $LAMP1^+$ population.

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

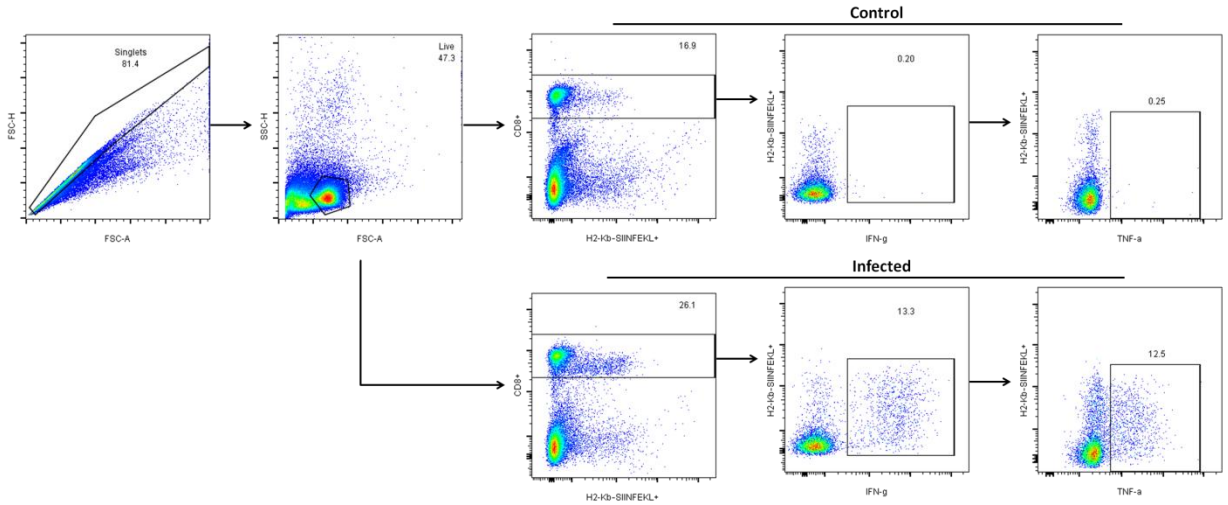

Figure 11: Gate strategy to observe IFN-γ and TNF-α expressing $CD8^+$ H2-K^b–$SIINFEKL^+$ population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris, H2-K^b–$SIINFEKL^+$ x $CD8^+$ to separate H2-K^b–$SIINFEKL^+$ x $CD8^+$ population, IFN-γ x H2-K^b–$SIINFEKL^+$ to separate IFN-γ^+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

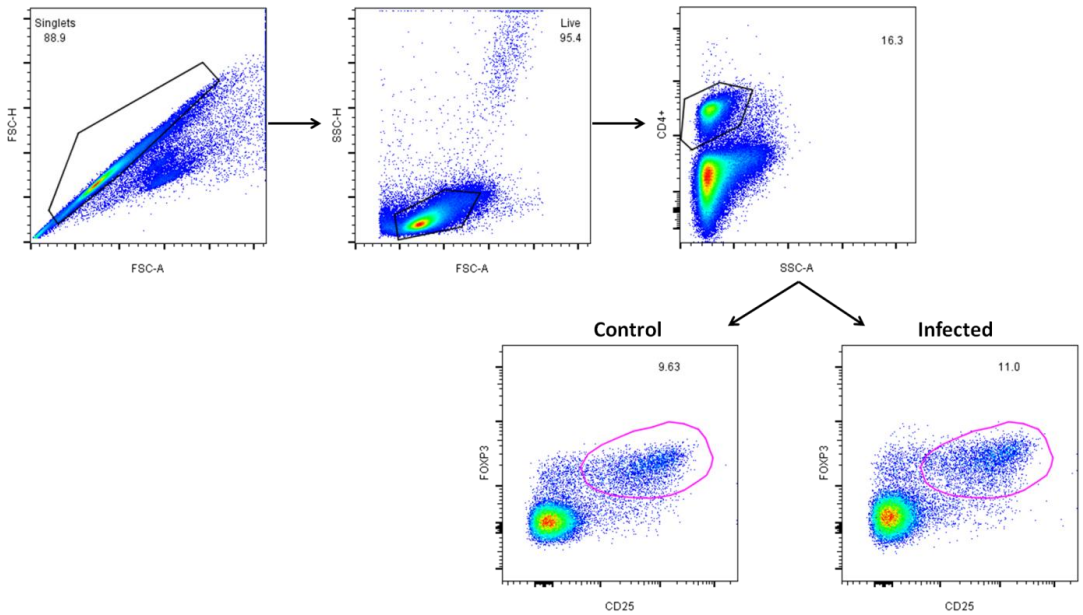

Figure 12: Gate strategy to evaluate CD4$^+$ CD25$^+$ FOXP3 T$_{reg}$ population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris, SSC-A x CD4$^+$ to separate CD4$^+$ T cell population and finally CD25 x FOXP3 to separate CD25$^+$ FOXP3 T$_{reg}$ cell population.

7.13. Adoptive transfer:

For adoptive transfer, splenocytes from OT-1 (CD45.1$^+$ and CD45.2$^+$) mice were obtained, processed and inoculated (10^7 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 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.

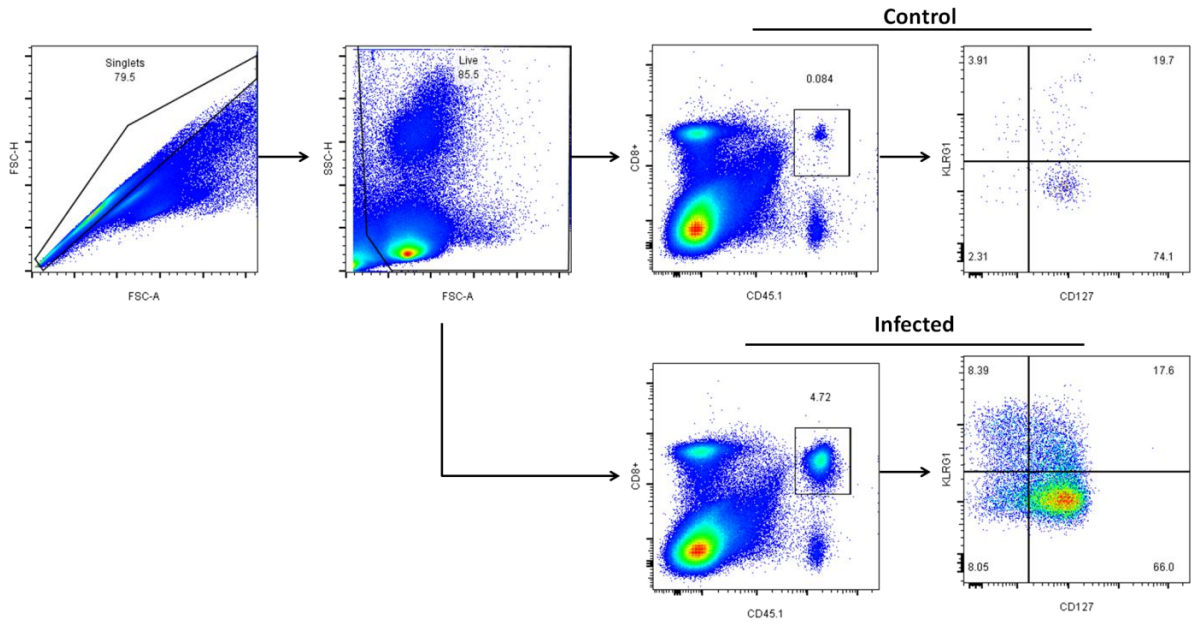

Figure 13: Gate strategy to evaluate KLRG1$^+$ CD127$^+$ OT-I CD8$^+$ (CD45.1 CD45.2$^+$) population. FSC-A x FSC-H to exclude doublets, then FSC-A x SSC-H to exclude debris, CD45.1 x CD8$^+$ to separate CD45.1$^+$ CD45.2$^+$ adoptively transferred CD8$^+$ OT-1 cells and finally CD127 x KLRG1 to observe 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 (5x10^6 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 20µl 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 2µg of RNA for total of 10µl 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: GAGCAACTCCCACTCTTCC

GAPGH 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-ΔΔCT calculation and each value was normalized by housekeeping GAPDH.

7.15. Protein electrophoresis and Western Blot analysis:

Total of 1 x 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 lystes 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 H_2O, 1ml Tris-HCL 1M pH 8.5, 22µl p-coumaric acid 90mM, 50µl Luminol 250µm and Solution **B**: 450µl H_2O and 50µl H_2O_2 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:

$2.5x10^5$ 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 10^6 B16 and B16.OVA cells. After 3 days of tumor challenge mice were infected intravenously with LM-OVA. Tumors were excised when the diameter reached to $1cm^3$. The diameter of tumor was measured after subsequent interval of 2 days by using the following formula.

$V= 0.5 \text{ a x } b^2$ (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).

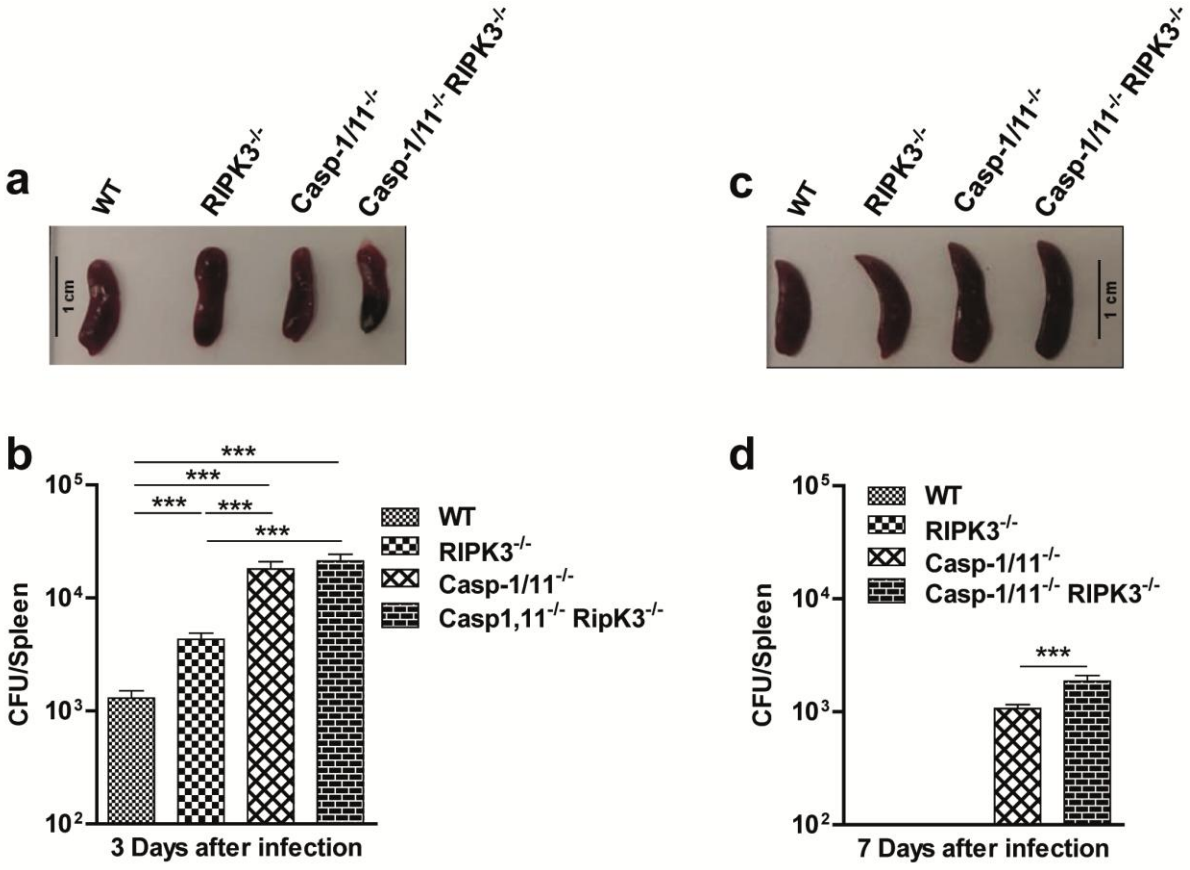

Figure 14: Casp-1/11 but not RIPK3 is necessary for the host ability to clear LM-OVA infection. Size of the spleens (a, c) and bacterial burden (b, d) were measured in infected mice at 3 (a, b) and 7 (c, d) days after LM-OVA vaccination. The data is expressed as means of five individual mice per group 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 OVA$_{257-264}$ (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 OVA$_{257-264}$ (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.

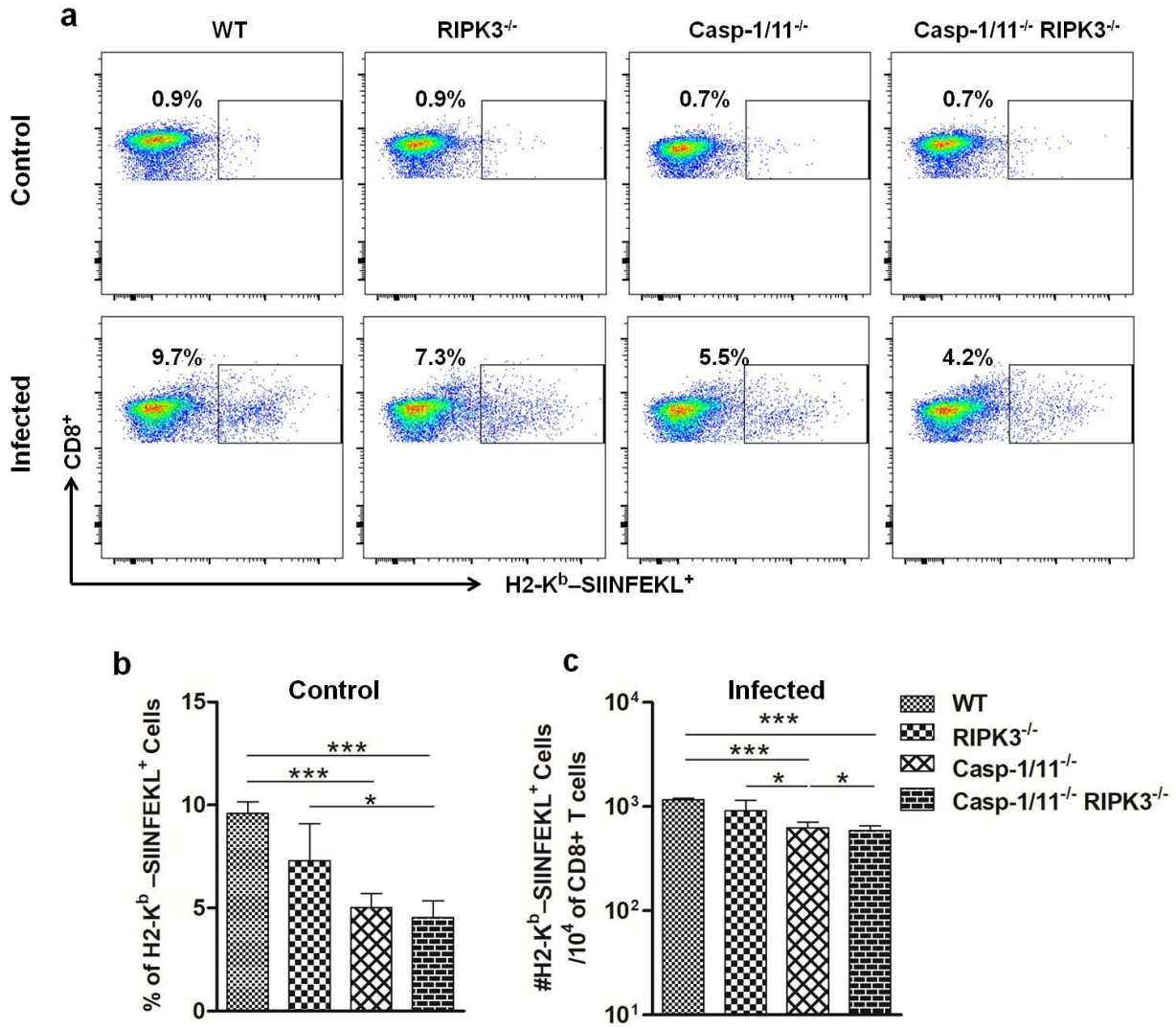

Figure 15: Casp-1/11 deficiency impairs *in vivo* antigen-specific CD8$^+$ T cell expansion in response to LM-OVA. (a,b) Frequency and (c) total number of $OVA_{257-264}$ (SIINFEKL)-specific CD8$^+$ T cells at 7 days of infection in the spleens of infected mice, revealed by staining with anti-CD8 antibody 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. ***($p<0.001$) *($p<0.5$)

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 highest 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.

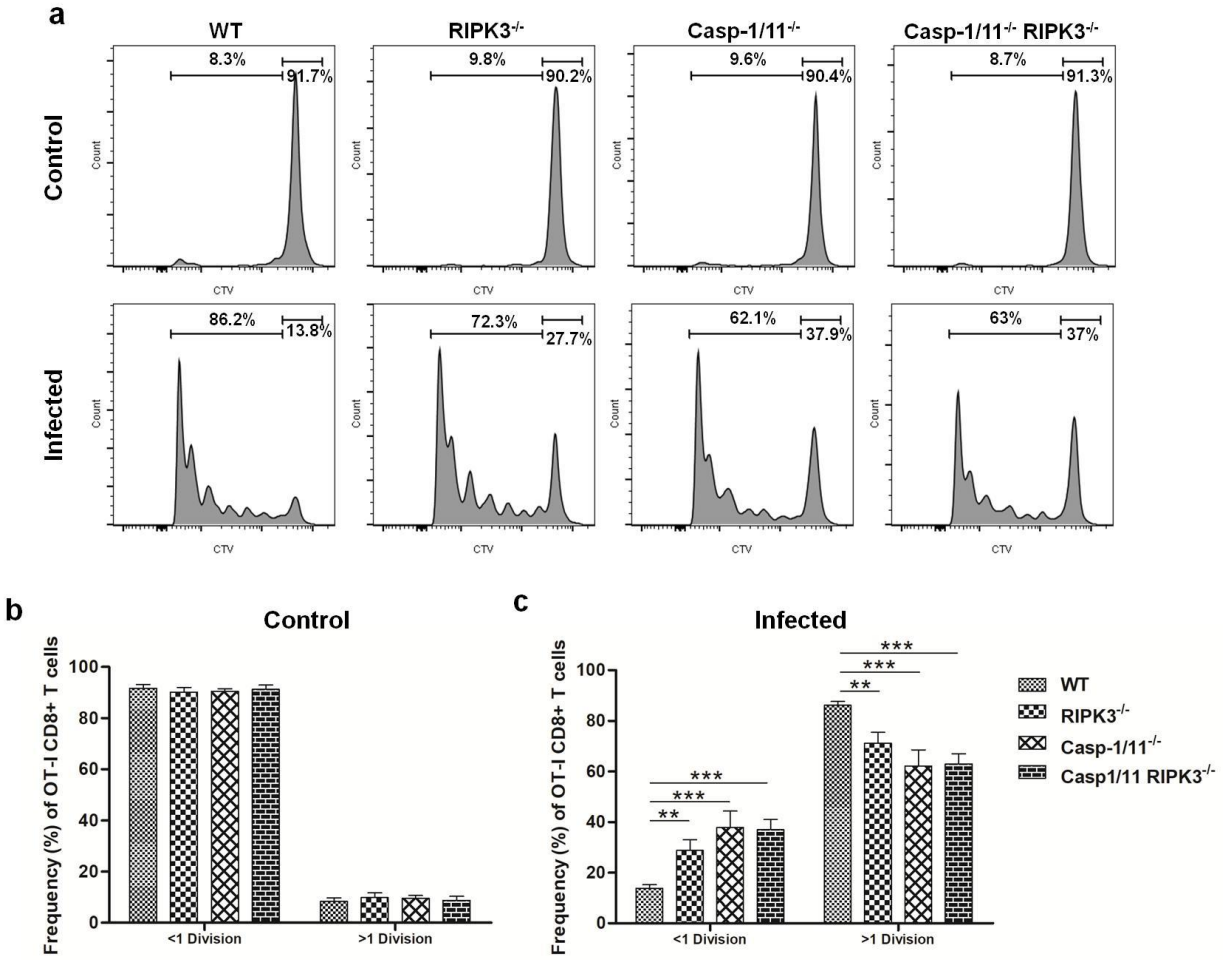

Figure 16: RIPK3 and/or Casp-1/11 deficiencies negatively impact on OVA-specific CD8$^+$ T cells priming and proliferation in response to LM-OVA. (a) *In vivo* proliferation of OT-I CD8$^+$ T cells in non-infected and LM-OVA-infected WT, RIPK3$^{-/-}$, Casp-1/11$^{-/-}$ and Casp-1/11$^{-/-}$/RIPK3$^{-/-}$ 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. ***($p<0.001$) *($p<0.5$)

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* cytotoxity to evaluate the CTL killing of targeted cells pulsed with

three different concentrations of $OVA_{257-264}$ 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).

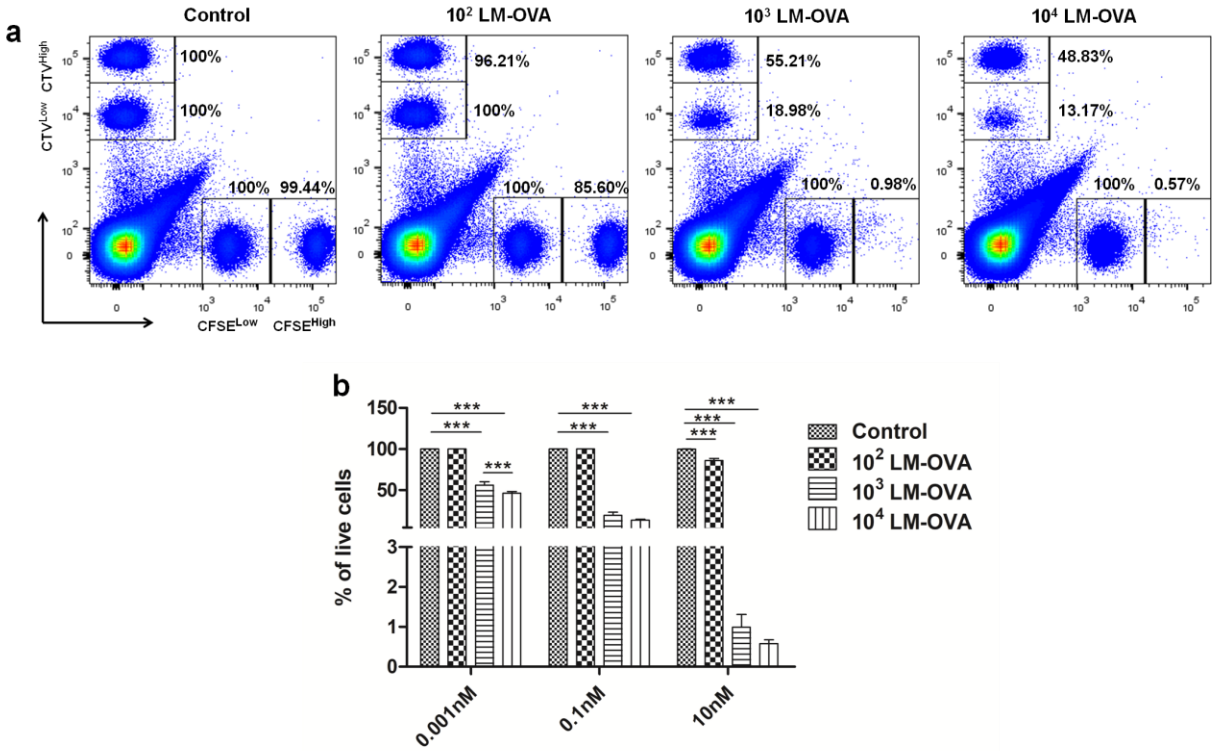

Figure 17: LM-OVA induce strong OVA-specific CTL response. (a-b) WT C57Bl/6 mice were infected or not with 10^2, 10^3 and 10^4 CFU of LM-OVA. *In vivo* cytoxic activity was estimated by injecting syngeneic target cells labeled with $CFSE^{High}$, $CFSE^{Low}$, CTV^{High} and CTV^{Low} and pulsed 0.001nM (CTV^{High}), 0.1nM (CTV^{Low}), 10nM ($CFSE^{High}$) or not ($CFSE^{Low}$) with $OVA_{257-264}$ 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.

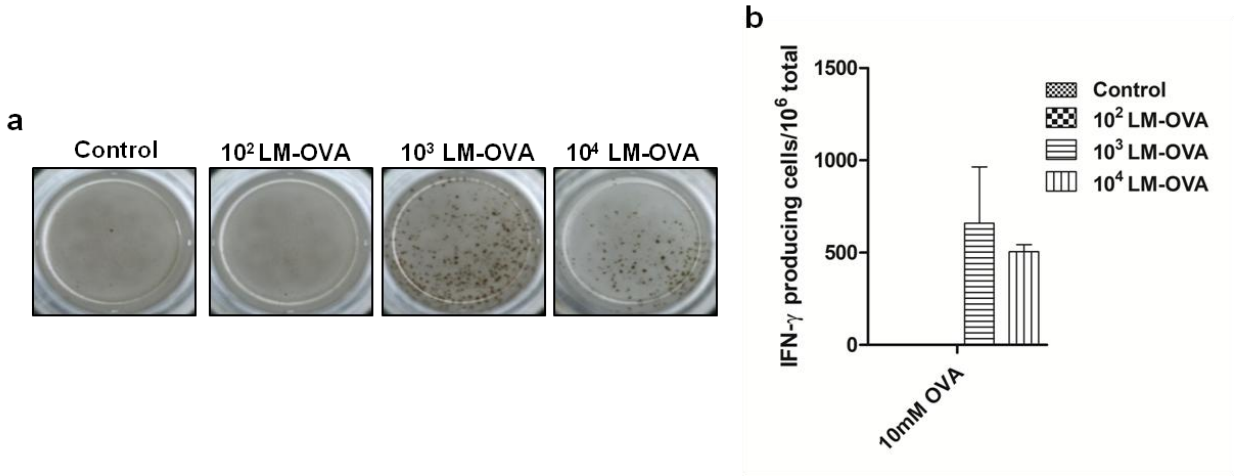

Figure 18: LM-OVA induces OVA-specific IFN-γ production by CD8+ T cells. (a, b) Splenocytes from non-infected and infected mice (10^2, 10^3 and 10^4 CFU of LM-OVA) were pulsed with 10mM of $OVA_{257-264}$ peptide. Frequency of IFN-γ producing OVA-specific $CD8^+$ T cells was determined by ELISPOT assay. 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. **($p<0.01$) *($p<0.05$)

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 $OVA_{257-264}$ 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 the cytolytic activity and cytokine production of antigen-specific CD8^{+} T cells are promoted RIPK3 and Casp-1/11.

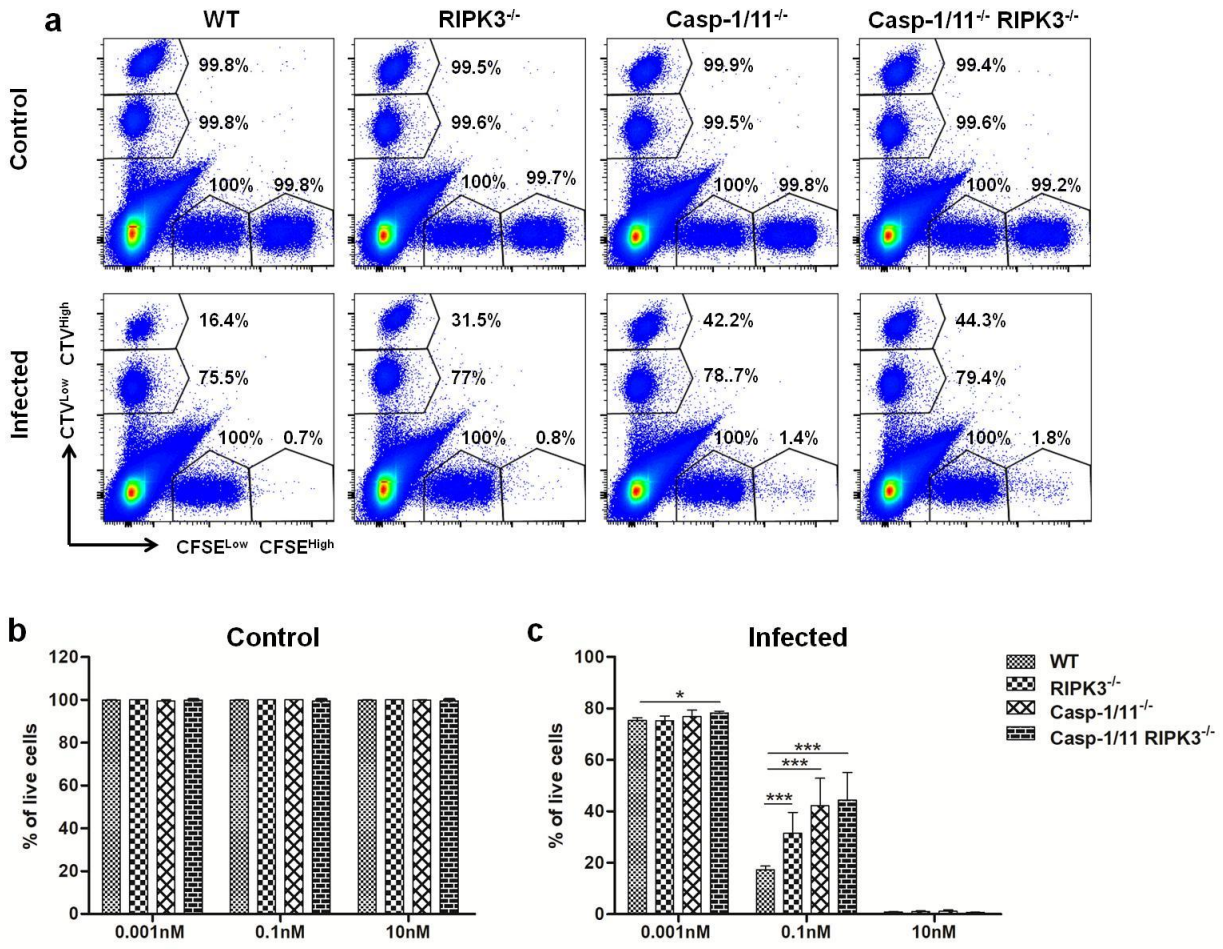

Figure 19: RIPK3 and/or Casp-1/11 deficiencies negatively impact on *in vivo* antigen-specific CTL activity. *In vivo* elimination of target cells pulsed with high (10nM), intermediate (0.1nM) and low (0.001nM) concentration of OVA$_{257-264}$ peptide at day 7 post-infection with LM-OVA. (a-c) Percentage 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. ***($p<0.001$) *($p<0.05$)

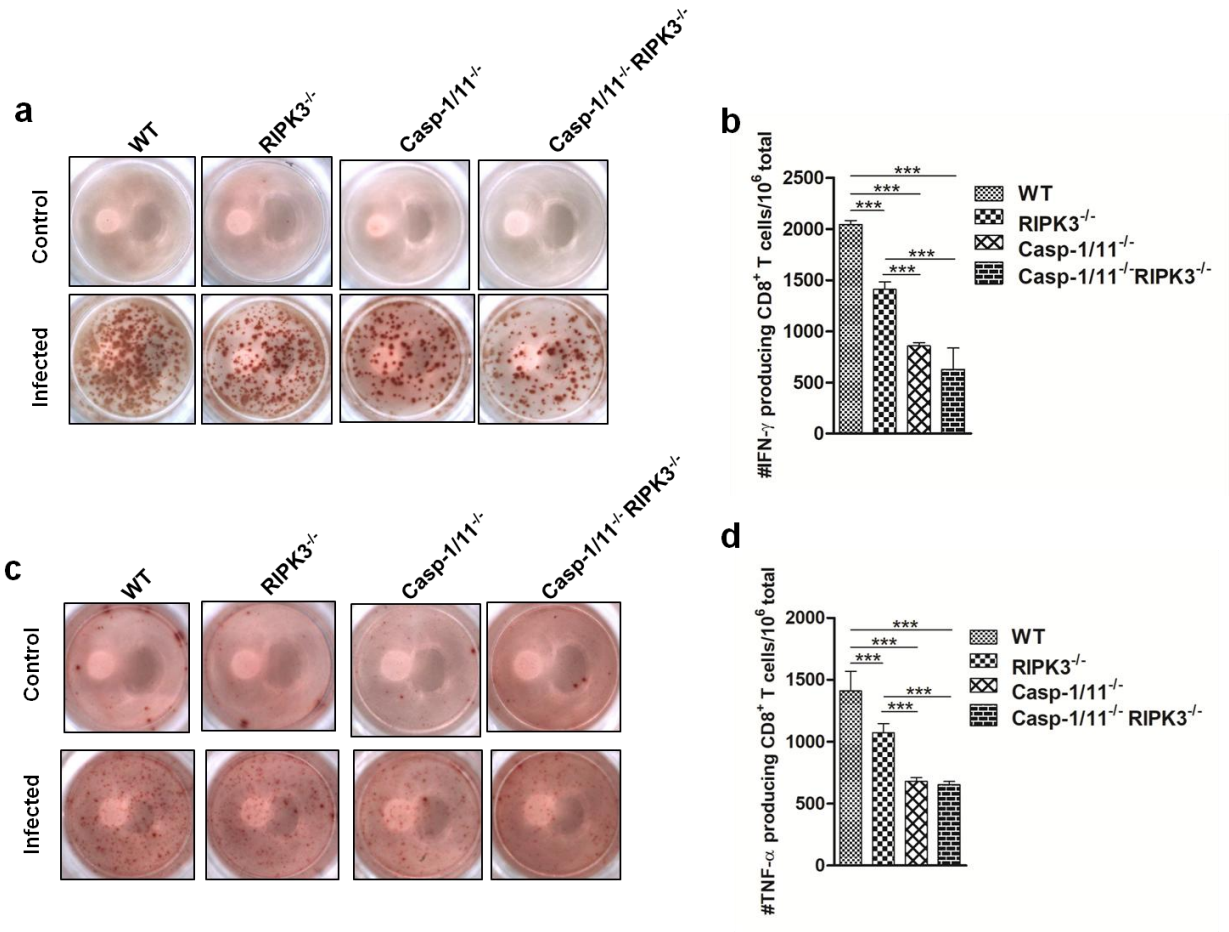

Figure 20: RIPK3 and/or Casp-1/11 deficiencies limit cytokine production by OVA-specific CD8$^+$ T cells in response to LM-OVA. ELISPOT assay was performed to determine the frequency of $OVA_{257-264}$ peptide specific (a, b) IFN-γ- or (c, d) TNF-α-producing CD8$^+$ T cells at day 7 post-infection with 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. ***($p<0.001$)

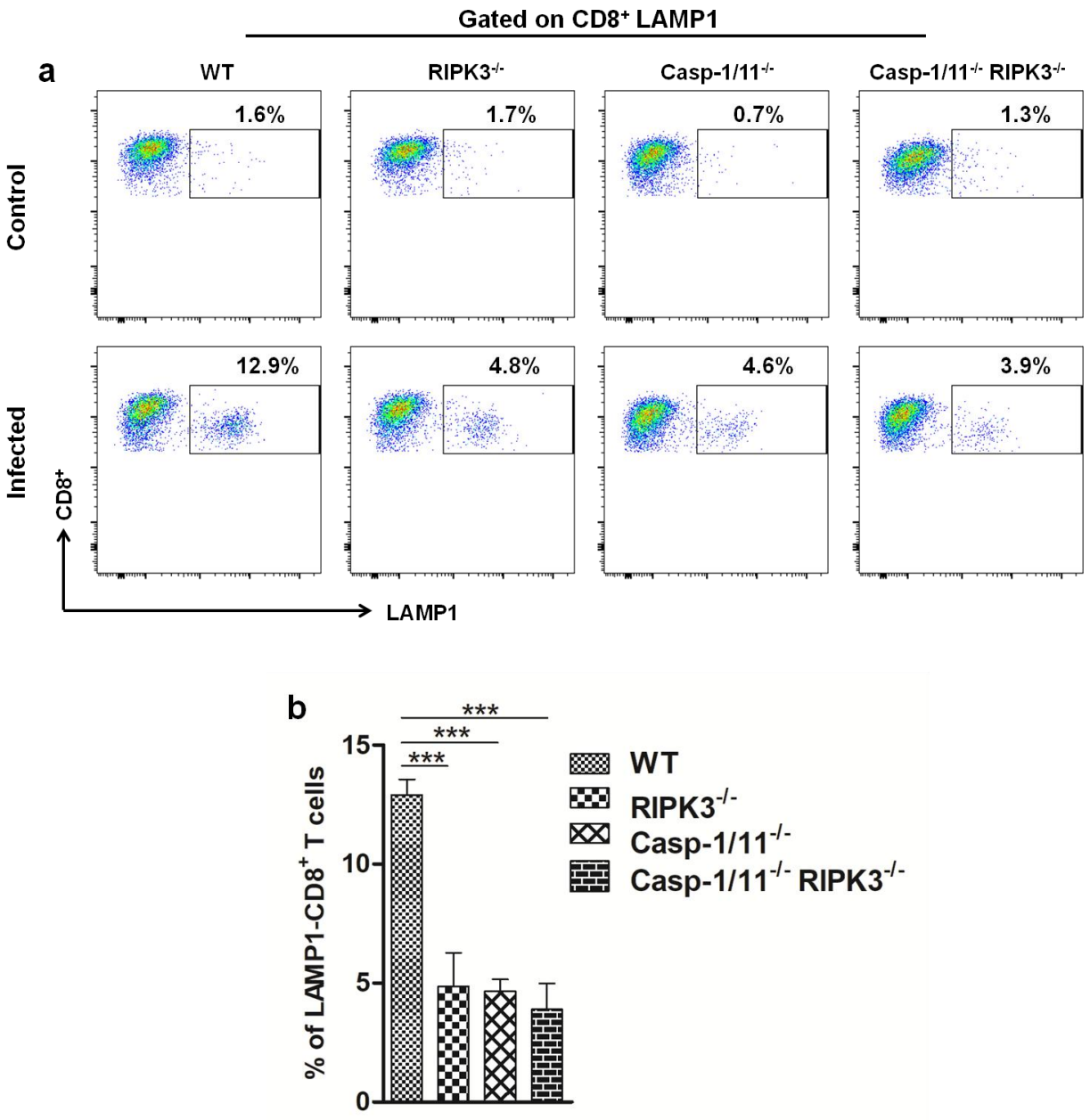

Figure 21: RIPK3 and/or Casp-1/11 deficiencies reduce antigen-specific CD8$^+$ T cells degranulation. (a) Surface expression of LAMP-1 on OVA-specific CD8$^+$ T cells (b) Frequency of LAMP-1$^+$-CD8$^+$ T cells at day 7 post-infection with LM-OVA. The data is expressed as means of five individual mice 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. ***($p<0.001$)

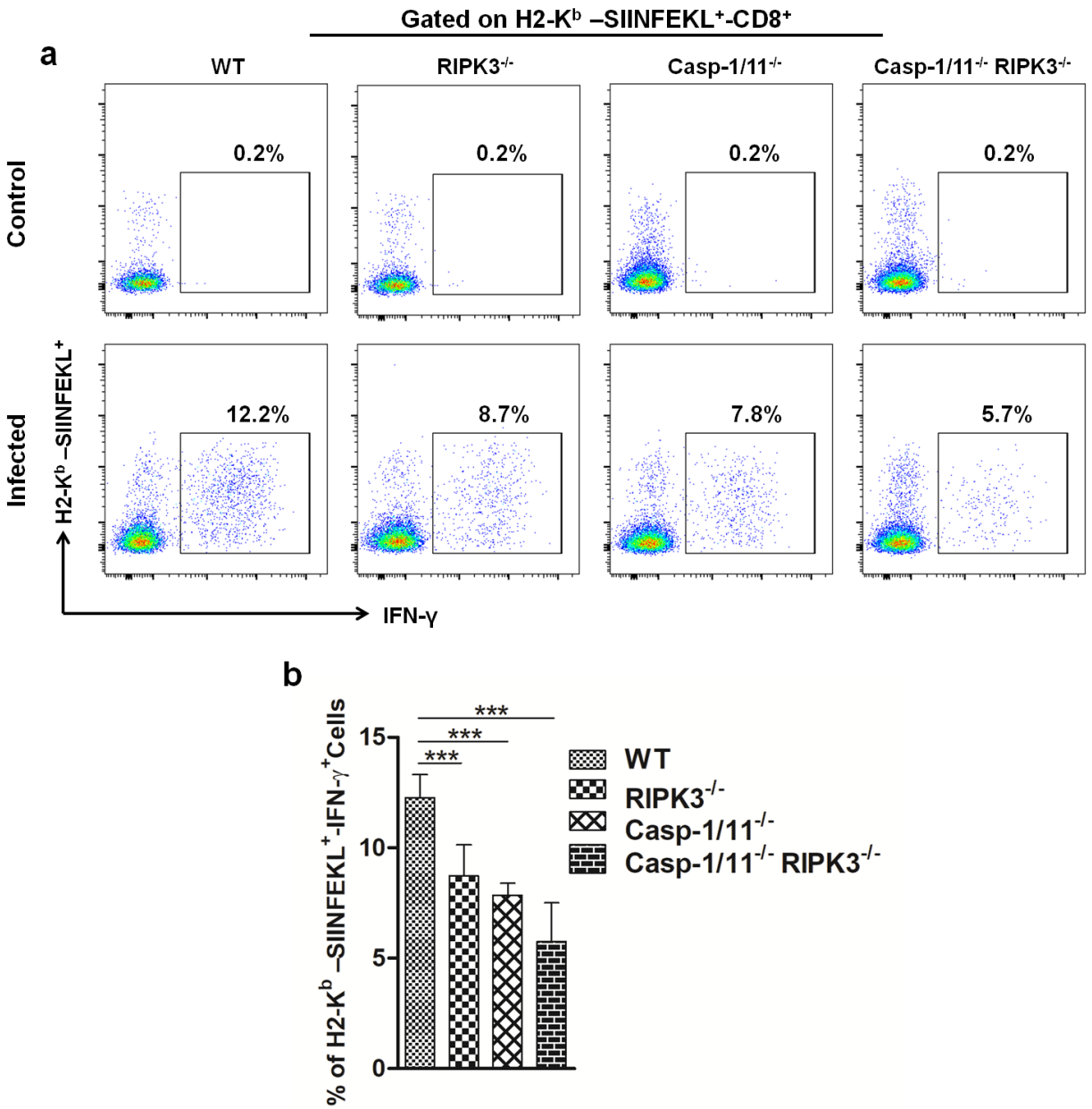

Figure 22: RIPK3 and/or Casp-1/11 deficiencies reduce antigen-specific CD8+ T cells intracellular IFN-γ production in response to LM-OVA. (a) Intracellular H2-K^b-SIINFEKL-specific IFN-γ producing CD8$^+$ T cell population (b) Frequency of H2-K^b-SIINFEKL-specific IFN-γ-producing CD8$^+$ T cells at day 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. ***($p<0.001$) **($p<0.01$) *($p<0.05$)

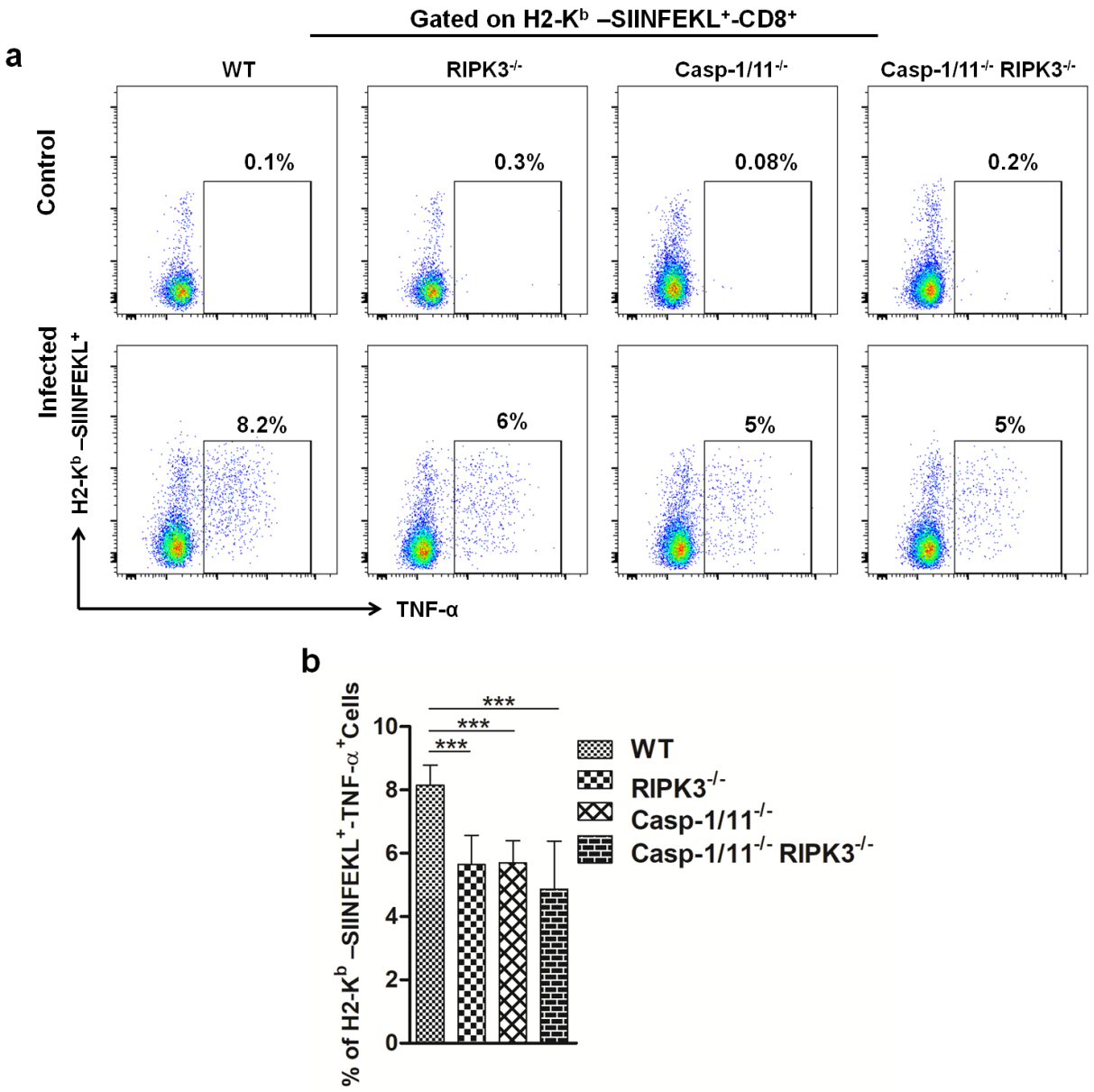

Figure 23: RIPK3 and/or Casp-1/11 deficiencies reduce antigen-specific CD8+ T cells intracellular TNF-α production in response to LM-OVA. (a) Intracellular H2-K^b-SIINFEKL-specific TNF-α-producing $CD8^+$ T cell population. (b) Frequency of H2-K^b-SIINFEKL-specific TNF-α producing $CD8^+$ T cells after 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. **($p<0.01$) *($p<0.05$)

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 $OVA_{257-264}$ 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.

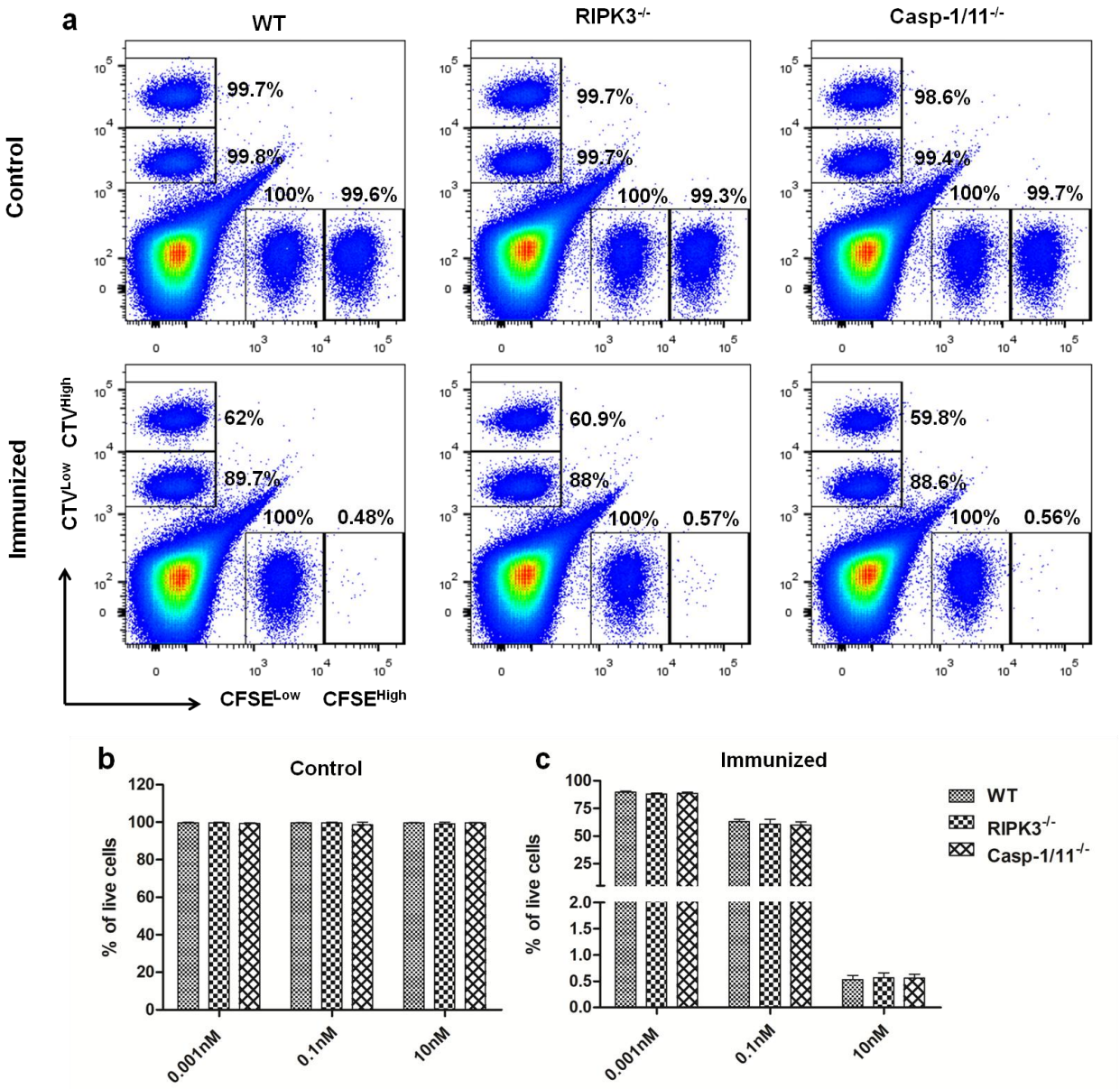

Figure 24: Antigen-specific CD8^{+} T cell response depends on antigen-delivery vector (*In vivo* cytotoxicity). (a-c) *In vivo* elimination of target cells pulsed with high (10nM), intermediate (0.1nM) and low (0.001nM) concentration of $OVA_{257-264}$ peptide at day 7 post-immunization with rhAd5-OVA. (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.

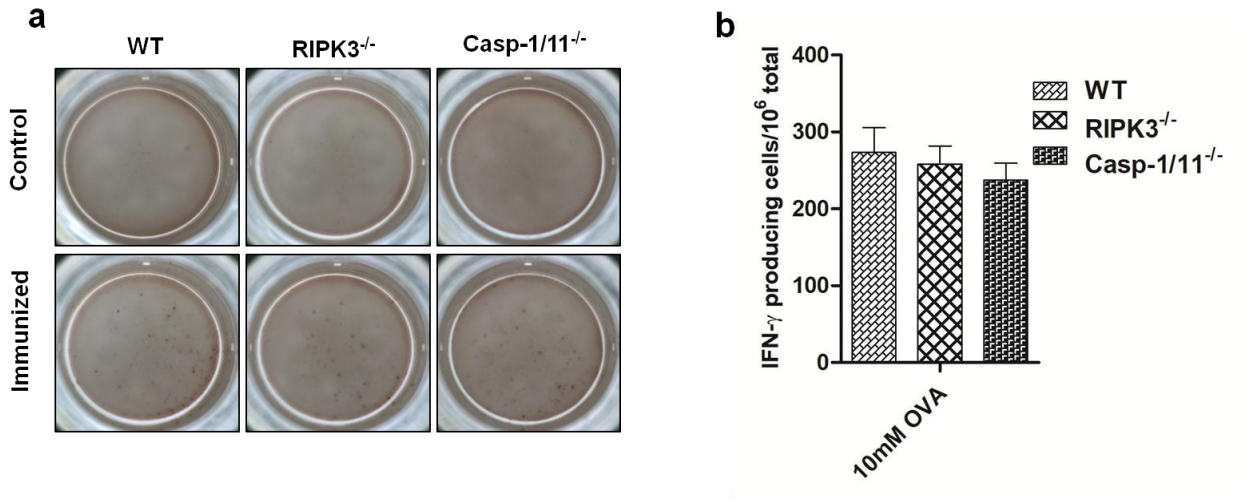

Figure 25: Antigen-specific CD8$^+$ T cell response depends on antigen-delivery vector (ELISPOT assay). (a-b) Frequency of IFN-γ producing cells: Splenocytes from WT, RIPK3$^{-/-}$ and Casp-1/11$^{-/-}$ immunized or non-immmunized mice pulsed with 10mM of OVA$_{257-264}$ peptide. Spots represent the 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 accessed 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.

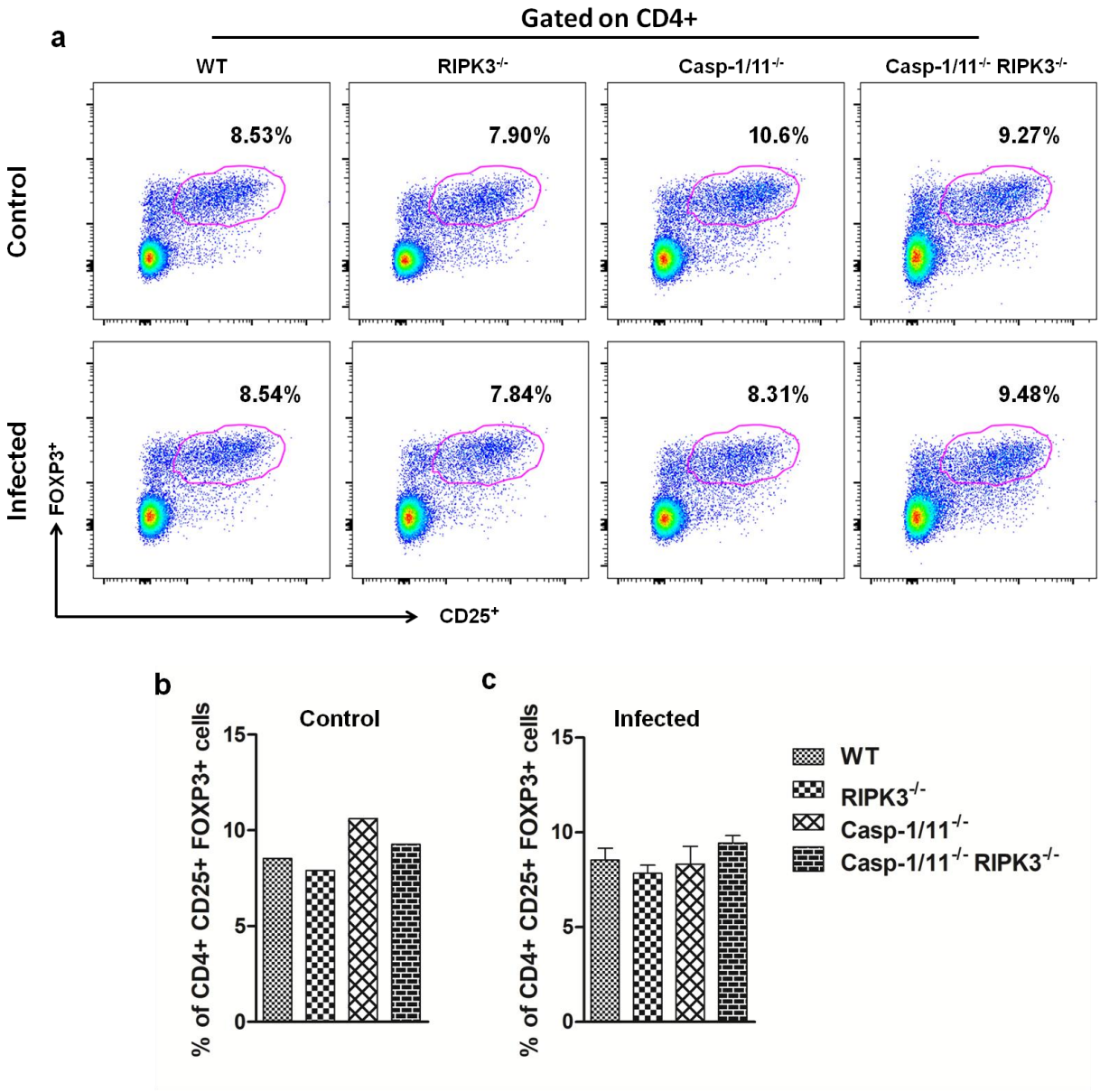

Figure 26: Regulatory T cell response is independent of RIPK3 and Casp-1/11 after 24 hours of LM-OVA infection. (a) Population of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in non-infected and infected mice. b) Percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in non infected mice. (b) Percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells after 24 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.

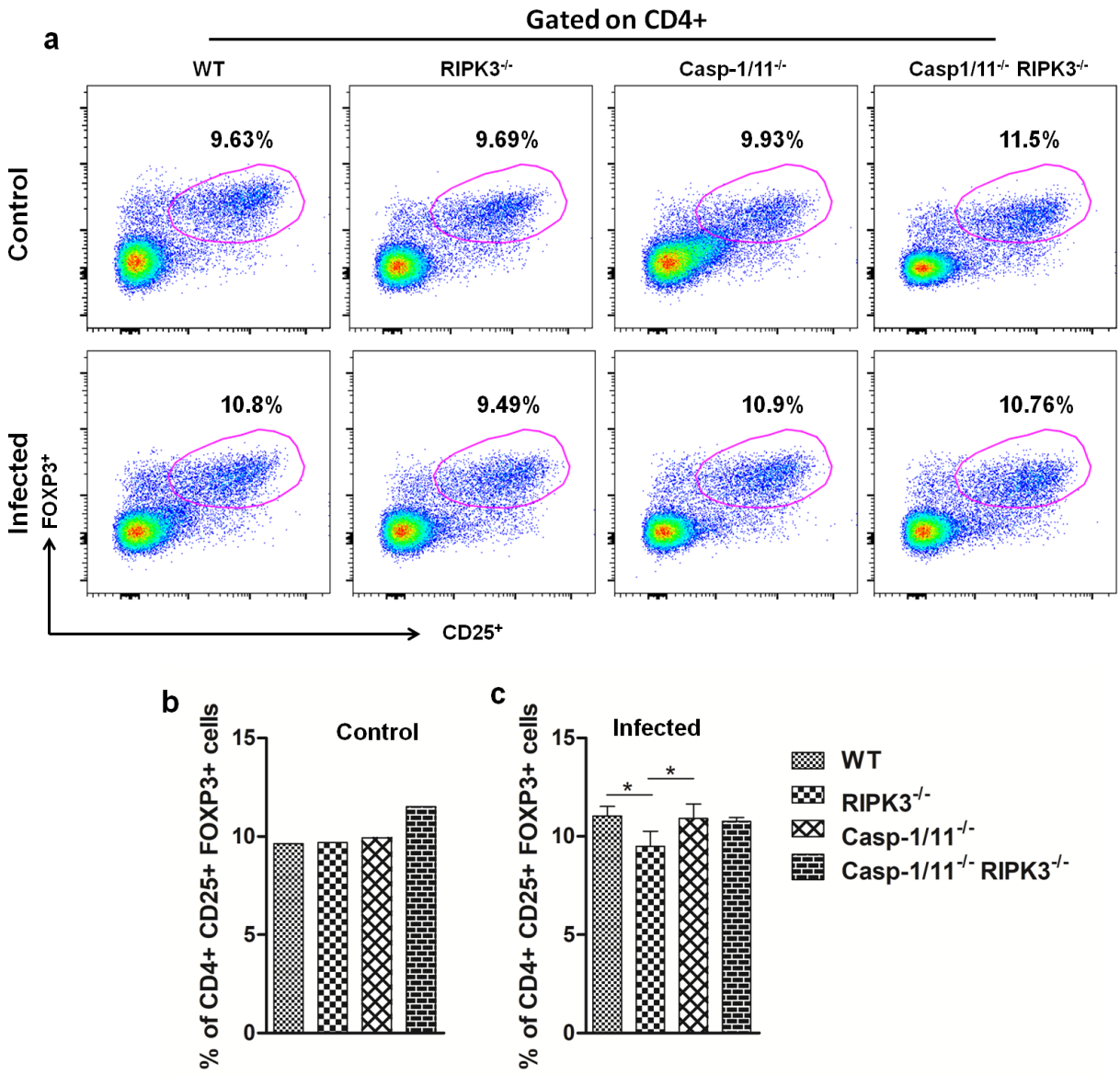

Figure 27: RIPK3 deficiency limits regulatory T cell expansion at peak of LM-OVA burden (after 72 hours of infection). (a) Population of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in non-infected and infected mice. b) Percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in non infected mice. (b) Percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells after 72 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.

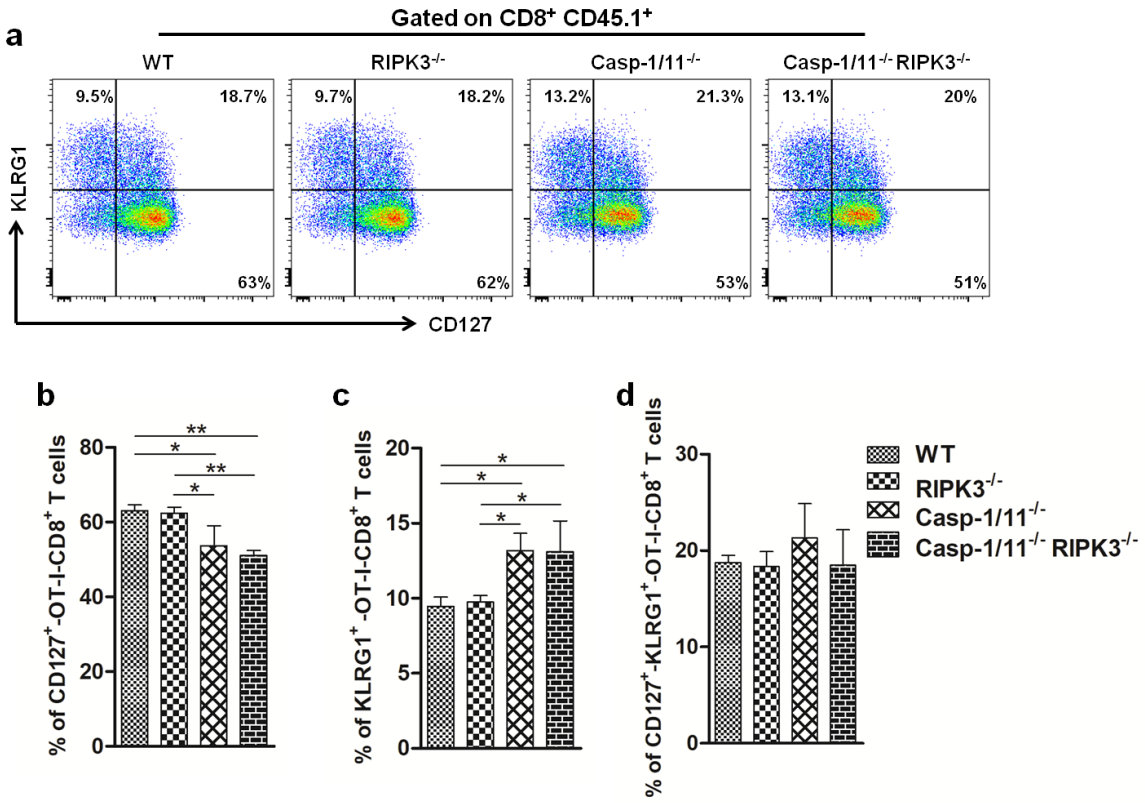

Figure 28: Casp-1/11 and Casp-1/11/RIPK-3 combined deficiencies restrict early OVA-specific CD8$^+$ T memory cells response. (a) Surface expression of CD127 and KLRG1 on adoptively transferred CD8$^+$ T cells after 7 days of infection. (b) Frequency of CD127 (c) KLRG1 (d) and CD127, KLRG1 positive OT-1 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 (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. All the experimental mice groups were challenged separately with B16 and B16.OVA. At day 3 post-tumor challenge, mice were infected or not with LM-OVA. We observed that RIPK3 and Casp-1/11 deficiencies do not affect the *in vivo* growth (Figure 31b, d) (Figure 32a) and weight (Figure 32c) of B16 by OVA-specific $CD8^+$ T cell response generated by LM-OVA. In comparison to B16, we found a significantly high growth (Figure 31c, e) (Figure 32b) and weight (Figure 32d) of B16.OVA in Casp-1/11 and Casp-1/11/RIPK3 double deficient mice (Figure 32e). Together, our results show that OVA-specific $CD8^+$ T cell response generated by LM-OVA is insufficient to control the *in vivo* growth of B16.OVA in Casp-1/11 and Casp-1/11/RIPK3 double deficient mice under our experimental conditions.

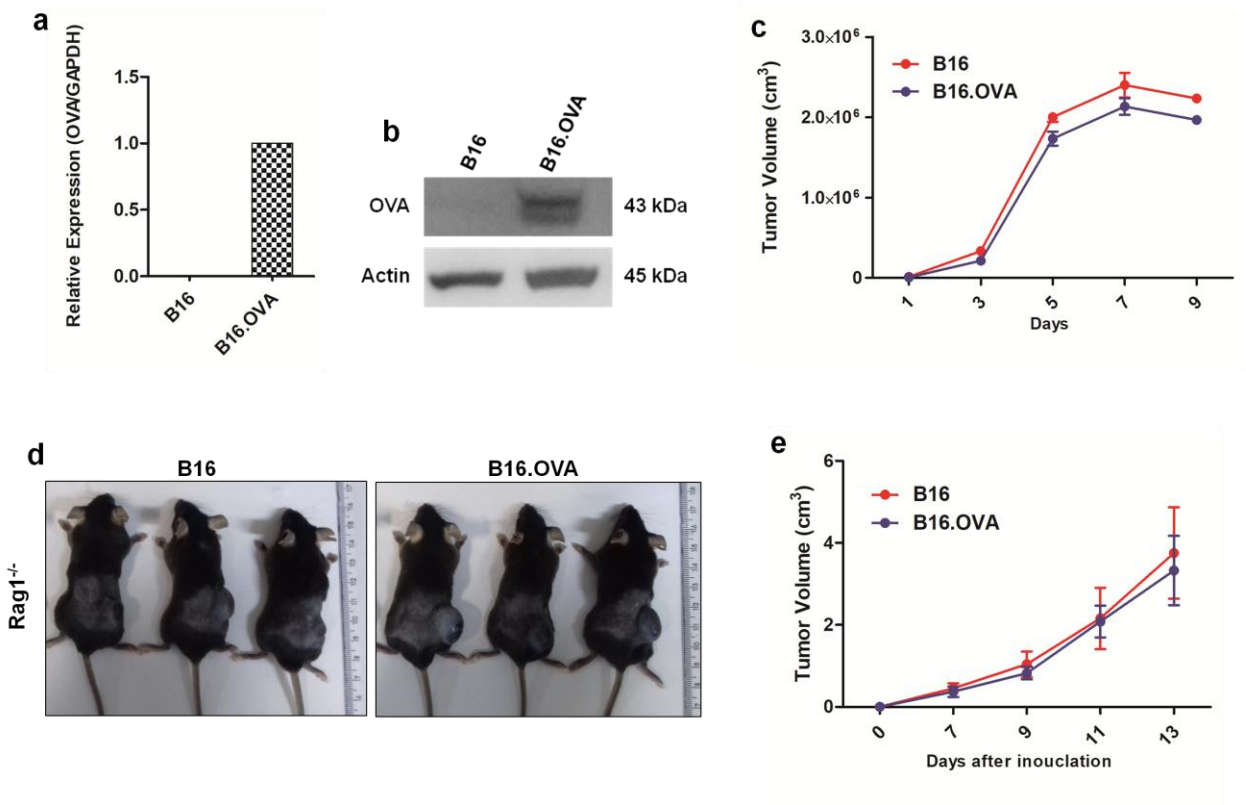

Figure 29: Ovalbumin expression does not affect B16 growth. Relative mRNA and protein expression of ovalbumin in B16.OVA: B16 was used as an experimental control (a) Relative mRNA expression of ovalbumin in B16.OVA was detected by quantitative-PCR Statistical analysis was performed by using one way ANOWA 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.

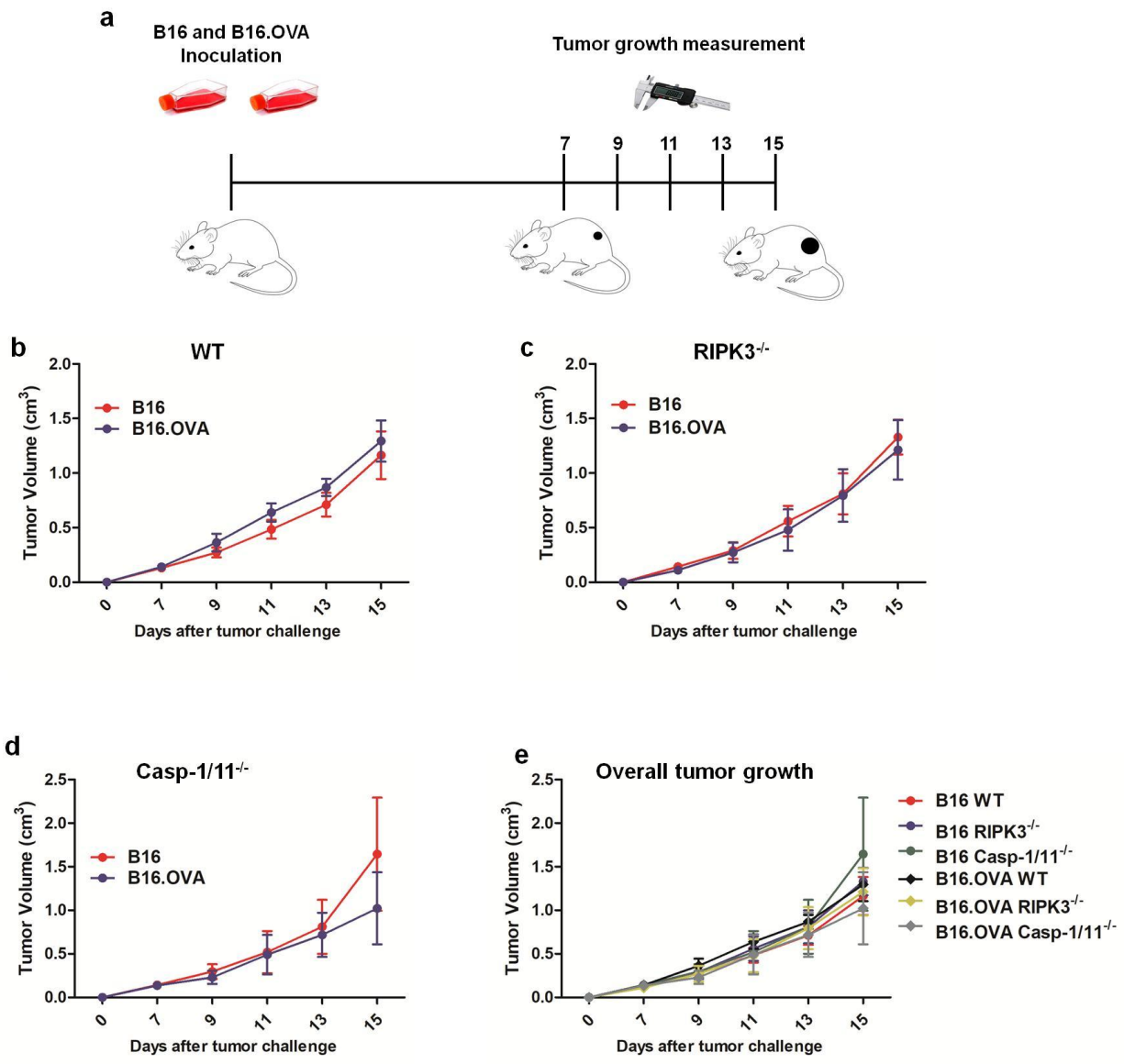

Figure 30: RIPK3 and Casp-1/11 do not regulate *in vivo* growth of B16 and B16.OVA melanoma cell lines without LM-OVA vaccination. WT, RIPK3$^{-/-}$ and Casp-1/11$^{-/-}$ mice were challenged subcutaneously with B16 and B16.OVA in the right and left (respectively) flank of each mouse. Tumor growth was 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. (d) *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.

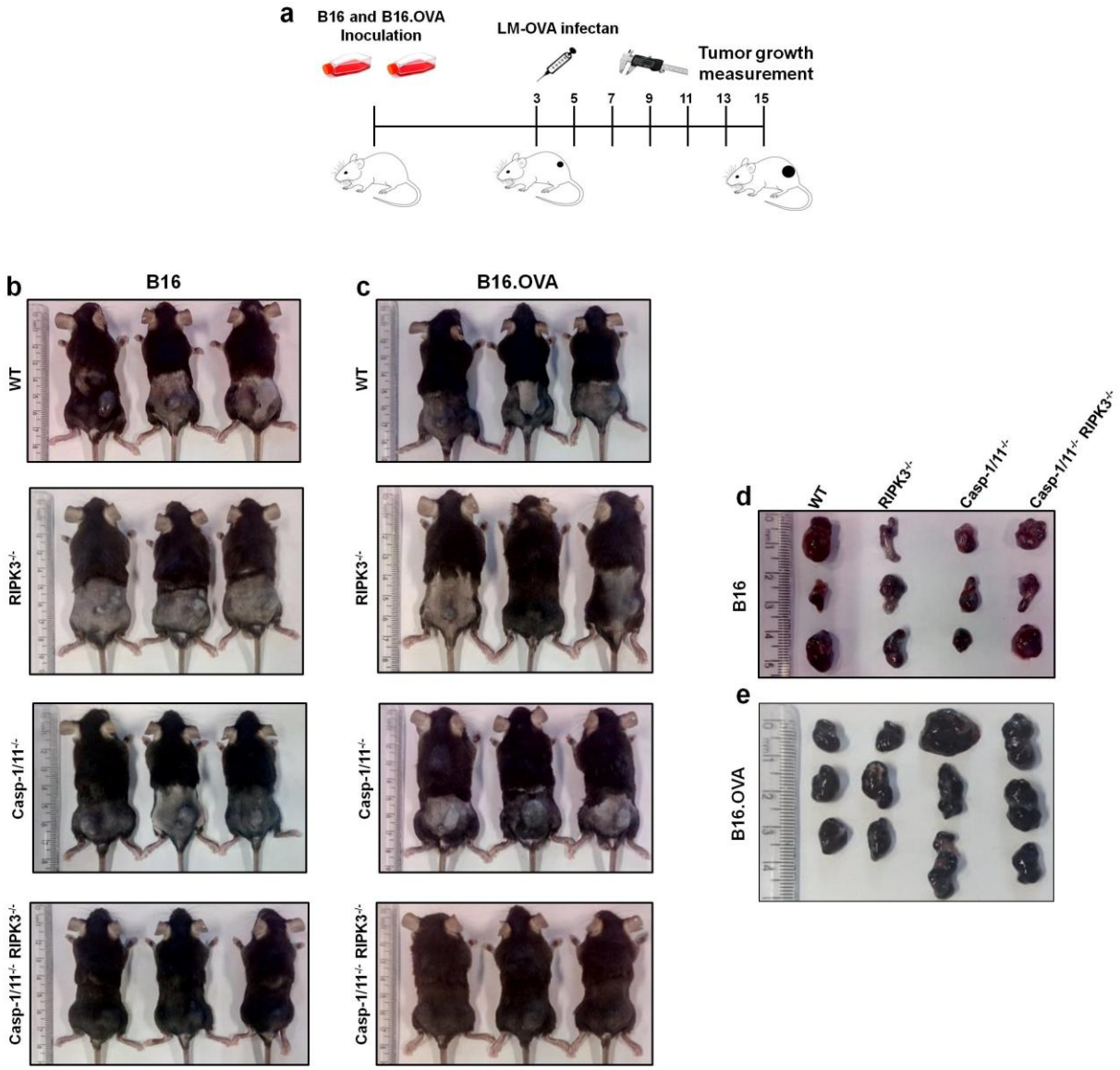

Figure 31: OVA-specific CD8^{+} T cell response generated by LM-OVA is insufficient to control tumor growth in Casp-1/11 and Casp-1/11/RIPK3 deficient mice. (a) Experimental design (b, d) *In vivo* growth of B16 in WT, RIPK3$^{-/-}$, Casp-1/11$^{-/-}$ and Casp1/11$^{-/-}$/RIPK3$^{-/-}$ mice. (c, e) *In 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.

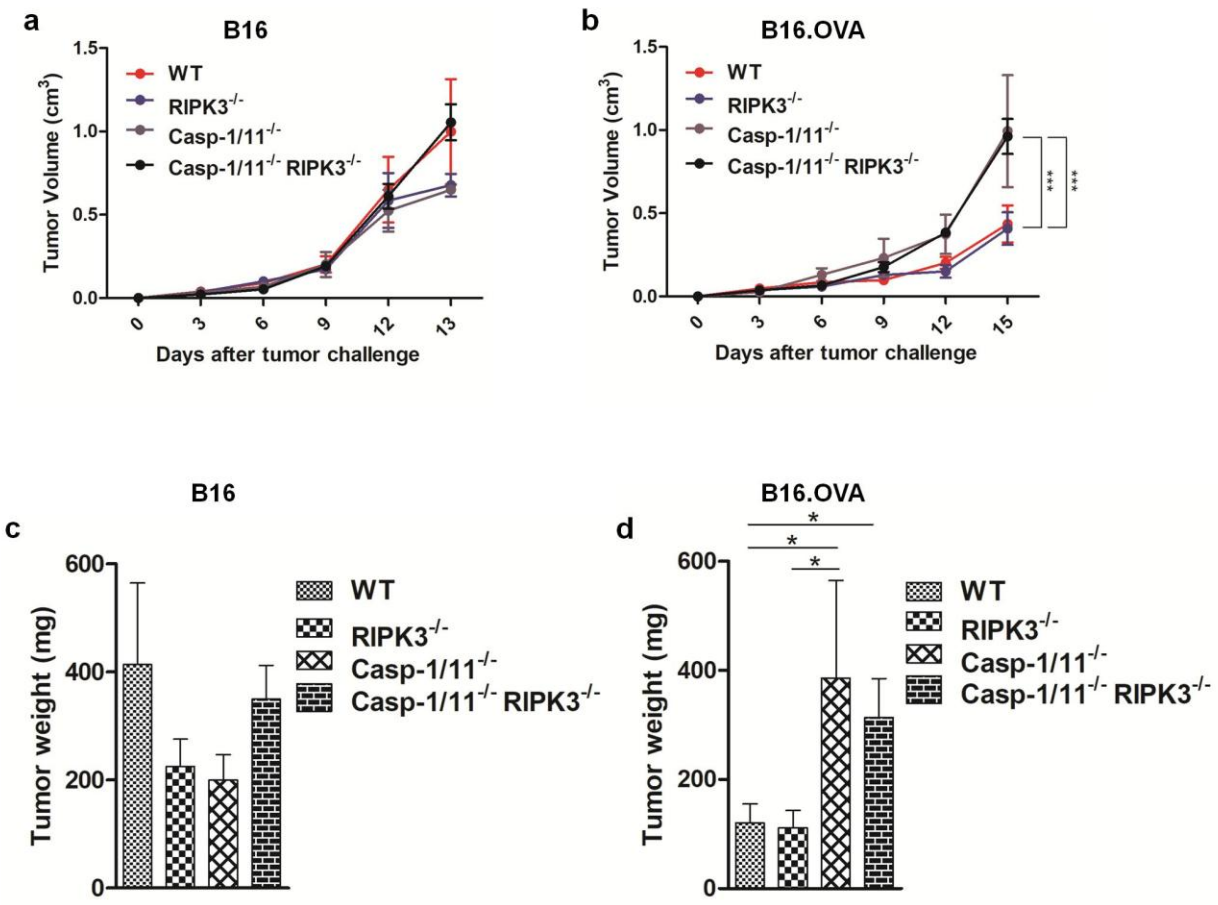

Figure 32: OVA-specific CD8$^+$ T cell response generated by LM-OVA is insufficient to control tumor growth in Casp-1/11 and Casp-1/11/RIPK3 deficient mice. (a) *In vivo* growth of B16 in WT, RIPK3$^{-/-}$, Casp-1/11$^{-/-}$ and Casp1/11$^{-/-}$/RIPK3$^{-/-}$ mice. (b) *In vivo* growth of B16.OVA in 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.(236) Among such strategies, the use of intracellular infectious bacteria carrying unique single or multiple therapeutic proteins holds promise.(237-239) *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.(145) Also, *Listeria* persists within tumors for longer time even after its clearance from spleen and liver.(146-148) 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.(240)

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.(241) 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.(242-244) 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.(203) *Listeria* induces rapid necroptotic cell death in macrophages (245,246), 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 (202). Interestingly enough, LM-induced activation of RIPK3 results in phosphorylation of MLKL without inducing necroptosis (202), suggesting a necroptosis-independent role or 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 (247,248). 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.(248) 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.(249) *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.(250) In response to *Listeria* infection $CD8^+$ T cell primary expansion peaks between 7-10 days.(251,252) Here, we established a dose-dependent response for LM-OVA and found out that 10^2 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 10^3 and 10^4 LM-OVA. Similarly, OVA-specific, IFN-γ-secreting $CD8^+$ T cells were observed only with 10^3 and 10^4 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).(252) 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.(253) 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 (254). 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.(255) $FOXP3^+$ 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.(256-258) 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 $OVA_{257–264}$ 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}.(259) 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.(260) 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.(261)

Previously, Szymczak-Workman *et al*., observed that T_{reg} cells mediated suppression of conventional T cells is independent on apoptosis and RIPK3-dependent necroptosis (262), 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.(241) 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.(263) 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).(202) 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.(264) Another group also delivered of mRNA-encoding MLKL intra-tumorally to promote necroptotic cell death and conferred protection in murine melanoma model.(265) 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.(266) 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 *lox*P, 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.(267) Anti-tumor $CD8^+$ T cell response highly depends on $CD8^+$ T cells differentiation and infiltration into the tumor microenvironment (TME).(112) 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.(268) 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.(269) 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.(240) Although, intratumoral activation necroptotic cell death (264,265) and the vaccination by recombinant *Listeria* (267) 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.(270-272) 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.(273,274) Recently, it has been reported that $CD103^+$ $CD8^+$ Trm cells protect against oral and intestinal infection of *Listeria monocytogenes*(275,276) and CD49a expressing Trm are more potent to control melanoma progression.(277) 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 phonotype 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 of LM-OVA infection.
- Casp-1/11 and Casp-1/11/RIPK3 combined deficiencies restrict antigen-specific $CD8^+$ T memory cell differentiation.
- RIPK3 and Casp-1/11 are essential for optimal $CD8^+$ T cell response to control tumor growth. Casp-1/11 and Casp-1/11/RIPK3 deficiencies restrict tumor regression by antigen-specific $CD8^+$ T cell response generated by LM-OVA vaccination.

11. REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018; 68(6):394-424.
2. National Cancer Institute, 2019, USA https://www.cancer.gov/about-cancer/understanding/statistics
3. de Oliveira Santos M. Estimativa 2018: incidência de câncer no Brasil. Revista Brasileira de Cancerologia. 2018 Mar 30;64(1):119-20.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 4;144(5):646-74.
5. Swann JB, Smyth MJ. Immune surveillance of tumors. The Journal of clinical investigation. 2007; 1;117(5):1137-46.
6. Safa AR. Resistance to cell death and its modulation in cancer stem cells. Critical Reviews™ in Oncogenesis. 2016; 21(3-4).
7. Lopez J and Tait SWG. Mitochondrial apoptosis: killing cancer using the enemy within. Br J Cancer 2015; 112: 957-962.
8. Juin P, Geneste O, Gautier F, Depil S and Campone M. Decoding and unlocking the BCL-2 dependency of cancer cells. Nat Rev Cancer 2013; 13: 455-465.
9. Green DR and Evan GI. A matter of life and death. Cancer Cell 2002; 1: 19-30.
10. Labi V and Erlacher M. How cell death shapes cancer. Cell Death Dis. 2015; 6: e1675.
11. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010; 19;140(6):883-99.
12. White E, Karp C, Strohecker AM, Guo Y, Mathew R. Role of autophagy in suppression of inflammation and cancer. Current opinion in cell biology. 2010; 1;22(2):212-7.
13. Vakkila J, Lotze MT. Inflammation and necrosis promote tumour growth. Nature Reviews Immunology. 2004; 4(8):641.
14. Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, *et al*. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer cell. 2006; 1;10(1):51-64.
15. Velho TR. Metastatic melanoma–a review of current and future drugs. Drugs in context. 2012;2012.
16. American Cancer Society, 2019, USA, http://www.cancer.org/cancer/melanoma-skin-cancer/about/what-is-melanoma.html
17. Schadendorf D, Hauschild A. Melanoma in 2013: Melanoma—the run of success continues. Nature Reviews Clinical Oncology. 2014; 14;11(2):75.
18. De Angelis R, Sant M, Coleman MP, Francisci S, Baili P, Pierannunzio D, *et al*. Cancer survival in Europe 1999–2007 by country and age: results of EUROCARE-5—a population-based study. The lancet oncology. 2014; 1;15(1):23-34.
19. Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, *et al*. International trends in the incidence of malignant melanoma 1953–2008—are recent generations at higher or lower risk?. International journal of cancer. 2013; 15;132(2):385-400.
20. American Cancer Society, 2019, USA, https://www.cancer.org/cancer/melanoma-skin-cancer/about/key-statistics.html

21. INCA - Instituto Nacional de Câncer, 2019, Brazil. https://www.inca.gov.br/tipos-de-cancer/cancer-de-pele-melanoma
22. WHO, 2019. http://www.who.int/uv/faq/skincancer/en/index1.html
23. Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, *et al*. Melanoma. Nature reviews Disease primers. 2015; 23;1:15003.
24. Chin L. The genetics of malignant melanoma: lessons from mouse and man. Nature Reviews Cancer. 2003; 3(8):559.
25. American Cancer Society, 2019, USA, https://www.cancer.net/cancer-types/melanoma/stages
26. Shain AH, Bastian BC. From melanocytes to melanomas. Nature Reviews Cancer. 2016 Jun;16(6):345.
27. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, Kiezun A. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013; 499(7457):214.
28. Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, *et al*. A landscape of driver mutations in melanoma. Cell. 2012; 20;150(2):251-63.
29. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. Science. 2013; 22;339(6122):957-9.
30. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, *et al*. TERT promoter mutations in familial and sporadic melanoma. Science. 2013; 22;339(6122):959-61.
31. Krauthammer M, Kong Y, Ha BH, Evans P, Bacchiocchi A, McCusker JP, *et al*. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nature genetics. 2012; 44(9):1006.
32. Mortarini R, Piris A, Maurichi A, Molla A, Bersani I, Bono A, *et al*. Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma. Cancer Research. 2003; 15;63(10):2535-45.
33. O'Donnell JS, Teng MW, Smyth MJ. Cancer immunoediting and resistance to T cell-based immunotherapy. Nature reviews Clinical oncology. 2018; 6:1.
34. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nature immunology. 2002; 3(11):991.
35. Fouad YA, Aanei C. Revisiting the hallmarks of cancer. American journal of cancer research. 2017; 7(5):1016.
36. Jarnicki AG, Lysaght J, Todryk S, Mills KH. Suppression of antitumor immunity by IL-10 and TGF-β-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. The journal of immunology. 2006; 15;177(2):896-904.
37. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, *et al*. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology. 2007; 1;132(7):2328-39.
38. Huang Y, Wang FM, Wang T, Wang YJ, Zhu ZY, Gao YT, *et al*. Tumor-infiltrating FoxP3+ Tregs and CD8+ T cells affect the prognosis of hepatocellular carcinoma patients. Digestion. 2012; 86(4):329-37.
39. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci. 2008 1;13(1):453-61.

40. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. Clinical Cancer Research. 2007; 15;13(2):721s-6s.
41. Hashimoto M, Kamphorst AO, Im SJ, Kissick HT, Pillai RN, Ramalingam SS, *et al*. CD8 T cell exhaustion in chronic infection and cancer: opportunities for interventions. Annual review of medicine. 2018; 29;69:301-18.
42. Kurachi M. CD8+ T cell exhaustion. InSeminars in immunopathology 2019; 1 (Vol. 41, No. 3, pp. 327-337). Springer Berlin Heidelberg.
43. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, *et al*. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity. 2007; 26;27(4):670-84.
44. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, *et al*. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nature immunology. 2009 Jan;10(1):29.
45. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, *et al*. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen–specific CD8+ T cell dysfunction in melanoma patients. Journal of Experimental Medicine. 2010 Sep 27;207(10):2175-86.
46. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, Bettini ML, Gravano DM, Vogel P, Liu CL, Tangsombatvisit S. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer research. 2012; 15;72(4):917-27.
47. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, *et al*. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001; 410:1107–11.
48. Mahmoud F, Shields B, Makhoul I, Avaritt N, Wong HK *et al*. Immune surveillance in melanoma: from immune attack to melanoma escape and even counterattack. Cancer Biology and Therapy. 2017; 3;18(7):451-69.
49. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nature Reviews Cancer. 2013; 13(10):714.
50. Couzin-Frankel, Jennifer. Cancer immunotherapy. 2013; 1432-1433.
51. Disis ML. Mechanism of action of immunotherapy. InSeminars in oncology 2014; 1 (Vol. 41, pp. S3-S13). WB Saunders.
52. Klemm F, Joyce JA. Microenvironmental regulation of therapeutic response in cancer. Trends in cell biology. 2015; 1;25(4):198-213.
53. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro-versus anti-tumor immunity. Cancer and Metastasis Reviews. 2010 Jun 1;29(2):309-16.
54. Gerritsen B, Pandit A. The memory of a killer T cell: models of CD8+ T cell differentiation. Immunology and cell biology. 2016; 94(3):236-41.
55. Zhang N, Bevan MJ. CD8+ T cells: foot soldiers of the immune system. Immunity 2011; 35: 161-168.
56. Badovinac VP, Haring JS, Harty JT. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8+ T cell response to infection. Immunity 2007; 26: 827–841.

57. Gerlach C, Rohr JC, Perié L, van Rooij N, van Heijst JW, Velds A *et al*. Heterogeneous differentiation patterns of individual CD8+ T cells. Science 2013; 340: 635–639.
58. Murphy K, Travers P and Walport M. Immunobiology: The Immune System in Health and Disease. 7th Edition. 365-367. Chapter 8-28. (2008).
59. Rousalova I, Krepela E. Granzyme B-induced apoptosis in cancer cells and its regulation. International journal of oncology. 2010; 1;37(6):1361-78.
60. Janeway C, Travers P and Walport M. Immunobiology: The Immune System in Health and Disease. 5th Edition Section 8-42. (2001).
61. Golstein P, Griffiths GM. An early history of T cell-mediated cytotoxicity. Nature Reviews Immunology. 2018; 18(8):527.
62. Peter ME, Krammer PH. The CD95 (APO-1/Fas) DISC and beyond. Cell Death & Differentiation. 2003 Jan;10(1):26.
63. Lee J, Dieckmann NM, Edgar JR, Griffiths GM, Siegel RM. Fas Ligand localizes to intraluminal vesicles within NK cell cytolytic granules and is enriched at the immune synapse. Immunity, inflammation and disease. 2018; 6(2):312-21.
64. Bennett MW, O'Connell J, O'Sullivan GC, Brady C, Roche D, Collins JK, *et al*. The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. The Journal of Immunology. 1998; 1;160(11):5669-75.
65. Andersen MH, Schrama D, thor Straten P, Becker JC. Cytotoxic T cells. Journal of Investigative Dermatology. 2006; 1;126(1):32-41.
66. Nagata S. Fas-mediated apoptosis. Adv Exp Med Biol, 1996; 406:119-124.
67. Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity. 2007; 21;27(3):393-405.
68. Williams MA, Bevan MJ. Shortening the infectious period does not alter expansion of CD8 T cells but diminishes their capacity to differentiate into memory cells. J Immunol 2004; 173: 6694–6702.
69. Joshi NS, Kaech SM. Effector CD8 T cell development: a balancing act between memory cell potential and terminal differentiation. J Immunol 2008; 180:1309-1315.
70. Hikono H, Kohlmeier JE, Takamura S, Wittmer ST, Roberts AD, Woodland DL. Activation phenotype, rather than central–or effector–memory phenotype, predicts the recall efficacy of memory CD8+ T cells. Journal of Experimental Medicine. 2007; 9;204(7):1625-36.
71. Arbonés ML, Ord DC, Ley K, Ratech H, Maynard-Curry C, Otten G, *et al*. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity. 1994; 1;1(4):247-60.
72. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, *et al*. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell. 1999; 1;99(1):23-33.
73. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999; 401(6754):708.

74. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: Function, generation, and maintenance. *Annu Rev Immunol*. 2004; 22:745-763.
75. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, *et al*. Phenotypic and functional separation of memory and effector human CD8+ T cells. Journal of Experimental Medicine. 1997; 3;186(9):1407-18.
76. Kaech SM, Hemby S, Kersh E, Ahmed R. Molecular and functional profiling of memory CD8 T cell differentiation. Cell. 2002; 111:837-851.
77. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nature immunology. 2003; 4(12):1191.
78. Schluns KS, Kieper WC, Jameson SC, Lefrançois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nature immunology. 2000; 1(5):426.
79. Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nature Reviews Immunology. 2012; 12(11):749.
80. Kaech SM, Wherry EJ. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. Immunity 2007; 27:393-405.
81. Broere F, Apasov SG, Sitkovsky MV, van Eden W. A2 T cell subsets and T cell-mediated immunity. InPrinciples of immunopharmacology 2011 (pp. 15-27). Birkhäuser Basel.
82. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. Journal of Experimental Medicine. 2008; 17;205(3):625-40.
83. Shipkova M, Wieland E. Surface markers of lymphocyte activation and markers of cell proliferation. Clinica chimica acta. 2012; 8;413(17-18):1338-49.
84. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith KA, Ahmed R. Prolonged interleukin-2Rα expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. Immunity. 2010; 29;32(1):91-103.
85. Arbonés ML, Ord DC, Ley K, Ratech H, Maynard-Curry C, Otten G, *et al*. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity. 1994; 1;1(4):247-60.
86. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E, *et al*. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell. 1999; 1;99(1):23-33.
87. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999; 401(6754):708.
88. Bachmann MF, Wolint P, Schwarz K, Jäger P, Oxenius A. Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor α and CD62L. The Journal of Immunology. 2005; 1;175(7):4686-96.
89. Masopust D, Vezys V, Marzo AL, Lefrançois L. Preferential localization of effector memory cells in nonlymphoid tissue. Science. 2001; 23;291(5512):2413-7.
90. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol.. 2004; 23;22:745-63.

91. Wherry EJ. T cell exhaustion. Nature immunology. 2011; 12(6):492.
92. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, *et al*. Viral immune evasion due to persistence of activated T cells without effector function. Journal of Experimental Medicine. 1998; 21;188(12):2205-13.
93. Wherry EJ, Blattman JN, Murali-Krishna K, Van Der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. Journal of virology. 2003; 15;77(8):4911-27.
94. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature. 1993; 362(6422):758.
95. Urbani S, Amadei B, Fisicaro P, Tola D, Orlandini A, Sacchelli L, *et al*. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. Hepatology. 2006; 44(1):126-39.
96. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, *et al*. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006; 439(7077):682.
97. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, *et al*. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proceedings of the National Academy of Sciences. 2010; 17;107(33):14733-8.
98. Crawford A, Wherry EJ. The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. Curr Opin Immunol 2009; 21: 179-186.
99. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, *et al*. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009; 10: 29–37.
100. Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C *et al*. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. Cancer Res 2012; 72:887–896.
101. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD *et al*. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol 2011; 186: 1338–1342.
102. Jiang Y, Li Y, Zhu B. T-cell exhaustion in the tumor microenvironment. Cell death & disease. 2015; 6(6):e1792.
103. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer Res 2013; 73: 3591–3603.
104. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C *et al*. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med 2010; 207: 2175–2186.
105. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ *et al*. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res 2012; 72: 917–927.
106. Chauvin JM, Pagliano O, Fourcade J, Sun Z, Wang H, Sander C *et al*. TIGIT and PD-1 impair tumor antigen-specific CD8+ T cells in melanoma patients. J Clin Invest 2015; 125: 2046–2058.

107. Lee N, Zakka LR, Mihm MC Jr, Schatton T. Tumour-infiltrating lymphocytes in melanoma prognosis and cancer immunotherapy. Pathology. 2016; 48:177-187.
108. Walliczek U, Grimm M, Rahbari NN, Koch M, Saadati M, *et al*. Tumor-specific cytotoxic T lymphocyte activity determines colorectal cancer patient prognosis. J Clin Invest. 2015 ;125:739-751.
109. Schalper KA, Brown J, Carvajal-Hausdorf D, McLaughlin J, Velcheti V, Syrigos KN, *et al*. Objective measurement and clinical significance of TILs in non–small cell lung cancer. Journal of the National Cancer Institute. 2015; 3;107(3):dju435.
110. Seo AN, Lee HJ, Kim EJ, Kim HJ, Jang MH, Lee HE, *et al*. Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. Br J Cancer. 2013;109:2705-2713.
111. Hussein MR. Tumour-infiltrating lymphocytes and melanoma tumorigenesis: an insight. British Journal of Dermatology. 2005; 153(1):18-21.
112. Nolz JC. Molecular mechanisms of CD8+ T cell trafficking and localization. Cell Mol Life Sci. 2016; 72:2461-73.
113. James KD, JenkinsonWE, Anderson G. T-cell egress from the thymus: should I stay or should I go? J Leukoc Biol 2017; 104:275-84.
114. Oelkrug C, Ramage JM. Enhancement of T cell recruitment and infiltration into tumours. Clin Exp Immunol 2014; 178:1-8.
115. James KD, JenkinsonWE, Anderson G. T-cell egress from the thymus: should I stayor should I go? J Leukoc Biol 2017;104:275-84.
116. Spranger S, Gajewski TF. Impact of oncogenic pathways on evasion of antitumour immune responses. Nature Reviews Cancer. 2018 Mar;18(3):139.
117. Borst J, Ahrends T, Bąbała N, Melief CJ, Kastenmüller W. CD4+ T cell help in cancer immunology and immunotherapy. Nature Reviews Immunology. 2018 Oct;18(10):635.
118. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adaptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science. 1986; 19;233(4770):1318-21.
119. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, *et al*. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. New England Journal of Medicine. 1988; 22;319(25):1676-80.
120. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, *et al*. IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001; 410(6832):1107.
121. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, *et al*. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science. 2002; 25;298(5594):850-4.
122. Peske JD, Thompson ED, Gemta L, Baylis RA, Fu YX, Engelhard VH. Effector lymphocyte-induced lymph node-like vasculature enables naive T-cell entry into tumours and enhanced anti-tumour immunity. Nature communications. 2015; 13;6:7114.
123. Wang T, Niu G, Kortylewski M, Burdelya L, Shain K, Zhang S, Bhattacharya R, Gabrilovich D, Heller R, Coppola D, Dalton W. Regulation of the innate and adaptive

immune responses by Stat-3 signaling in tumor cells. Nature medicine. 2004; 10(1):48.
124. Mullins DW, Sheasley SL, Ream RM, Bullock TN, Fu YX, Engelhard VH. Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. Journal of Experimental Medicine. 2003; 6;198(7):1023-34.
125. Kawakami Y, Rosenberg SA. Immunobiology of human melanoma antigens MART-1 and gp100 and their use for immuno-gene therapy. Int Rev Immunol 1997; 14:173-92.
126. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 14;299(5609):1057-61.
127. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. Nature immunology. 2003; 4(4):330.
128. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. The Journal of Immunology. 1995; 1;155(3):1151-64.
129. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, *et al*. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nature genetics. 2001; 27(1):68.
130. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nature Reviews Immunology. 2010; 10(7):490.
131. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nature immunology. 2010; 11(1):7.
132. Adeegbe DO, Nishikawa H. Natural and induced T regulatory cells in cancer. Frontiers in immunology. 2013; 11;4:190.
133. Togashi Y, Shitara K, Nishikawa H. Regulatory T cells in cancer immunosuppression-implications for anticancer therapy. Nature Reviews Clinical Oncology. 2019; 31:1.
134. Sarris M, Andersen KG, Randow F, Mayr L, Betz AG. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. Immunity. 2008; 14;28(3):402-13.
135. Getnet D, Grosso JF, Goldberg MV, Harris TJ, Yen HR, Bruno TC, *et al*. A role for the transcription factor Helios in human CD4+ CD25+ regulatory T cells. Molecular immunology. 2010; 1;47(7-8):1595-600.
136. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. Cancer research. 1999; 1;59(13):3128-33.
137. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+ CD4+ T cells: a common basis between tumor immunity and autoimmunity. The Journal of Immunology. 1999; 15;163(10):5211-8.
138. Togashi Y, Nishikawa H. Regulatory T cells: Molecular and cellular basis for immunoregulation. InEmerging Concepts Targeting Immune Checkpoints in Cancer and Autoimmunity 2017 (pp. 3-27). Springer, Cham.
139. Tada Y, Togashi Y, Kotani D, Kuwata T, Sato E, Kawazoe A, *et al*. Targeting VEGFR2 with Ramucirumab strongly impacts effector/activated regulatory T cells

and CD8+ T cells in the tumor microenvironment. Journal for immunotherapy of cancer. 2018; 6(1):106.
140. Saito T, Nishikawa H, Wada H, Nagano Y, Sugiyama D, Atarashi K, Maeda Y, Hamaguchi M, Ohkura N, Sato E, Nagase H. Two FOXP3+ CD4+ T cell subpopulations distinctly control the prognosis of colorectal cancers. Nature medicine. 2016 Jun;22(6):679.
141. Brockstedt DG, Giedlin MA, Leong ML, Bahjat KS, Gao Y, Luckett W, *et al*. Listeria-based cancer vaccines that segregate immunogenicity from toxicity. Proceedings of the National Academy of Sciences. 2004; 21;101(38):13832-7.
142. Clynes R, Takechi Y, Moroi Y, Houghton A, Ravetch JV. Fc receptors are required in passive and active immunity to melanoma. Proceedings of the National Academy of Sciences. 1998; 20;95(2):652-6.
143. Jain RK. New approaches for the treatment of cancer. Adv Drug Delivery Rev. (2001); 46:149-168.
144. Silva-Valenzuela CA, Desai PT, Molina-Quiroz RC, Pezoa D, Zhang Y. Solid tumors provide niche-specific conditions that lead to preferential growth of Salmonella. Oncotarget. 2016; 7;7(23):35169.
145. Chandra D, Jahangir A, Quispe-Tintaya W, Einstein MH, Gravekamp C. Myeloid-derived suppressor cells have a central role in attenuated Listeria monocytogenes-based immunotherapy against metastatic breast cancer in young and old mice. British journal of cancer. 2013; 108(11):2281.
146. Yong AY, Shabahang S, Timiryasova TM, Zhang Q, Beltz R, Gentschev I, Goebel W, Szalay AA. Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins. Nature biotechnology. 2004; 22(3):313.
147. Yu YA, Zhang Q, Szalay AA. Establishment and characterization of conditions required for tumor colonization by intravenously delivered bacteria. Biotechnol. Bioeng. 2008; 100(3), 567-578.
148. Huang B, Zhao J, Shen S, Li H, He KL, Shen GX, Mayer L, Unkeless J, Li D, Yuan Y, Zhang GM. Listeria monocytogenes promotes tumor growth via tumor cell toll-like receptor 2 signaling. Cancer Research. 2007; 1;67(9):4346-52.
149. Kramer MG, Masner M, Ferreira FA, Hoffman RM. Bacterial therapy of cancer: Promises, limitations, and insights for future directions. Frontiers in microbiology. 2018; 23;9:16.
150. Biron CA, Gazzinelli RT. Effects of IL-12 on immune responses to microbial infections: a key mediator in regulating disease outcome. Current opinion in immunology. 1995; 1;7(4):485-96.
151. Stavru F, Archambaud C, Cossart P. Cell biology and immunology of Listeria monocytogenes infections: novel insights. Immunological reviews. 2011; 240(1):160-84.
152. Gaillard JL, Berche P, Frehel C, Gouln E, Cossart P. Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell. 1991; 28;65(7):1127-41.
153. Gaillard JL, Berche P, Frehel C, Gouln E, Cossart P. Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell. 1991; 28;65(7):1127-41.

154. Pentecost M, Otto G, Theriot JA, Amieva MR. Listeria monocytogenes invades the epithelial junctions at sites of cell extrusion. PLoS pathogens. 2006; 27;2(1):e3.
155. Freitag NE, Rong LI, Portnoy DA. Regulation of the prfA transcriptional activator of Listeria monocytogenes: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. Infection and immunity. 1993; 1;61(6):2537-44.
156. Mengaud J, Chenevert J, Geoffroy C, Gaillard JL, Cossart P. Identification of the structural gene encoding the SH-activated hemolysin of Listeria monocytogenes: listeriolysin O is homologous to streptolysin O and pneumolysin. Infection and immunity. 1987; 1;55(12):3225-7.
157. Noor S, Goldfine H, Tucker DE, Suram S, Lenz LL, Akira S, *et al*. Activation of cytosolic phospholipase A2α in resident peritoneal macrophages by Listeria monocytogenes involves listeriolysin O and TLR2. Journal of Biological Chemistry. 2008; 22;283(8):4744-55.
158. Edelson BT, Unanue ER. MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity. The Journal of Immunology. 2002; 1;169(7):3869-75.
159. Sauer JD, Witte CE, Zemansky J, Hanson B, Lauer P, Portnoy DA. Listeria monocytogenes triggers AIM2-mediated pyroptosis upon infrequent bacteriolysis in the macrophage cytosol. Cell host & microbe. 2010; 20;7(5):412-9.
160. Hasegawa M, Yang K, Hashimoto M, Park JH, Kim YG, Fujimoto Y, *et al*. Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments. Journal of Biological Chemistry. 2006; 29;281(39):29054-63.
161. Brunt LM, Portnoy DA, Unanue ER. Presentation of Listeria monocytogenes to CD8+ T cells requires secretion of hemolysin and intracellular bacterial growth. The Journal of Immunology. 1990; 1;145(11):3540-6.
162. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, *et al*. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. Journal of Experimental Medicine. 2005; 19;202(12):1691-701.
163. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annual review of immunology. 2013 Mar 21;31:51-72.
164. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013; 25;39(1):1-0.
165. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nature Reviews Immunology. 2009; 9(5):353.
166. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, *et al*. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell death and differentiation. 2012; 19(1):107.
167. Vandenabeele P, Galluzzi L, Berghe TV, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature reviews Molecular cell biology. 2010; 11(10):700.
168. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002; 418(6894):191.

169. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010; 464(7285):104.
170. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity. 2013;21;38(2):209-23.
171. Vanlangenakker N, Berghe TV, Vandenabeele P. Many stimuli pull the necrotic trigger, an overview. Cell death and differentiation. 2012; 19(1):75-86.
172. Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, *et al*. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Molecular cell. 2008; 20;30(6):689-700.
173. Vucic D. IAP antagonists induce autoubiquitination of c-IAPs. NF-kappaB activation, and. 2007.
174. Geserick P, Hupe M, Moulin M, Wong WW, Feoktistova M, Kellert B, *et al*. Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. The Journal of cell biology. 2009; 28;187(7):1037-54.
175. Moquin DM, McQuade T, Chan FK. CYLD deubiquitinates RIP1 in the TNFα-induced necrosome to facilitate kinase activation and programmed necrosis. PloS one. 2013; 2;8(10):e76841.
176. Wertz IE, O'rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. Nature. 2004; 430(7000):694.
177. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M, *et al*. cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Molecular cell. 2011; 5;43(3):449-63.
178. Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F *et al*. The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 2011; 43: 1-18.
179. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S *et al*. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity 2013; 39: 443–453.
180. Sun L, Wang H, Wang Z, He S, Chen S, Liao D *et al*. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 2012; 148: 213–227.
181. He S, Liang Y, Shao F, Wang X. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3–mediated pathway. Proceedings of the National Academy of Sciences. 2011; 13;108(50):20054-9.
182. Kaiser WJ, Sridharan H, Huang C, Mandal P, Upton JW, Gough PJ, *et al*. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. Journal of Biological Chemistry. 2013 Oct 25;288(43):31268-79.
183. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell host & microbe. 2012; 15;11(3):290-7.

184. Chen D, Yu J, Zhang L. Necroptosis: an alternative cell death program defending against cancer. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer. 2016; 1;1865(2):228-36.
185. Lalaoui N, Brumatti G. Relevance of necroptosis in cancer. Immunology and cell biology. 2017; 95(2):137-45.
186. Aaes TL, Kaczmarek A, Delvaeye T, De Craene B, De Koker S, Heyndrickx L *et al*. Vaccination with necroptotic cancer cells induces efficient anti-tumor immunity. Cell Rep 2016; 15: 274–287.
187. Kang YJ, Bang BR, Han KH, Hong L, Shim EJ, Ma J, *et al*. Regulation of NKT cell-mediated immune responses to tumours and liver inflammation by mitochondrial PGAM5-Drp1 signalling. Nature communications. 2015; 18;6:8371.
188. Thapa RJ, Ingram JP, Ragan KB, Nogusa S, Boyd DF, Benitez AA, *et al*. DAI senses influenza A virus genomic RNA and activates RIPK3-dependent cell death. Cell host & microbe. 2016; 9;20(5):674-81.
189. Cho Y, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell. 2009; 12;137(6):1112-23.
190. Nogusa S, Thapa RJ, Dillon CP, Liedmann S, Oguin III TH, Ingram JP, *et al*. RIPK3 activates parallel pathways of MLKL-driven necroptosis and FADD-mediated apoptosis to protect against influenza A virus. Cell host & microbe. 2016; 13;20(1):13-24.
191. Lu JV, Weist BM, van Raam BJ, Marro BS, Nguyen LV, Srinivas P, Bell BD, Luhrs KA, Lane TE, Salvesen GS, Walsh CM. Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity. Proceedings of the National Academy of Sciences. 2011; 13;108(37):15312-7.
192. Upton JW, Kaiser WJ, Mocarski ES. Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. Journal of Biological Chemistry. 2008; 20;283(25):16966-70.
193. Omoto S, Guo H, Talekar GR, Roback L, Kaiser WJ, Mocarski ES. Suppression of RIP3-dependent necroptosis by human cytomegalovirus. Journal of biological chemistry. 2015; 1;290(18):11635-48.
194. Wang X, Li Y, Liu S, Yu X, Li L, Shi C, *et al*. Direct activation of RIP3/MLKL-dependent necrosis by herpes simplex virus 1 (HSV-1) protein ICP6 triggers host antiviral defense. Proceedings of the National Academy of Sciences. 2014 Oct 28;111(43):15438-43.
195. Weng D, Marty-Roix R, Ganesan S, Proulx MK, Vladimer GI, Kaiser WJ, *et al*. Caspase-8 and RIP kinases regulate bacteria-induced innate immune responses and cell death. Proceedings of the National Academy of Sciences. 2014; 20;111(20):7391-6.
196. Kitur K, Wachtel S, Brown A, Wickersham M, Paulino F, Peñaloza HF, *et al*. Necroptosis promotes Staphylococcus aureus clearance by inhibiting excessive inflammatory signaling. Cell reports. 2016; 23;16(8):2219-30.

197. Kitur K, Parker D, Nieto P, Ahn DS, Cohen TS, Chung S. *et al*. Toxin-induced necroptosis is a major mechanism of Staphylococcus aureus lung damage. PLoS pathogens. 2015 Apr 16;11(4):e1004820.
198. González-Juarbe N, Gilley RP, Hinojosa CA, Bradley KM, Kamei A, Gao G, *et al*. Pore-forming toxins induce macrophage necroptosis during acute bacterial pneumonia. PLoS pathogens. 2015; 11;11(12):e1005337.
199. Robinson N, McComb S, Mulligan R, Dudani R, Krishnan L, Sad S. Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. Nature immunology. 2012; 13(10):954.
200. Autheman D, Wyder M, Popoff M, D'herde K, Christen S, Posthaus H. Clostridium perfringens beta-toxin induces necrostatin-inhibitable, calpain-dependent necrosis in primary porcine endothelial cells. PloS one. 2013; 29;8(5):e64644.
201. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell. 2013; 25;153(3):521-34.
202. Sai K, Parsons C, House JS, Kathariou S, Ninomiya-Tsuji J. Necroptosis mediators RIPK3 and MLKL suppress intracellular Listeria replication independently of host cell killing. The Journal of cell biology. 2019; 3;218(6):1994-2005.
203. Zhang T, Balachandran S. Bayonets over bombs: RIPK3 and MLKL restrict Listeria without triggering necroptosis. The Journal of cell biology. 2019; 3;218(6):1773-5.
204. Xia X, Wang X, Zheng Y, Jiang J, Hu J. What role does pyroptosis play in microbial infection?. Journal of cellular physiology. 2019; 234(6):7885-92.
205. Mathan MM, Mathan VI. Morphology of rectal mucosa of patients with shigellosis. Reviews of infectious diseases. 1991; 1;13(Supplement_4):S314-8.
206. Zychlinsky A, Prevost MC, Sansonetti PJ. Shigella flexneri induces apoptosis in infected macrophages. Nature. 1992; 358(6382):167.
207. Chen Y, Smith MR, Thirumalai K, Zychlinsky A. A bacterial invasin induces macrophage apoptosis by binding directly to ICE. The EMBO journal. 1996 Aug 1;15(15):3853-60.
208. Hilbi HU, Chen YA, Thirumalai KA, Zychlinsky AR. The interleukin 1beta-converting enzyme, caspase 1, is activated during Shigella flexneri-induced apoptosis in human monocyte-derived macrophages. Infection and immunity. 1997; 1;65(12):5165-70.
209. Hilbi H, Moss JE, Hersh D, Chen Y, Arondel J, Banerjee S, *et al*. Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. Journal of Biological Chemistry. 1998; 4;273(49):32895-900.
210. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, Sarkar A, *et al*. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. Nature immunology. 2010 Dec;11(12):1136.
211. Danelishvili L, Bermudez LE. Analysis of pyroptosis in bacterial infection. InNecrosis 2013 (pp. 67-73). Humana Press, Totowa, NJ.
212. Cookson BT, Brennan MA. Pro-inflammatory programmed cell death. Trends in microbiology. 2001; 1;9(3):113-4.
213. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, *et al*. Pore-forming activity and structural autoinhibition of the gasdermin family. Nature. 2016; 535(7610):111.

214. Shi J, Gao W, Shao F. Pyroptosis: gasdermin-mediated programmed necrotic cell death. Trends in biochemical sciences. 2017; 1;42(4):245-54.
215. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, *et al*. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature. 2015; 526(7575):660.
216. Jorgensen I, Miao EA. Pyroptotic cell death defends against intracellular pathogens. Immunological reviews. 2015; 265(1):130-42.
217. Wen H, Miao EA, Ting JP. Mechanisms of NOD-like receptor-associated inflammasome activation. Immunity. 2013; 19;39(3):432-41.
218. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, *et al*. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nature immunology. 2010; 11(5):395.
219. Jakobsen MR, Bak RO, Andersen A, Berg RK, Jensen SB, Jin T, Laustsen A, Hansen K, Østergaard L, Fitzgerald KA, Xiao TS. IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. Proceedings of the National Academy of Sciences. 2013; 26;110(48):E4571-80.
220. Xu H, Yang J, Gao W, Li L, Li P, Zhang L, Gong YN, Peng X, Xi JJ, Chen S, Wang F. Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. Nature. 2014; 513(7517):237.
221. Zhao Y, Yang J, Shi J, Gong YN, Lu Q, Xu H, *et al*. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature. 2011; 477(7366):596.
222. Kayagaki N, Warming S, Lamkanfi M, Walle LV, Louie S, Dong J, *et al*. Non-canonical inflammasome activation targets caspase-11. Nature. 2011; 479(7371):117.
223. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science. 2013; 13;341(6151):1250-3.
224. Aglietti RA, Estevez A, Gupta A, Ramirez MG, Liu PS, Kayagaki N, et al. GsdmD p30 elicited by caspase-11 during pyroptosis forms pores in membranes. Proceedings of the National Academy of Sciences. 2016; 12;113(28):7858-63.
225. Liu X, Zhang Z, Ruan J, Pan Y, Magupalli VG, Wu H, *et al*. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature. 2016; 535(7610):153.
226. Wang J, Sahoo M, Lantier L, Warawa J, Cordero H, Deobald K, *et al*. Caspase-11-dependent pyroptosis of lung epithelial cells protects from melioidosis while caspase-1 mediates macrophage pyroptosis and production of IL-18. PLoS pathogens. 2018; 23;14(5):e1007105.
227. Kip E, Naze F, Suin V, Berghe TV, Francart A, Lamoral S, *et al*. Impact of caspase-1/11,-3,-7, or IL-1β/IL-18 deficiency on rabies virus-induced macrophage cell death and onset of disease. Cell death discovery. 2017; 6;3:17012.
228. Regan T, Nally K, Carmody R, Houston A, Shanahan F, MacSharry J, *et al*. Identification of TLR10 as a key mediator of the inflammatory response to Listeria monocytogenes in intestinal epithelial cells and macrophages. The Journal of Immunology. 2013; 15;191(12):6084-92.
229. Broz P. Recognition of Intracellular Bacteria by Inflammasomes. Microbiology spectrum. 2019; 7(2).

230. Kim S, Bauernfeind F, Ablasser A, Hartmann G, Fitzgerald KA, Latz E, Hornung V. Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome. European journal of immunology. 2010 Jun;40(6):1545-51.
231. Wu J, Fernandes-Alnemri T, Alnemri ES. Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. Journal of clinical immunology. 2010 Sep 1;30(5):693-702.
232. Williams MA, Schmidt RL, Lenz LL. Early events regulating immunity and pathogenesis during Listeria monocytogenes infection. Trends in immunology. 2012 Oct 1;33(10):488-95.
233. Jorgensen I, Rayamajhi M, Miao EA. Programmed cell death as a defence against infection. Nature reviews immunology. 2017 Mar;17(3):151.
234. Clemente T, Dominguez MR, Vieira NJ, Rodrigues MM, Amarante-Mendes GP. In vivo assessment of specific cytotoxic T lymphocyte killing. Methods 2013; 61:105-109.
235. Boscardin SB, Kinoshita SS, Fujimura AE, Rodrigues MM. Immunization with cDNA expressed by amastigotes of Trypanosoma cruzi elicits protective immune response against experimental infection. Infection and immunity. 2003; 1;71(5):2744-57.
236. Comber JD, Philip R. MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines. Ther Adv Vaccines 2014; 2: 77-89.
237. Wing EJ, Gregory SH. From hot dogs to CD8+ T cells: Listeria monocytogenes. Transactions of the American Clinical and Climatological Association. 2000;111:76.
238. Darji A, zur Lage S, Garbe AI, Chakraborty T, Weiss S. Oral delivery of DNA vaccines using attenuated Salmonella typhimurium as carrier. FEMS Immunology & Medical Microbiology. 2000; 1;27(4):341-9.
239. Pamer EG, Sijts AJ, Villanueva MS, Busch DH, Vijh S. MHC class I antigen processing of Listeria monocytogenes proteins: implications for dominant and subdominant CTL responses. Immunological reviews. 1997; 158(1):129-36.
240. Deng W, Lira V, Hudson TE, Lemmens EE, Hanson WG, Flores R, *et al*. Recombinant Listeria promotes tumor rejection by CD8+ T cell-dependent remodeling of the tumor microenvironment. Proceedings of the National Academy of Sciences. 2018 Aug 7;115(32):8179-84.
241. Stark FC, Sad S, Krishnan L. Intracellular bacterial vectors that induce CD8+ T cells with similar cytolytic abilities but disparate memory phenotypes provide contrasting tumor protection. Cancer Res. 2009; 69: 4327-4334.
242. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. Biochemical and biophysical research communications. 2009; 30;388(4):621-5.
243. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature Reviews Immunology. 2010; 10(2):131.
244. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature. 2006; 442(7098):39.
245. Blériot C, Dupuis T, Jouvion G, Eberl G, Disson O, Lecuit M. Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. Immunity. 2015; 20;42(1):145-58.

246. González-Juarbe N, Gilley RP, Hinojosa CA, Bradley KM, Kamei A, Gao G, Dube PH, Bergman MA, Orihuela CJ. Pore-forming toxins induce macrophage necroptosis during acute bacterial pneumonia. PLoS pathogens. 2015; 11;11(12):e1005337.
247. Warren SE, Duong H, Mao DP, Armstrong A, Rajan J, Miao EA, *et al*. Generation of a Listeria vaccine strain by enhanced caspase-1 activation. *Eur J Immunol* 2011; 41: 1934-1940.
248. Tsuji NM, Tsutsui H, Seki E, Kuida K, Okamura H, Nakanishi K, *et al*. Roles of caspase-1 in Listeria infection in mice. *Int Immunol* 2004; 16: 335-343.
249. Wherry EJ, Puorro KA, Porgador A, Eisenlohr LC. The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. The Journal of Immunology. 1999; 1;163(7):3735-45.
250. Mercado R, Vijh S, Allen SE, Kerksiek K, Pilip IM, Pamer EG. Early programming of T cell populations responding to bacterial infection. J Immunol. 2000; 165:6833-6839.
251. Porter BB, Harty JT. The onset of CD8+-T-cell contraction is influenced by the peak of Listeria monocytogenes infection and antigen display. Infection and immunity. 2006; 1;74(3):1528-36.
252. Pearce VQ, Bouabe H, MacQueen AR, Carbonaro V, Okkenhaug K. PI3Kδ regulates the magnitude of CD8+ T cell responses after challenge with Listeria monocytogenes. The Journal of Immunology. 2015; 1;195(7):3206-17.
253. Kolb-Mäurer A, Gentschev I, Fries HW, Fiedler F, Bröcker EB, Kämpgen E, *et al*. Listeria monocytogenes-infected human dendritic cells: uptake and host cell response. Infect and Immun 2000; 68 :3680-3688.
254. Patel R, Kim K, Shutinoski B, Wachholz K, Krishnan L, Sad S. Culling of APCs by inflammatory cell death pathways restricts TIM3 and PD-1 expression and promotes the survival of primed CD8 T cells. *Cell Death Differ* 2017; **24**: 1900-1911.
255. Mohammadnia-Afrouzi M, Shahbazi M, Damavandi SB, Ganji GF, Ebrahimpour S. Regulatory T-cell: regulator of host defense in infection. Journal of Molecular Biology Research. 2017; 1;7(1):9.
256. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA. *et al*. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nature genetics. 2001; 27(1):68.
257. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+ CD25+ T regulatory cells. Nature immunology. 2003; 4(4):337.
258. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. Nature immunology. 2003 Apr;4(4):330.
259. Ertelt JM, Rowe JH, Mysz MA, Singh C, Roychowdhury M, Aguilera MN, *et al*. Foxp3+ regulatory T cells impede the priming of protective CD8+ T cells. The Journal of Immunology. 2011; 1;187(5):2569-77.
260. Ertelt JM, Rowe JH, Johanns TM, Lai JC, McLachlan JB, Way SS. Selective priming and expansion of antigen-specific Foxp3− CD4+ T cells during Listeria monocytogenes infection. The Journal of Immunology. 2009; 1;182(5):3032-8.
261. Kursar M, Bonhagen K, Fensterle J, Köhler A, Hurwitz R, Kamradt T, *et al*. Regulatory CD4+ CD25+ T cells restrict memory CD8+ T cell responses. Journal of Experimental Medicine. 2002; 16;196(12):1585-92.

262. Szymczak-Workman AL, Delgoffe GM, Green DR, Vignali DA. Cutting edge: regulatory T cells do not mediate suppression via programmed cell death pathways. The Journal of Immunology. 201; 1;187(9):4416-20.
263. Daniels BP, Snyder AG, Olsen TM, Orozco S, Oguin III TH, Tait SW, *et al*. RIPK3 restricts viral pathogenesis via cell death-independent neuroinflammation. Cell. 2017 Apr 6;169(2):301-13
264. Snyder AG, Hubbard NW, Messmer MN, Kofman SB, Hagan CE, Orozco SL, *et al*. Intratumoral activation of the necroptotic pathway components RIPK1 and RIPK3 potentiates antitumor immunity. *Sci Immunol*. 2019; 21;4(36):eaaw2004.
265. Van Hoecke L, Van Lint S, Roose K, Van Parys A, Vandenabeele P, Grooten J, Tavernier J, De Koker S, Saelens X. Treatment with mRNA coding for the necroptosis mediator MLKL induces antitumor immunity directed against neo-epitopes. Nature communications. 2018; 24;9(1):3417.
266. Mackaness GB. The immunological basis of acquired cellular resistance. Journal of Experimental Medicine. 1964; 1;120(1):105-20.
267. Hanson WG, Benanti EL, Lemmens EE, Liu W, Skoble J, Leong ML, *et al*. A potent and effective suicidal Listeria vaccine platform. Infect Immun. 2019; 87:e00144-19.
268. Poggi A, Zocchi MR. Mechanisms of tumor escape: role of tumor microenvironment in inducing apoptosis of cytolytic effector cells. Arch Immunol Ther Exp (Warsz). 2006; 1;54(5):323-33.
269. Jarnicki AG, Lysaght J, Todryk S, Mills KH. Suppression of antitumor immunity by IL-10 and TGF-β-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. *J Immunol*. 2006; 15;177(2):896-904.
270. Goff SL, Dudley ME, Citrin DE, Somerville RP, Wunderlich JR, Danforth DN, *et al*. Randomized, prospective evaluation comparing intensity of lymphodepletion before adaptive transfer of tumor-infiltrating lymphocytes for patients with metastatic melanoma. J Clin Oncol. 2016; 34:2389-2397.
271. Lee N, Zakka LR, Mihm MC Jr., Schatton T. Tumour-infiltrating lymphocytes in melanoma prognosis and cancer immunotherapy. Pathology. 2016; 48:177–187.
272. Schatton T, Scolyer RA, Thompson JF, Mihm MC Jr. Tumorinfiltrating lymphocytes and their significance in melanomaprognosis. Methods Mol Biol. 2014;1102:287-32.
273. Jameson SC, Masopust D. Understanding subset diversity in T cell memory. Immunity. 2018; 20;48(2):214-26.
274. Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol. 2016; 16(2):79.
275. Sheridan BS, Pham QM, Lee YT, Cauley LS, Puddington L, Lefrançois L. Oral infection drives a distinct population of intestinal resident memory CD8+ T cells with enhanced protective function. Immunity. 2014; 15;40(5):747-57.
276. Romagnoli PA, Fu HH, Qiu Z, Khairallah C, Pham QM, Puddington L, *et al*. Differentiation of distinct long-lived memory CD4 T cells in intestinal tissues after oral Listeria monocytogenes infection. Mucosal Immunol. 2017; 10(2):520.
277. Murray T, Fuertes Marraco SA, Baumgaertner P, Bordry N, Cagnon L, Donda A, *et al*. Very late antigen-1 marks functional tumor-resident CD8 T cells and correlates with survival of melanoma patients. Frontiers in immunology. 2016; 12;7:573.