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MOLECULAR DETERMINANTS GOVERNING CD8+ T CELL MEDIATED ANTI-TUMORIMMUNITY

Martinez-Usatorre Amaia

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Faculté de biologie
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par

Amaia Martínez-Usatorre

Master de l'Université de Lausanne

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Prof. Pascal Bovet, Président
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pour le Doyen
de la Faculté de biologie et de médecine

Prof. Pascal Bovet

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RÉSUMÉ LARGE PUBLIC

Les lymphocytes T $CD8^+$ sont des cellules du système immunitaire qui reconnaissent et tuent les cellules infectées ou transformées. Cette reconnaissance se produit par l'interaction entre le récepteur des lymphocytes T (TCR) exprimé par la cellule T $CD8^+$ et un peptide d'origine virale ou tumorale chargé sur les molécules de classe I du complexe majeur d'histocompatibilité (pMHC) sur la cellule cible. Malgré la présence de cellules T $CD8^+$ spécifiques contre la tumeur, chez de nombreux patients cancéreux, les cellules cancéreuses parviennent tout de même à échapper à leur attaque menant à la progression du cancer. Ceci est dû à deux raisons principales: 1) la force de l'interaction généralement faible entre les TCR spécifiques aux tumeurs et les molécules pMHC tumoraux et 2) le microenvironnement tumoral immunosuppresseur. L'interaction entre le récepteur inhibiteur PD-1 sur les cellules T $CD8^+$ et son ligand PD-L1 sur les cellules cancéreuses et les cellules myéloïdes est l'un des nombreux facteurs qui suppriment les réponses des cellules T $CD8^+$ dans la tumeur.

Dans le cadre de ma thèse de doctorat, j'ai étudié l'impact de la force d'interaction TCR-pMHC sur: 1) la différenciation des cellules T $CD8^+$ et la réponse anti-tumorale, 2) la réponse au blocage PD-1 médié par des anticorps et 3) l'expression du microRNA-155 (miR-155), qui régule l'expression de différentes protéines et s'est avéré précédemment important pour les réponses des lymphocytes T $CD8^+$.

En utilisant des tumeurs de mélanome de souris B16 exprimant un antigène tumoral d'affinité élevée ou faible, nous avons montré que, en effet, la force d'interaction TCR-pMHC influence fortement le résultat de la réponse anti-tumorale des lymphocytes T $CD8^+$. La vaccination avec un peptide qui interagit fortement avec le TCR spécifique de la tumeur est nécessaire pour permettre une activation et une prolifération efficaces des lymphocytes T $CD8^+$ dans la périphérie et contrôler la croissance des tumeurs exprimant un peptide faiblement reconnu par le TCR spécifique de la tumeur. Fait important, le contrôle de ces tumeurs de faible affinité peut être encore amélioré avec le traitement par l'anticorps anti-PD-1.

Nous avons également remarqué que la force d'interaction TCR-pMHC et la quantité de molécules pMHC reconnue déterminent les niveaux d'expression de miR-155 dans les lymphocytes T $CD8^+$ humains et de souris. Fait intéressant, nous avons observé une corrélation négative entre les niveaux d'expression de miR-155 dans les cellules T $CD8^+$ infiltrant la tumeur et le volume des tumeurs exprimant un peptide de faible affinité. Dans la même lignée, nous avons mis en évidence une corrélation positive entre les niveaux d'expression de miR-155 dans les cellules T $CD8^+$ effectrices des ganglions lymphatiques infiltrés par cellules tumorales (TILNs) et les fréquences de ces cellules T $CD8^+$ effectrices dans les TILN, ce qui est positivement corrélé avec la survie globale des patients. Ainsi, nous proposons que les niveaux d'expression de miR-155 reflètent la réactivité et l'affinité pour le peptide tumoral et peuvent être utilisés comme marqueur de la qualité fonctionnelle des cellules T $CD8^+$ des tumeurs.

RÉSUMÉ

Malgré la capacité des cellules T $CD8^+$ à reconnaître et tuer les cellules cancéreuses, les tumeurs parviennent à échapper au contrôle immunitaire par des mécanismes multiples. Les stratégies immunothérapeutiques ont montré des avantages cliniques chez certains types de cancer, mais pas chez tous les patients. Par conséquent, une meilleure compréhension des paramètres clés régissant la relation complexe entre les cellules T $CD8^+$ et les tumeurs est nécessaire pour assurer le succès de l'immunothérapie chez la plupart des patients du cancer.

La réponse des cellules T anti-tumorales implique principalement les lymphocytes T $CD8^+$ avec une haute affinité pour les antigènes du soi mutés et une faible affinité pour les antigènes associés aux tumeurs. Par conséquent, la première partie de mon projet de thèse a porté sur l'impact de la force de reconnaissance entre le TCR et son antigène dans les réponses anti-tumorales induites par les lymphocytes T $CD8^+$. Nous avons remarqué que l'activation périphérique des lymphocytes T $CD8^+$ avec un vaccin peptidique de haute affinité était nécessaire pour contrôler la croissance des tumeurs de mélanome B16 exprimant un antigène de faible affinité. En outre, malgré une activation périphérique de haute affinité, l'affinité que les lymphocytes T $CD8^+$ ont pour l'antigène qu'ils rencontrent dans la tumeur influe sur la qualité de leur réponse : la reconnaissance d'un antigène de faible affinité conduit à une faible expansion des lymphocytes T $CD8^+$ et à un contrôle réduit de la croissance tumorale, mais induit une expression plus faible de récepteurs inhibiteurs et une meilleure production de cytokines lors d'une restimulation *ex vivo*. De manière intéressante, les cellules T $CD8^+$ infiltrant la tumeur répondaient également au traitement par l'anti-PD-1 indépendamment de leur affinité pour l'antigène tumoral. De plus, lorsque les lymphocytes T $CD8^+$ infiltrés dans les tumeurs ont été retransférés dans des hôtes sans tumeur, ils ont démontré une capacité de ré-expansion *in vivo* similaire à celle des lymphocytes T $CD8^+$ isolés des organes lymphoïdes secondaires, ce qui suggère que la fonction des lymphocytes T $CD8^+$ peut être restaurée lorsque l'immunosuppression induite de la tumeur est absente.

Notre groupe avait précédemment démontré l'importance du micro-ARN-155 (miR-155) lors des réponses anti-tumorales des cellules T $CD8^+$, car sa surexpression augmentait leur capacité à contrôler des tumeurs murines. Cependant, les aspects quantitatifs de la régulation des niveaux d'expression de miR-155 étaient peu caractérisés. Ainsi, la deuxième partie de ma thèse a porté sur la régulation de l'expression de miR-155 dans les cellules T $CD8^+$ humaines et murines provenant de tissus sains et de tumeurs de mélanome. J'ai démontré que l'affinité des cellules T et la dose d'antigène déterminent le niveau d'expression de miR-155 dans les cellules T $CD8^+$. De plus, un taux d'expression élevé de miR-155 était corrélé avec un contrôle tumoral accru dans les tumeurs B16 exprimant l'antigène de faible affinité et un traitement par anti-PD-1 augmentait l'expression de miR-155 des cellules T $CD8^+$. De plus, les cellules T $CD8^+$ surexprimant miR-155 présentaient une persistance accrue dans un contexte d'infection chronique. En accord avec ces observations dans les modèles murins, le taux d'expression de miR-155 dans les cellules T $CD8^+$ effectrices de mémoire (EM) humaines est corrélé avec leurs fréquences dans les ganglions lymphatiques infiltrés par la tumeur. Ainsi, nous suggérons que le niveau d'expression de miR-155 reflète la réactivité et l'affinité des lymphocytes T $CD8^+$ pour leurs antigènes et peut être utilisé comme un marqueur de leur qualité fonctionnelle.

Finalement, nous démontrons que la combinaison de vaccination avec ligands peptidiques altérés en combinaison avec un traitement anti-PD-1 peut permettre le contrôle de tumeurs exprimant un antigène de faible affinité, ce qui peut être révélé par une surexpression du miR-155 dans les cellules T $CD8^+$ infiltrant la tumeur.

SUMMARY

Despite the ability of $CD8^+$ T-cells to recognize and kill cancer cells, tumors manage to evade immune control through multiple mechanisms. Immunotherapeutic strategies have shown clinical benefit in some but not all cancer types or patients. Hence, a better understanding of the key parameters governing the complex relationship between $CD8^+$ T-cells and tumors is necessary to ensure the success of immunotherapy in most cancer patients.

Anti-tumor T-cell responses mostly involve $CD8^+$ T-cells with high affinity for mutated self-antigens and of low affinity for tumor-associated antigens. Therefore, the first part of my thesis project addressed the impact of the recognition strength between the TCR and its antigen in $CD8^+$ T-cell mediated anti-tumor responses. We found that $CD8^+$ T-cell peripheral priming with a high affinity peptide vaccine was necessary to control low affinity antigen expressing B16 melanoma tumors. In addition, despite a high affinity peripheral priming, the affinity for the antigen that $CD8^+$ T-cells encountered later in the tumor influenced on the $CD8^+$ T-cell response: low affinity antigen recognition led to decreased $CD8^+$ T-cell expansion and tumor control while induced lower expression levels of inhibitory receptors and increased cytokine production upon *ex vivo* restimulation. Interestingly, tumor infiltrating $CD8^+$ T-cells regardless of their affinity for the tumor antigen, responded equally to PD-1 blocking monoclonal antibodies. Moreover, when tumor infiltrating $CD8^+$ T-cells were re-transferred to tumor-free hosts, they showed similar *in vivo* re-expansion capacity irrespectively of the affinity for the tumor antigen. Interestingly, the re-expansion capacity of these T-cells was similar to that of their counterparts isolated from secondary lymphoid organs, suggesting that $CD8^+$ T-cells in tumors may be rekindled upon relief of tumor immunosuppression.

Our group had previously shown the importance of micro-RNA-155 (miR-155) for $CD8^+$ T cells anti-tumor responses, as its overexpression enhanced tumor control by $CD8^+$ T cells. However, the quantitative aspects of the regulation of miR-155 expression levels were poorly characterized. Thus, the second part of my thesis addressed miR-155 expression regulation in mouse and human $CD8^+$ T cells from healthy tissues and melanoma tumors. I demonstrated that T cell affinity and antigen dose are two important factors determining miR-155 expression levels in $CD8^+$ T cells. Interestingly, high miR-155 expression levels correlated with increased tumor control in low affinity antigen expressing B16 tumors and anti-PD-1 treatment increased $CD8^+$ T cells miR-155 expression levels. Moreover, $CD8^+$ T cells overexpressing miR-155 showed enhanced persistence in chronic infections. In line with these observations in mouse model systems, miR-155 expression levels in human effector memory $CD8^+$ T cells positively correlated with their frequencies in tumor infiltrated lymph nodes. Thus, we propose that miR-155 expression levels reflect responsiveness and affinity for the antigen and may be used as an overall marker of tumor infiltrating $CD8^+$ T cells fitness.

Altogether, we suggest that combination of high affinity peripheral priming by altered peptide ligands and checkpoint blockade may enable tumor control of even low affinity antigen expressing tumors, which can be revealed by miR-155 upregulation in tumor infiltrating $CD8^+$ T cells.

ABBREVIATIONS

Ab: Antibody
ACT: Adoptive cell therapy
APC: Antigen-presenting cell
B6: C57BL/6 mouse
BCR: B-cell receptor
BrdU: 5-Bromo-2'-deoxyuridine
CAR: Chimeric antigen receptor
CFU: Colony forming units
CM: Central memory
cTEC: Cortical thymic epithelial cell
DC: Dendritic cell
dLN: Draining lymph nodes
DN: Double negative
DP: Double positive
EAE: Experimental autoimmune encephalomyelitis
EM: Effector-memory
EMA: European medicines agency
EMRA: $CD45RA^+$ effector memory
FCS: Fetal calf serum
FDA: Food and drug administration
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HD: Healthy donor
HLA: Human leukocyte antigen
ICB: Immune checkpoint blockade
ILC: Innate-lymphoid cell
i.p: Intraperitoneal
ISO: Isotype control
ITAM: Immunoreceptor tyrosine-based activation motif
i.v: Intravenous
K_D: Dissociation constant
LCMV: Lymphocytic choriomeningitis virus
Lm: *Listeria monocytogenes*
MDSC: Myeloid-derived suppressor cell
MFI: Mean fluorescence intensity
MHC: Major histocompatibility complex
MiR: Micro-RNA
MPEC: Memory precursor effector cell
mTEC: Medullary thymic epithelial cell
N4: SIINFEKL
NK: Natural killer
NLN: Non-tumor-infiltrated lymph node
NTAmer: Ni (2+)-nitrilotriacetic acid histidine tag-containing multimers
OVA: Ovalbumin
PB: Peripheral blood
Pfu: Plaque forming unit
PMA: Phorbol 12-myristate 13-acetate
pMHC: Peptide-loaded major histocompatibility complex**PRR:** Pattern-recognition receptor
RM: Repeated measurement
RT: Room temperature
s.c: Subcutaneous
SCM: Memory stem cell
SD: Standard deviation
SLEC: Short-lived effector cell

SP: Single positive

SPR: Surface plasmon resonance

SSC: Saline-sodium citrate buffer

T4: SIITFEKL

TAA: Tumor-associated antigen

TCR: T-cell receptor

TE: Terminal effector

TF: Transcription factor

Th: T helper

TILN: Tumor-infiltrated lymph node

TIL: Tumor-infiltrating lymphocyte

Treg: Regulatory T cell

TRM: Tissue resident memory

WT: Wild-type

GENERAL INTRODUCTION

1. The immune system

1.1. Innate and adaptive immune system

The immune system protects the host from infection by pathogens and development of tumors. It is composed by various cell types which develop from pluripotent hematopoietic stem cells in the bone marrow and then patrol through the blood and lymphatic system to guard the peripheral tissues (Janeway, 2001). Upon an infection, cells from the innate system are the first ones to respond by sensing danger signals. Four cell types belong to the innate immune system: granulocytes (neutrophils, eosinophils and basophils), macrophages, innate-lymphoid cells (ILCs) (Spits & Di Santo, 2011) and dendritic cells (DCs). Macrophages phagocyte and destroy pathogens through the recognition of pattern-recognition receptors (PRRs). Once activated they secrete inflammatory cytokines and chemokines that initiate the so-called inflammation process. Local inflammation will activate the complement system and recruit other cells of the innate immune system such as neutrophils, who are also able to engulf and destroy pathogens. The innate immune response might clear the infection, but not always. In those cases, the adaptive immune response will be activated thanks to tissue resident DCs. Phagocytosis of invading microorganisms via PRRs recognition induces activation and maturation of DCs that will migrate to a nearby secondary lymphoid organ. There, mature DCs present pathogen-derived antigens on major histocompatibility complex (MHC) molecules to T and B cells as well as secrete cytokines that will tune the innate and adaptive immune response. T and B cells are lymphocytes which express antigen-specific receptors (TCRs and BCRs respectively) and form the adaptive immune system. Upon specific antigen recognition in secondary lymphoid organs, they expand and differentiate. More specifically, T cells mainly differentiate into antigen-specific effector cells that migrate to the site of infection to eradicate the infection by killing infected cells or secreting cytokines that activate other immune cells, while B cells differentiate into antibody-secreting cells. Antibodies recognize specific epitopes of the pathogen allowing its neutralization. In addition, antibody bound pathogens can be recognized by other immune cells and induce antibody-dependent cell-mediated cytotoxicity (ADCC) as well as by the complement system and trigger complement-dependent cytotoxicity (CDC) (Janeway, 2001).

1.2. T cell development

All cells of the blood including immune cells derive from hematopoietic stem cells in the bone marrow. During hematopoiesis, these pluripotent cells divide and produce more specialized stem cells: common lymphoid progenitors, common myeloid progenitors, erythrocytes and megakaryocytes. T and B cells both derive from a common lymphoid progenitor. However, whereas B cell maturation occurs in the bone marrow, naïve T cells develop in the thymus. Common lymphoid progenitors migrate from the bone marrow to the thymus where they lose the capacity to become B cells (Pui et al., 1999; Radtke et al., 1999) and natural-killer (NK) cells (Michie et al., 2000). These early committed T cells still lack the expression of the T cell receptor (TCR) as well as CD4 or CD8 co-receptors and are referred as double negative (DN) thymocytes. DN thymocytes can give rise to T cells expressing αβ or γδ TCR chain pairs. For αβ T cell development, DN thymocytes rearrange the TCR β and α-chain by recombination-activating gene 1 (RAG1) (Mombaerts et al., 1992) and RAG2 (Shinkai et al., 1993) dependent somatic DNA recombination. They also initiate the expression of CD4 and CD8 co-receptors becoming double-positive (DP) thymocytes. DP thymocytes interact with cortical thymic epithelia cells (cTECs) that express high levels of self-peptide loaded MHC I and MHC II molecules. DP thymocytes that recognize self-peptide MHC molecules (pMHC) bellow a minimal affinity do not get enough TCR signalling to survive and die by apoptosis (death by neglect). Those with enough affinity for the MHC molecules go to the medulla and further interact with medullary thymic epithelial cells (mTECs) that express MHC molecules loaded with a range of self-antigens thanks to the expression of the Autoimmune Regulator (AIRE) transcription factor. Neighbouring thymic dendritic cells may also capture self-antigens from mTECs and interact with DP thymocytes (Anderson

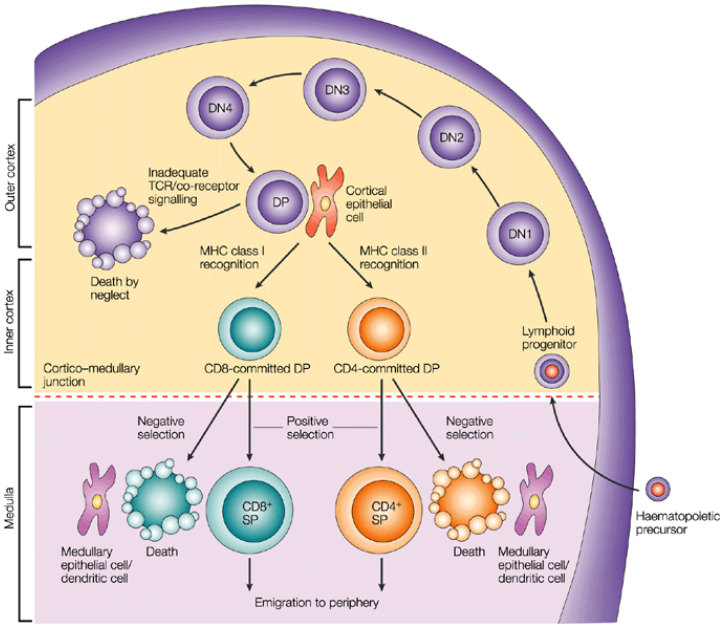

Figure 1. T cell development in the thymus. Lymphoid progenitors undergo a positive and negative selection process during their maturation into naïve $CD4^+$ or $CD8^+$ single positive (SP) lymphocytes. Such selection process enables the elimination of cells that recognize too weakly or too strongly self-peptide loaded MHC molecules. DP: Double positive (Germain, 2002).

& Takahama, 2012). Strong interaction between TCRs of DP thymocytes and these self-pMHC molecules invokes thymocyte death (negative selection). Only intermediate TCR signalling allows complete maturation of DP thymocytes (positive selection), which become conventional single positive (SP) $CD4^+$ or $CD8^+$ T-cells depending on the recognition of MHC II or MHC I molecules respectively (Germain, 2002; von Boehmer, Teh, & Kisielow, 1989) (Figure 1). This selection process generates a TCR repertoire that recognizes self MHC I and MHC II molecules but only with low affinity when loaded with self-peptides, with the exception of TCRs of regulatory T cells (Josefowicz, Lu, & Rudensky, 2012). This highly sophisticated thymic selection system prevents possible activation of self-antigen specific T cells in the periphery and consequent autoimmunity. This mechanism contributes to the central tolerance initially described by Joshua Lederberg for B cells (Lederberg, 1959). Finally, naïve mature $CD4^+$ and $CD8^+$ T-cells exit the thymus and circulate through the blood and lymphatic system ready to get activated and mount an immune response against their specific antigen.

1.3. T cell activation

Naïve ($CD44^{low}CD62L^{high}CCR7^{high}$) T cell activation is a complex process in which many molecules in addition to the TCR and pMHC enable the signal transduction of the extracellular antigen recognition stimuli into an intracellular signalling cascade and cell reprogramming. The TCR is associated to CD3ε, γ, δ, and ζ polypeptides which contain immunoreceptor tyrosine-based activation motifs (ITAMs) on their cytoplasmic tails. Upon TCR-pMHC interaction, the TCR-CD3 complex undergoes a conformational change and the cytoplasmic tails of CD3ε and CD3ζ become accessible to the LCK tyrosine kinase (Gil, Schamel, Montoya, Sanchez-Madrid, & Alarcon, 2002; Joshi et al., 2011; Mingueneau et al., 2008; H. Zhang, Cordoba, Dushek, & van der Merwe, 2011). LCK is associated to CD4 and CD8 co-receptors, which also participate in the recognition of pMHC molecules. CD3 ITAM phosphorylation by LCK (Straus & Weiss, 1992) leads to the recruitment, phosphorylation and consequent activation of ZAP-70 tyrosine kinase (Chan et al., 1995; Iwashima, Irving, van Oers, Chan, & Weiss, 1994) that will then phosphorylate LAT and SLP-76 adaptor proteins (Malissen & Bongrand, 2015). Phosphorylated LAT will then recruit phospholipase C γ (PLCγ) that will be activated by interleukin-2-inducible-T-cell kinase (ITK), a kinase that is activated by LCK and binds phosphorylated SLP-76 (Smith-Garvin, Koretzky, & Jordan, 2009). Active PLCγ generates diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) secondary

messengers that ultimately lead to the activation of NFAT and NFkB transcription factors. Although T cells can be activated *in vitro* by TCR stimulation alone, physiological T cell activation requires two additional signals: costimulation via surface molecules such as CD28 (Lenschow, Walunas, & Bluestone, 1996) and/or 4-1BB (Tan, Whitmire, Ahmed, Pearson, & Larsen, 1999) and inflammatory signals: IL-12 (Trinchieri, 2003) and type I IFNs (Curtsinger, Valenzuela, Agarwal, Lins, & Mescher, 2005). Combination of the three signals induces T cell proliferation and transcriptional, epigenetic and metabolic reprogramming that ultimately give rise to effector and memory cells (Kaech & Cui, 2012; Lanzavecchia & Sallusto, 2002).

1.4. CD4+ T cell differentiation

Naïve $CD4^+$ T cells get activated by antigen presenting cells in secondary lymphoid organs upon recognition of their specific peptide antigen presented on MHC-II molecules and interaction with costimulatory molecules. Once activated, $CD4^+$ T cells clonally expand and differentiate into different T helper (Th) subsets. $CD4^+$ T cell polarization is controlled by specific transcription factors, whose expression depends on several signals including soluble factors. Figure 2 summarizes the main Th subsets described up to now, and characterized by the expression of defined transcription factors. Th1, Th2 and Th17 boost other lymphocytes' responses thanks to the secretion of IFN-γ and TNF (Th1), IL-4, IL-5 and IL-13 (Th2), and IL-17 and IL-22 (Th17) respectively. In addition, follicular helper T cells (T_{FH}) are necessary for optimal B cell responses through their secretion of IL-6, and regulatory T cells (Tregs) dampen T cell responses by different mechanisms such as IL-10 secretion. Finally, more recently discovered, cytolytic $CD4^+$ T cells are able to kill infected cells similarly to $CD8^+$ T cells (Swain et al., 2012).

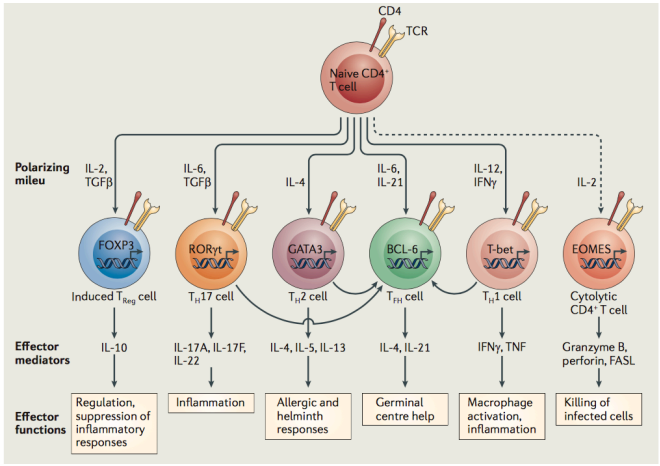

Figure 2. $CD4^+$ T cell subsets. Depending on the cytokines present in the milieu, $CD4^+$ T cells polarize into distinct cell subsets. Each subset is characterized by the expression of a master TF and secretion of determined cytokines (Swain, McKinstry, & Strutt, 2012).

1.5. CD8$^+$ T cell differentiation

Among the cells of the adaptive immune system, CD8$^+$ T cells play a critical role in the immune response against intracellular pathogens and cancer cells. Upon pMHC I antigen recognition in secondary lymphoid organs, the majority of activated CD8$^+$ T cells differentiate into cytotoxic effector cells, which migrate to the site of infection or proliferation of malignant transformed cells to exert their effector functions (N. Zhang & Bevan, 2011). They can induce apoptosis of target cells via three different mechanisms: 1) exocytosis of granules containing lytic molecules such as perforin, granzymes and granulysin (Faroudi et al., 2003; Wiedemann, Depoil, Faroudi, & Valitutti, 2006) 2) Fas-FasL cell-cell contact and 3) cross-linking of TNF and TNFR type I (Chavez-Galan, Arenas-Del Angel, Zenteno, Chavez, & Lascurain, 2009). As the antigen is cleared and the inflammation resolves, most effector CD8$^+$ T cells undergo apoptosis. Only some of the expanded cells will differentiate into memory CD8$^+$ T cells that self-renew in an antigen independent manner and may persist for a lifetime ready to initiate a rapid and potent specific recall response against a second encounter with the antigen.

In acute viral and bacterial infections, at least two effector CD8$^+$ T cell subsets can be distinguished. CD127^{high}KLRG1^{low} memory-precursor effector cells (MPECs) and CD127^{low}KLRG1^{high} short-lived or terminal effector cells (SLECs or TEs). Distinction of these two cell subsets is particularly relevant as it has been shown in acute infections that MPECs have increased potential to form memory cells compared to TEs (Joshi et al., 2007; Kaech et al., 2003).

The formation of effector and memory CD8$^+$ T cells is tightly regulated by transcription factors (TF). In particular, T-bet and Eomesodermin (Eomes) T-box TFs are key for CD8$^+$ T cell effector and memory formation. Both TFs contribute to the induction of IFNγ, Granzyme B and perforin in early effector cells (Banerjee et al., 2010; Joshi et al., 2007; Pipkin et al., 2010) and IL2-Rβ (CD122) in memory cells that enables IL-15 dependent homeostatic proliferation (Banerjee et al., 2010; Intlekofer et al., 2005; Yajima et al., 2006). T-bet expression is directly induced by TCR signaling and amplified by IL-12 and mammalian target of rapamycin (mTOR) activity (Joshi et al., 2011; Rao, Li, Odunsi, & Shrikant, 2010; Takemoto, Intlekofer, Northrup, Wherry, & Reiner, 2006). Its deficiency leads to impaired CD8$^+$ TE cell formation (Joshi et al., 2007). In contrast, Eomes expression is induced after T-bet in early effector cells, is amplified by IL-2 (Pipkin et al., 2010) but repressed by IL-12 and mTOR activity (Rao et al., 2010; Takemoto et al., 2006). Lack of Eomes leads to efficient primary expansion and MPEC formation but

fewer central memory cells with impaired long-term persistence (Banerjee et al., 2010). Despite some redundant functions, the expression pattern of these two TFs differ. While highest T-bet expression levels are reached in effector cells and decline in memory cells (Joshi et al., 2011), Eomes expression increases from effector to memory cells, probably in response to TCF-1 TF (Banerjee et al., 2010; Zhou et al., 2010). Thus, the ratio of T-bet versus Eomes expression level may define effector $CD8^+$ T cells fate (Banerjee et al., 2010; Joshi et al., 2007; Joshi et al., 2011). Although the exact mechanism regulating this ratio remains to be elucidated, it is known that pro-inflammatory cytokines (IL-12, type I IFNs, IL-2) and other immunomodulatory factors (IL-4, IL-10, and IL-21) are involved (Pipkin et al., 2010; Takemoto et al., 2006; Wiesel et al., 2012).

B lymphocyte-induced maturation protein (BLIMP1) and BCL-6 TFs also participate in $CD8^+$ T cell effector and memory formation. BLIMP1 is a transcriptional repressor that, like T-bet, is highly expressed in effector cells and downregulated in memory $CD8^+$ T cells (Kallies, Xin, Belz, & Nutt, 2009; Rutishauser et al., 2009). On the contrary, BCL-6 expression increases in memory cells and is particularly crucial for central memory $CD8^+$ T cell formation and maintenance (Cui, Liu, Weinstein, Craft, & Kaech, 2011; Ichii et al., 2007; Ichii et al., 2002). Additional TFs such as ID2, ID3 and members of the STAT family (reviewed by Kaech and Cui (Kaech & Cui, 2012)) also participate in $CD8^+$ T cell fate decision. Thus, $CD8^+$ T cell effector and memory formation is a complex process finely regulated by multiple transcription factors that respond to cell intrinsic and environmental cues.

1.6. Memory $CD8^+$ T cells

Memory formation is a hallmark of the adaptive immunity. Acute infection and vaccination models have broadly been used to describe $CD8^+$ differentiation and memory formation. Single cell transfer and barcoding experiments revealed that a single naïve T cell can give rise to both effector and memory cells (Gerlach et al., 2010; Stemberger et al., 2007). However, several mechanisms have been proposed to explain T cell fate decision: the asymmetric cell division during APC and T cell interaction (Chang et al., 2007), the signal strength during T cell priming (Joshi et al., 2007; Masopust, Kaech, Wherry, & Ahmed, 2004; Sallusto, Geginat, & Lanzavecchia, 2004; Wiesel et al., 2012) and the overall accumulation of signals over the T cell response (Badovinac, Porter, & Harty, 2004; Joshi et al., 2007; Sarkar et al., 2008).

Based on their function, anatomical location and phenotypic markers, three different memory T cell subsets can be distinguished: effector-memory (EM) ($CD44^{high}CD62L^{low}CCR7^{low}$), central-memory (CM) ($CD44^{high}CD62L^{high}CCR7^{high}$) and tissue-resident-memory (TRM) ($CD44^{high}$ $CD62L^{low}$ $CD103^{high}$ $CD69^{high}$ $CD27^{low}$) T cells. While EM and CM T cells circulate through the blood vessels as well as lymphoid and non-lymphoid tissues, TRM T cells show very limited recirculation and reside in the brain and mucosal tissues. TRM and EM T cells confer immediate effector functions at the pathogen entry site, whereas CM T cells tend to mount a more robust recall response by generating many secondary effector cells (Kaech & Cui, 2012). More recently, a forth memory T cell subset has been described from the original naïve T cell pool defined as $CCR7^{high}CD62L^{high}SCA1^{+}IL\text{-}2R\beta^{+}CXCR3^{+}$ in mice and $CD95^{+}IL\text{-}2R\beta^{+}CXCR3^{+}CD58^{+}CD11a^{+}$ in humans. They are referred as T memory stem cells (SCM) and are characterized by long persistence, high proliferative potential and ability to generate a diverse range of T cell subsets (Gattinoni, Speiser, Lichterfeld, & Bonini, 2017). In humans, an additional $CCR7^{low}$ $CD45RA^{+}$ $CD8^{+}$ T cell subset has been identified, characterized by larger amounts of perforin than EM $CD8^{+}$ T cells. It is highly differentiated and defined as $CD45RA^{+}$ effector memory (EMRA) T cells (Appay, van Lier, Sallusto, & Roederer, 2008).

1.7. T cell affinity

TCR pMHC recognition is essential for the activation and differentiation of T cells. In fact, the strength of the interaction between the TCR and pMHC directly correlates with the amplitude of T cell expansion (Vigano et al., 2012; Zehn, Lee, & Bevan, 2009). Three terms can be defined to describe the strength of the T cell interaction with an APC or target cell: T cell affinity, avidity and functional avidity (Figure 3). To assess each of these parameters, different techniques have been developed as described below.

T cell receptor affinity

It defines the physical binding strength between a single TCR and pMHC molecule (Figure 3, left panel). The dissociation constant (K_D) between soluble TCRs and pMHCs is measured by surface plasmon resonance (SPR) and has mainly been used to define T cell affinity. K_D measures the dissociation rate of two molecules. Therefore, the higher the K_D, the lower the T cell affinity. More recently, NTA-His tag-containing multimers (NTAmers) have been developed. These multimers, as conventional multimers, bind to

TCRs, but upon imidazole addition they rapidly dissociate into pMHC monomers allowing the measurement of monomeric TCR –pMHC dissociation rates (K_{off} and $t_{1/2}$). Unlike SPR experiments which involve recombinant soluble TCR and pMHC complexes, NTAmers measure TCR – pMHC interactions on the cell surface, and so far, only MHC I NTAmers are available (Schmidt, Dojcinovic, Guillaume, & Luescher, 2013). Therefore, NTAmers but not SPR, include the contribution of the CD8 co-receptor, known to stabilize TCR –pMHC complexes. Nonetheless, NTAmer based dissociation kinetics generally correlate well with SPR measured affinities (Hebeisen et al., 2015).

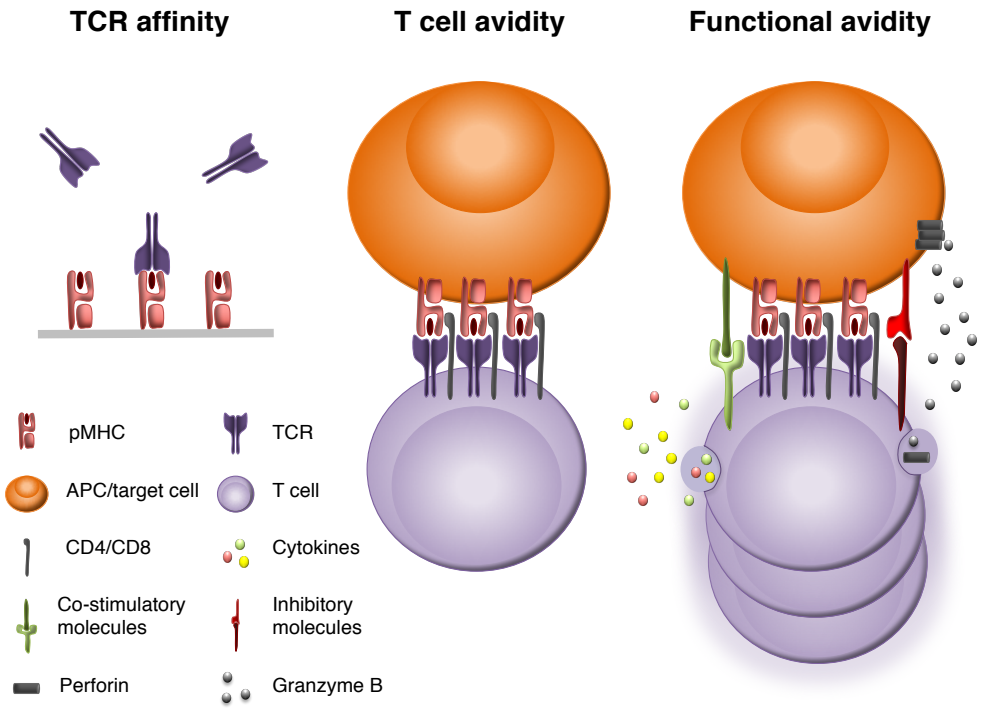

Figure 2. Illustration of T cell affinity, T cell avidity and functional avidity of T cells. The three parameters measure the interaction strength between the TCR and pMHC. However, while the TCR affinity defines the strength between single pMHC-TCR molecules, the T cell avidity measures the strength of the multimeric TCR-pMHC interaction and the contribution of the CD8/CD4 co-receptor. The functional avidity determines the amount of antigen needed for a T cell response and therefore depends on the TCR avidity but also the presence of co-stimulatory and co-inhibitory receptors as well as the differentiation state of the cell (Jandus, Martínez-Usatorre, Vigano, Zhang, & Romero, 2017).

T cell avidity

It defines the interaction strength between several TCRs and pMHC molecules (Figure 3, middle panel). It can be measured by pMHC multimer staining on the cell surface of T cells. The binding of multimeric pMHC to T cells correlates with monomeric TCR – pMHC affinity (Crawford, Kozono, White, Marrack, & Kappler, 1998). Therefore, the fluorescence intensity of pMHC multimer staining can be used to have an idea of the affinity of a T cell for its ligand, avoiding the laborious and expensive production of

soluble TCRs for SPR measurements. Of note, pMHC multimer staining also reflects the contribution of the CD8 or CD4 co-receptor (Crawford et al., 1998).

Functional avidity

It defines the sensitivity of a T cell to the antigen as translated in effector functions (Figure 3, right panel). It inversely correlates with the antigen dose needed for the triggering of a T-cell response. The antigen concentration needed to reach half of a maximum response (EC_{50}) is the parameter used to define the functional avidity of a T cell. The main functional readouts are the EC_{50} for proliferation, cytokine production, and target cell lysis. As the functional avidity is a cellular measurement, it is influenced by many parameters. T cell affinity normally correlates with functional avidity, yet not always. Expression levels of the TCR, co-receptors and adhesion molecules also influence the binding strength between a T cell and an antigen presenting cell/target cell. Moreover, expression of inhibitory and co-stimulatory molecules modulates the T cell response and sensitivity to the antigen.

High functional avidity T cell responses mediate efficient virus clearance (Berger et al., 2011). In addition, since many tumors are characterized by low antigen presentation and absence of co-stimulatory signals, higher-avidity $CD8^+$ T cells promote more efficient tumor rejection (Dutoit et al., 2001). However, low avidity antigen recognition may be useful to better discriminate self-antigens overexpressing tumors from healthy tissues (D. J. Morgan et al., 1998), thus preventing autoimmunity. Moreover, when T cells are chronically exposed to the antigen as in chronic infections, low functional avidity T cells may be less sensitive to activation induced cell death, exhaustion, and senescence (Anderton, Radu, Lowrey, Ward, & Wraith, 2001; Utzschneider, Alfei, et al., 2016). Thus, in the first part of this thesis we study the impact of T cell avidity on $CD8^+$ T cells anti-tumor responses.

1.8. T cell exhaustion

Upon acute infections, $CD8^+$ T cells massively expand. However, when the antigen is cleared and inflammation is resolved most of them die and only memory $CD8^+$ T cells remain. In contrast, in chronic infections and cancer, there is a persistent inflammation and antigen exposure which alters the conventional $CD8^+$ T differentiation and memory formation described in acute responses (Wherry, 2011). For instance, studies on HIV infected patients and on mice chronically infected with the LCMV cl13, revealed that

$CD8^+$ T cells progressively and hierarchically loose effector functions, first the ability to kill target cells and produce IL-2, followed by TNFα secretion and finally IFNγ production (Wherry, Blattman, Murali-Krishna, van der Most, & Ahmed, 2003). In addition, they up-regulate multiple inhibitory receptors (PD-1, CTLA-4, LAG-3, 2B4, CD160 and TIM-3) (Blackburn et al., 2009), show poor proliferative capacity and are unable to persist in the absence of antigen (Altfeld et al., 2002; Wherry, Barber, Kaech, Blattman, & Ahmed, 2004). This differentiation state is commonly known as T cell exhaustion and characterized by a transcriptional state that differs from the one of effector and memory cells during acute infections. Transcriptional changes are observed in cell metabolism pathways, cell cycle regulation and transcription factor expression (Doering et al., 2012; Wherry et al., 2007).

Chronic antigen stimulation has been identified as the major driver leading to this differentiation state (Utzschneider, Alfei, et al., 2016; Wherry et al., 2003). Nonetheless, lack of $CD4^+$ T cell help (Matloubian, Concepcion, & Ahmed, 1994), signaling through inhibitory receptors (Quigley et al., 2010) and immunosuppressive cytokines (Brooks et al., 2006; Teijaro et al., 2013) also contribute to this status. As chronic infections and tumors are commonly characterized by chronic antigen stimulation and inflammation, tumor infiltrating $CD8^+$ T lymphocytes ($CD8^+$ TILs) also show exhausted T cell characteristics such as increased expression of inhibitory receptors (Baitsch et al., 2011) and decreased capacity to produce IFNγ, perforin and Granzyme B upon *ex vivo* restimulation (Zippelius et al., 2004). However, less is known about the exact mechanisms leading to this impairment. Chronic antigen stimulation may be one of the reasons as tumor specific $CD8^+$ T cells from blood of patients show robust inflammatory and cytotoxic functions and have low expression levels of inhibitory receptors (Baitsch et al., 2011; Zippelius et al., 2004). In addition, $CD4^+$ T cells are important for $CD8^+$ T cell mediated anti-tumor responses as they enhance recruitment and cytolytic activity of tumor specific $CD8^+$ T cells (Bos & Sherman, 2010) and prevent $CD8^+$ T cells peripheral tolerance (Kirberg, Bruno, & von Boehmer, 1993). In fact, central tolerance of the majority of tumor-associated antigens results on the maturation of only low affinity self/tumor-specific $CD8^+$ T cells that are poorly primed by antigen presenting cells (APCs) due to the lack of innate stimulators needed for APC maturation in the immunosuppressive tumor microenvironment (Gabrilovich, 2004). Therefore, $CD8^+$ TILs are found in a mixed anergic and exhausted hyporesponsive state.

Despite the inability of exhausted $CD8^+$ T cells to clear the tumor or infection, they are not inert but retain some functionality that limits pathogen replication and tumor progression. It is now accepted that this exhausted state is a way of minimizing tissue damage while still mediating some pathogen/tumor control (Speiser et al., 2014). In fact, a memory-like TCF-1-expressing $CD8^+$ T cell population has recently been described as able to sustain the $CD8^+$ T cell response in chronic viral infections (Utzschneider, Charmoy, et al., 2016). In addition, T cell exhaustion seems to be reversible, at least at the bulk population level, as seen by the success of immunotherapies blocking inhibitory receptors and targeting co-stimulatory molecules expressed by exhausted T cells, which rescue T cell functionality (Sharma & Allison, 2015). This suggests that high inhibitory signals in combination with lack of costimulation is the major constraint of tumor infiltrating $CD8^+$ T cell functionality.

2. Micro-RNAs

2.1. Biogenesis and roles of microRNAs

MicroRNAs (miRs) are endogenous noncoding ~22nt long RNAs found in multicellular organisms which regulate the expression of proteins at post-transcriptional level. They target mRNAs, mostly for their degradation or translational repression (Bartel, 2004). Each miR can target numerous mRNAs, and each single mRNA is commonly targeted by multiple miRs (Bartel, 2009). Thus, miRs have an enormous combinatorial complexity and regulatory potential. The majority of miRs are processed from longer primary transcripts (pri-miRs), which are either encoded in independent miR genes or are matured from introns or exons of protein-coding mRNAs (V. N. Kim, Han, & Siomi, 2009).

Pri-miRs are divided into two major classes, canonical and non-canonical, depending on their maturation process. The canonical pri-miRs (Figure 4) are transcribed by RNA polymerase II and recognized by the Microprocessor complex. This complex is mainly composed by the RNase type III endonuclease Drosha and the double-stranded RNA-binding protein DiGeorge critical region 8 (DGCR8). DGCR8 recognizes the RNA substrate that Drosha will cleave in a processed known as cropping, producing ~70 nucleotide hairpin structured precursor miRs (pre-miRs) (Denli, Tops, Plasterk, Ketting, & Hannon, 2004). Pre-miRs exit the nucleus by exportin5 via a Ran-GTP dependent mechanism. Once in the cytoplasm, pre-miRs are processed by the RNase type III enzyme Dicer (Yi, Qin, Macara, & Cullen, 2003). An argonaute (AGO) protein and a glycine–

tryptophan repeat-containing protein of 182 kDa (GW182) bind the resulting ~22 basepair miR, forming the core of a multi-subunit complex called miR-induced silencing complex (miRISC) (Fabian & Sonenberg, 2012). While one of the miR strands is discarded from the complex, the remaining one determines which mRNAs will be targeted. Partial complementarity of the miR seed region (nucleotides 2-8 from the 5' end) to the target mRNA typically induces translational repression followed by de-adenylation and mRNA decay (Bethune, Artus-Revel, & Filipowicz, 2012; Djuranovic, Nahvi, & Green, 2012; Fabian & Sonenberg, 2012), whereas perfect base pairing leads to endonucleolytic cleavage of the target mRNA by AGO2 in vertebrates (J. Liu et al., 2004). Nonetheless, in specific cellular conditions such as cell cycle arrest, miR-mediated mRNA upregulation has also been observed (Vasudevan, Tong, & Steitz, 2007).

The non-canonical pri-miRs differ from the canonical pri-miRs on their maturation process. They do not require all the protein factors required by the canonical pri-miRs to become mature miRs. For example, some pre-miRs are produced by splicing, not by Drosha cleavage (Okamura, Hagen, Duan, Tyler, & Lai, 2007). However, most of the ~22 nt long mammalian RNAs are canonical miRs (Graves & Zeng, 2012).

Over 2500 miRs have been identified in humans (http://www.mirbase.org, miRBase 21 release,(Kozomara & Griffiths-Jones, 2014)) which play an important role in several biological processes by targeting multiple components of regulatory networks (Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). Combinations of bioinformatic, biochemical and genetic approaches have revealed the role of miRs as regulatory elements in the control of cellular development, homeostasis and response of the immune system (Xiao & Rajewsky, 2009). For instance, Dicer$^{flox/flox}$ CD4cre mice, whose T cells lose the expression of Dicer in the double

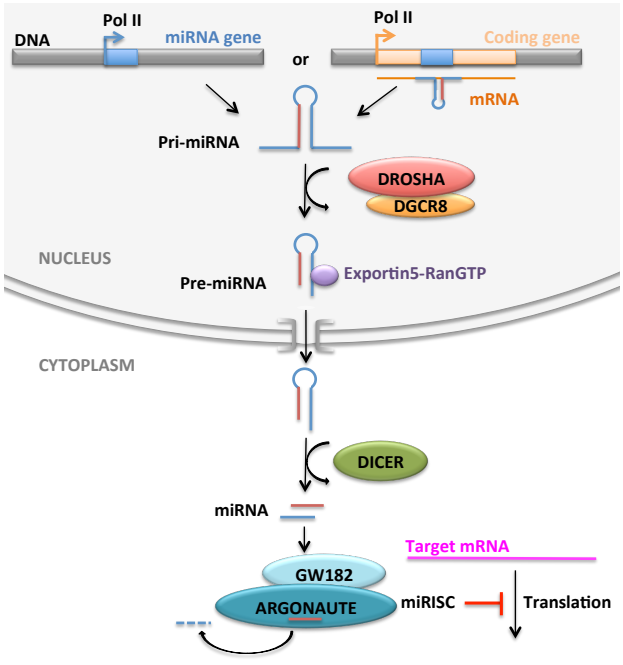

Figure 4. Biogenesis of canonical miRs. RNA polymerase transcribed pri-miRNAs are processed in the nucleus by the DROSHA and DGCR8 containing Microprocessor complex generating pre-miRNAs. These pre-miRNAs exit the nucleus, where they are further processed by DICER, producing mature miRNAs. One of the miRNA strands is discarded while the other binds an Argonaute protein generating the miRNA-induced silencing complex (miRISC) that cleaves or represses the translation of target mRNAs.

positive stage during thymic development, show reduced numbers of peripheral T cells, suggesting the requirement of Dicer for maturation and homeostasis of peripheral T lymphocytes. Moreover, Dicer deficient T cells show decreased proliferation and increased apoptosis in response to activation (Muljo et al., 2005). Nonetheless, the roles of individual miRs in T cell biology are just beginning to emerge.

2.2. miR-155

The human and mouse miR-155 resides in the exon 3 of B-cell integration cluster (BIC) transcript, originally identified as a gene transcriptionally activated by promoter insertion at a common retroviral integration site in B cell lymphomas (Tam, Ben-Yehuda, & Hayward, 1997). In fact, overexpression of miR-155 is found not only in human B cell lymphomas (Eis et al., 2005) but also in non-hematopoietic cancers such as breast (Iorio et al., 2005), pancreatic (Lee et al., 2007) and lung cancer (Yanaihara et al., 2006). In addition, transgenic mice overexpressing miR-155 develop B cell malignancies (Costinean et al., 2006). Thus, miR-155 is considered as an oncomiR. However, the recently assembled list of miR-155 targets by Neilsen et al. comprise 140 genes including regulatory proteins for myelopoiesis and leukemogenesis (AICDA, ETS1, JARID2 etc.), inflammation (BACH1, FADD, IKBKE, INPP5D, MYD88, SPI1, SOCS1 etc.) and tumor suppressor genes (C/EBPβ, IL17RB, PCCD4, TCF12 etc) (Neilsen et al., 2013), indicating that miR-155 plays an important role in many biological processes. It has been suggested that miR-155 plays a role in TNF-α dependent adipogenesis inhibition (S. Liu, Yang, & Wu, 2011), and since the human miR-155 resides on chromosome 21 (http://www.mirbase.org), it has been hypothesized that certain characteristics of individuals with trisomy 21 are due to reduced expression of miR-155 target proteins. For instance, reduced blood pressure and risk for cardiovascular diseases in individuals with trisomy 21 may be due to increased miR-155 expression levels and consequent reduced angiotensin II receptor type 1 (AT_1R) expression in fibroblasts (Draheim, McCubbin, & Williams, 2002; Sethupathy et al., 2007). Moreover, the defective DC mediated antigen presentation and impaired T and B cell responses observed in BIC deficient mice indicates that miR-155 is essential for immune responses (Rodriguez et al., 2007).

Altogether, through the transcriptional repression of different mRNAs, miR-155 plays an important role in many biological functions including the immune system (Figure 5).

2.3. Role of miR-155 in the immune system

miR-155 is upregulated upon antigen receptor stimulation in mouse and human B and T cells (Haasch et al., 2002; Rodriguez et al., 2007; Stahl et al., 2009), as well as upon stimulation of Toll-like receptors in macrophages and dendritic cells (Taganov, Boldin, Chang, & Baltimore, 2006) via NF-κB and AP-1 transcription factors (Kluiver et al., 2007; O'Connell, Taganov, Boldin, Cheng, & Baltimore, 2007; Tili et al., 2007; Yin et al., 2008).

However, soluble factors also modulate miR-155 expression. While IL-2 and IL-15 induce miR-155 expression in T cells in a STAT5 dependent manner (Kopp et al., 2013), IL-10 has been shown to suppress miR-155 expression in bone marrow derived macrophages. This suppression depends on STAT3 and Ets1 transcription factor binding site, located in the miR-155 promoter (McCoy et al., 2010).

MiR-155 expression is also controlled by FoxP3, the essential transcription factor for Tregs (Cobb et al., 2006). In fact, miR-155 deficiency results in diminished Treg numbers, suggesting that it is an important miR for Treg development (Kohlhaas et al., 2009). In addition, miR-155 deficient Tregs show reduced proliferation in the presence of limiting amounts of IL-2 due to increased expression of suppressors of cytokine signaling 1 (SOCS1), a miR-155 target that negatively regulates IL-2 signaling (L. F. Lu et al., 2009). Not only Tregs, other $CD4^+$ T cell subsets also require miR-155. Th1 and Th17 $CD4^+$ T cell differentiation is controlled by miR-155 as miR-155 deficient $CD4^+$ T cells show skewed differentiation towards Th2. Such imbalanced differentiation is probably due to increased expression of the c-Maf miR-155 target TF (Rodriguez et al., 2007). In addition, CD4-cre $MiR155^{fl/fl}$ mice have increased resistance to Th17-dependent experimental autoimmune encephalomyelitis (EAE) (O'Connell et al., 2010), suggesting that miR-155 could be a therapeutic target for the treatment of autoimmune diseases.

MiR-155 also plays a role in T cell dependent B cell responses as it is important for Tfh $CD4^+$ T cell differentiation (Hu et al., 2014) and B cells. MiR-155 deficiency in B cells leads to reduced generation of germinal centers (GC) and diminished production of high affinity isotype switched Abs (Thai et al., 2007; Vigorito et al., 2007). MiR-155 GC regulation is, at least impart, through PU.1 targeting, which at the same time regulates

proteins involved in T-B cell interactions and down-regulates Pax5, a negative regulator of B cell terminal differentiation (D. Lu et al., 2014).

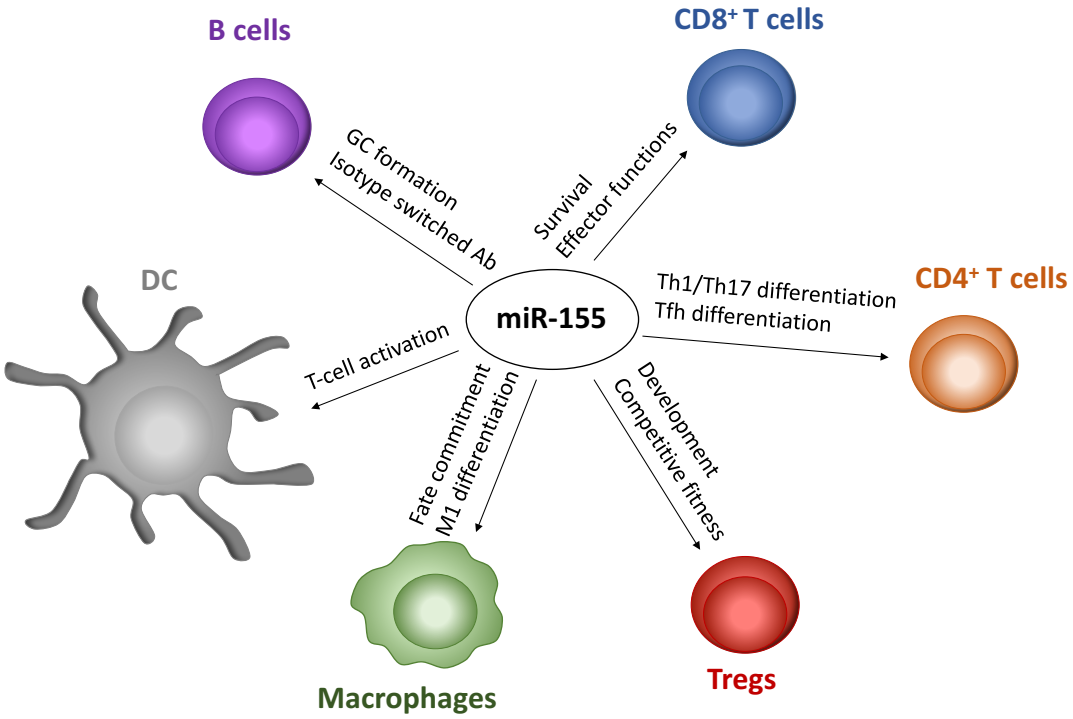

Figure 5. Role of miR-155 in immune cells. miR-155 plays important and specific roles in cells of the innate (macrophages and DCs) and adaptive immune system (T cells and B cells). Therefore, it is essential to mount effective immune responses.

Not only the adaptive immune system, miR-155 is also important for cells of the innate immune system such as macrophages (Squadrito, Etzrodt, De Palma, & Pittet, 2013). It is involved in the macrophage-fate commitment from progenitors, as its upregulation drives differentiation of RAW264.7 into macrophages by inhibition of osteoclast transcriptional machinery (Mann, Barad, Agami, Geiger, & Hornstein, 2010). In addition, miR-155 enhances TNFα production by stabilizing TNFα mRNA (Bala et al., 2011) and promotes classical inflammatory M1 macrophage differentiation (Jablonski, Gaudet, Amici, Popovich, & Guerau-de-Arellano, 2016) by downregulating SOCS1 (Wang et al., 2010), BCL-6 (Nazari-Jahantigh et al., 2012) and IL13 receptor, which promotes alternative/M2 macrophage differentiation (Martinez-Nunez, Louafi, & Sanchez-Elsner, 2011). In fact, miR-155 delivery in alternatively activated macrophages was shown to be sufficient to repolarize the cells into proinflammatory macrophages (Cai et al., 2012). Moreover, miR-155 deficiency in myeloid cells led to a skewed differentiation into a M2/Th2 phenotype and accelerated spontaneous breast cancer development (Zonari et

al., 2013). Finally, the key players in the cross-talk between the innate and adaptive immune system, DCs, also require miR-155 for the efficient activation of T cells (Rodriguez et al., 2007).

2.4. miR-155 and $CD8^+$ T cell function

The differential expression of miR-155 observed in healthy individuals' peripheral blood $CD8^+$ T cell subsets (Salaun et al., 2011) indicate that miR-155 plays a role in $CD8^+$ T cell function. In fact, studies in our lab using miR-155-deficient murine T cells have shown that miR-155 is required for effector $CD8^+$ T cell responses against viruses and tumors. MiR-155 deficiency leads to curtailed proliferation and $CD8^+$ T cell expansion upon acute LCMV and WSN acute viral infections (Dudda et al., 2013; Gracias et al., 2013). Gracias et al. observed that miR-155 deficiency led to enhanced susceptibility to type I interferon's antiproliferative signaling (Gracias et al., 2013). However, Dudda et al. showed that reduced proliferation and increased apoptosis of miR-155 deficient $CD8^+$ T cells was due to increased expression of the miR-155 target SOCS-1 (Dudda et al., 2013), a negative regulator of γc cytokine signalling (Cornish et al., 2003). In fact, the increased anti-tumor efficacy of miR-155 overexpressing $CD8^+$ T cells also depended on homeostatic γc cytokine signalling. Not only SOCS-1 but another negative regulator of STAT5, PTPN2, was downregulated in miR-155 overexpressing $CD8^+$ T cells and contributed to their enhanced survival (Ji et al., 2015). Of note, $CD8^+$ T cells effector functions, such as IFNγ and TNF-α production, are also increased upon miR-155 overexpression (Gracias et al., 2013; Ji et al., 2015).

Altogether, extensive studies on murine $CD8^+$ T cells have demonstrated that miR-155 is important for $CD8^+$ T cell responses but less is known about how this is translated into human $CD8^+$ T cell responses, in particular, in cancer patients. Therefore, one of the aims of this thesis, addressed in part 2, is to study the role and regulation of miR-155 in cancer patients' $CD8^+$ T cells.

3. Cancer immunity

3.1. Cancer

Cancer is a major public health problem with an increasing global burden due to the aging and growth of the world population, as well as to cancer-causing behaviors (Jemal et al., 2011). Tumor cell transformation is driven by the accumulation of somatic mutations

that ultimately lead to the acquisition of the hallmarks of cancer: genomic instability, sustained cell proliferation, resistance to cell death, evasion of growth suppressors, replicative immortality, metabolic deregulation, promotion of inflammation, angiogenesis induction, evasion of immune regulation, invasion and metastasis (Hanahan & Weinberg, 2011). Conventional surgery, radiotherapy and chemotherapy efficiently induce remission of certain cancer types. However, cancer is still one of the most difficult humankind affecting disease to cure as it is a highly dynamic disease that constantly adapts and eventually progresses most of the time. One of the reasons is that many different genes are implicated in the development of cancer, which differ between types of cancers, tumor sites, cancer cells within the same tumor and over time in response to selective pressure induced by treatments. Moreover, cancer cells recruit stromal cells that also contribute to tumorigenesis. Hence, tumors are heterogeneous tissues composed by distinct types of cells that interact with one another creating a complex tumor microenvironment that evolves during the course of the disease (Hanahan & Weinberg, 2011). Such dynamic complexity makes it difficult to treat. Thus, efforts are being made to understand cancer biology and develop new therapies against cancers like melanoma, which is generally considered resistant to irradiation and conventional chemotherapy (Trinh, 2008).

3.2. Cancer immunosurveillance

Opposing roles of the immune system in cancer have been described: support of tumor growth and anti-tumor immune response. Certain types of immune cells can induce proliferation, survival and invasion of cancer cells as well as angiogenesis (Hanahan & Weinberg, 2011). However, already 50 years ago, the idea that the adaptive immune system recognizes and destroys tumor cells was conceived (Burnet, 1957). In the last decade, several laboratories have demonstrated that the immune system can protect mice from outgrowth of many different types of primary and transplantable tumors (Shankaran et al., 2001; Swann & Smyth, 2007). Moreover, Shankaran et al. observed that tumors from immunocompetent mice were less immunogenic than tumors from immunodeficient mice when transplanted into secondary immunocompetent hosts, demonstrating that the immune system not only prevents tumor formation but also edits tumor immunogenicity (Shankaran et al., 2001). Nonetheless, tumors arise in immunocompetent hosts. Thus, the interaction between the immune system and tumors is a complex and evolving process, nowadays conceived as tumor immunoediting. This process can be divided into

three phases: elimination, equilibrium and escape, in which cells of the innate and adaptive immune system participate. In the first phase, immune cells prevent tumor formation by recognizing and destroying arising cancer cells. The equilibrium or tumor dormancy phase is a dynamic balance between the immune system and the tumor. The immune system can still recognize and destroy cancer cells but some of them already acquire immune evasion properties preventing the complete eradication of cancer cells. Finally, in the escape phase the immune system is no longer able to control the tumor growth and may even select more aggressive tumor cell variants and promote tumor cell proliferation (Vesely, Kershaw, Schreiber, & Smyth, 2011).

3.3. Tumor antigens

Based on the observation that T cells can recognize and specifically kill tumor cells, genetic and biochemical approaches have been used to identify tumor associated antigens that trigger anti-tumor immune response (Cox et al., 1994; Sahin et al., 1995; van der Bruggen et al., 1991). As a result, a relatively long list of MHC I and MHC II restricted tumor antigens is now available under http://cancerimmunity.org/peptide/. Two major groups of tumor antigens can be distinguished: non-mutated shared antigens and unique neoantigens originated from non-synonymous somatic mutations. Shared antigens are present on many independent tumors and can be further divided into three groups: 1) cancer-germline antigens such as NY-ESO-1 whose expression are restricted to germ cells and trophoblast tissues but frequently overexpressed in cancer cells due to epigenetic dysregulation (Akers, Odunsi, & Karpf, 2010), 2) differentiation antigens like Melan-A in melanoma, which are expressed in the tumor and the normal tissue of origin and 3) overexpressed antigens such as telomerase or HER2, which are antigens expressed in a wide variety of normal tissues and overexpressed in tumors. This classification is useful to determine the potential usefulness of a given tumor antigen for cancer immunotherapy such as vaccine based immunotherapy (Speiser et al., 2008; Valmori et al., 2007; J. Weber et al., 2008; Weide et al., 2008; Yamada, Sasada, Noguchi, & Itoh, 2013) or adoptive transfer of engineered T cells (Kalos & June, 2013). In melanoma, NY-ESO-1 and Melan-A have been for a long-time priority targets for immunotherapy (Balasse, Gatouillat, Patigny, Andry, & Madoulet, 2009; Caballero & Chen, 2009).

MHCI and MHCII restricted neoantigens have more recently been identified thanks to advances in deep sequencing of nucleic acids. They result from point mutations in genes that are expressed ubiquitously and are unique to the tumor of an individual patient or

restricted to some patients. Two studies using mouse tumor models demonstrated for the first time the possibility to identify neoantigens that are recognized by T cells. The technological pipeline used to identify such neoantigens involved deep exome sequencing of tumor material, followed by *in silico* prediction of major histocompatibility complex-binding capacity of the identified mutated peptides and final query of T cell reactivity (Castle et al., 2012; Matsushita et al., 2012). Following these studies, cancer exome based neoantigen identification has also been reported in human malignancies (Robbins et al., 2013; van Rooij et al., 2013). However, most non-synonymous mutations identified by cancer exome sequencing are not recognized by autologous T cells. Thus, additional filtering of neoantigen candidates is crucial. In preclinical models, cancer exome sequencing data has been used to guide neoantigen discovery by mass spectrometry (Gubin et al., 2014; Yadav et al., 2014). However, implementation of this approach as standard care in clinical settings is nowadays technically and economically unfeasible. Nonetheless, mass spectrometry data of MHC-bound peptides, immunoproteasome processing predictive algorithms (Gubin et al., 2014), MHCI binding algorithms and information about gene expression levels (Fortier et al., 2008) could be combined to optimize MHC presentation algorithms and facilitate the identification of immunogenic neoantigens.

As neoantigens arise from somatic mutations it is reasonable to expect that tumors with high mutational load (Alexandrov et al., 2013) are more likely to present neoantigens. In fact, neoantigens are frequent in tumors with mutational loads above 10 somatic mutations per Mbp such as melanoma, lung carcinoma and colorectal cancer (Alexandrov et al., 2013; Schumacher & Schreiber, 2015). Somatic mutations are consequence of exogenous factors such carcinogens (Pfeifer et al., 2002), ultraviolet exposure and anticancer drugs (Hunter et al., 2006) as well as defects in cell intrinsic mechanisms: altered activity of the error-prone polymerase Pol ε (Cancer Genome Atlas, 2012), over activity of APOBEC family of cytidine deaminases (Alexandrov et al., 2013), inactivation of DNA mismatch repair machinery (Boland & Goel, 2010) and defects in DNA-double-strand break repair machinery. However, the presence of somatic mutation derived neoantigens does not guarantee the existence of reactive T cells (Schumacher & Schreiber, 2015).

3.4. Immunotherapy

Although tumor-specific $CD8^+$ T cells can be detected in cancer patients, they display an exhausted phenotype in the tumor (Baitsch et al., 2011). Several factors contribute to this dysfunctional state such as impaired antigen-presentation, lack of costimulation, low affinity antigen recognition, T cell inhibition and the presence of immunosuppressive soluble factors. Therefore, different immunotherapies are being developed to overcome such hurdles and boost the immune system to eliminate the tumor and cure patients that are refractory to conventional chemotherapy and radiotherapy.

Therapeutic cancer vaccines

One of the strategies to improve antigen presentation and $CD8^+$ T cell priming is vaccination. The field of therapeutic cancer vaccines has greatly evolved in the past years, whereby vaccines with different antigenic composition, delivery system, adjuvants and administration modalities have been designed. Administration of native tumor antigen peptides in combination with adjuvants (Incomplete Freund's adjuvant and CpG) elicited some specific T cell responses in melanoma patients but failed to confer clinical benefit (Parkhurst et al., 2004; Speiser et al., 2008). Vaccination with an analog tumor peptide ($gp100_{209\text{-}217}$) in combination with IL-2, however, showed clinical benefit in melanoma patients (Schwartzentruber et al., 2009), indicating that antigens that are not subject to central tolerance may be better candidates for therapeutic cancer vaccines. In fact, vaccination with idiotypes, epitopes found in the hypervariable regions of the Ig variable domain of B cells, elicited humoral and cellular responses and increased disease-free survival in follicular lymphoma patients (Inoges et al., 2006). Moreover, vaccination with neoantigen peptides in combination with poly-ICLC (Caskey et al., 2011) and percutaneous injection of neoantigen coding RNA into lymph nodes induced polyfunctional $CD4^+$ and $CD8^+$ specific T cell responses and prolonged progression-free survival in melanoma patients (Ott et al., 2017; Sahin et al., 2017).

In an attempt to enhance tumor-antigen presentation, vaccines containing peptides, proteins, whole tumor lysates or mRNA loaded DCs have also been used in clinical trials and induced specific T cell responses (Chiang et al., 2013; Linette et al., 2005). For instance, vaccination with autologous APCs loaded with GM-CSF-prostatic acid phosphatase (PAP) fusion protein (Sipuleucel-T, Provenge) has prolonged overall survival of castrate-resistant metastatic prostate cancer patients and was approved by the FDA in 2010 (Kantoff et al., 2010). Neoantigens have also been combined with DCs in

vaccine formulations and elicited neoantigen-specific T cell responses in three malignant melanoma patients (Carreno et al., 2015). Tumor antigen delivery with viral vectors or by intramuscular injection of coding DNA have also been applied, yet with poor clinical benefit (Jager et al., 2006; Lienard et al., 2004; van Baren et al., 2005; Yuan et al., 2009). Thus, cancer vaccines have shown capacity to induce specific $CD8^+$ T cell responses but still need to be improved to increase their so far modest clinical efficacy. International consortiums to converge efforts may be necessary to fully understand the human antitumor immune responses and develop clinically effective therapeutic cancer vaccines (Romero et al., 2016).

Adoptive cell therapy

Despite the detection of naturally occurring tumor specific $CD8^+$ T cells in cancer patients, they are outnumbered in tumors. That led to the development of adoptive cell therapy (ACT), which consists on isolating lymphocytes from fresh tumors, expanding them *in vitro* and transferring them back to the patients. Already in 1988, metastatic melanoma patients were treated with *in vitro* expanded autologous tumor infiltrating lymphocytes and objective regression was achieved in 60% of the patients (Rosenberg et al., 1988). Selection and expansion of tumor-reactive TILs was later applied and induced clinical remission in 46% of metastatic melanoma patients (Dudley et al., 2002). This selection process however is limited as it requires between 21 and 36 days of *in vitro* culture, not always available autologous melanoma cell lines and IFNγ-secreting TILs, that are only found in half of the patients (Besser et al., 2009). Therefore, a short-term (10-18 days), tumor reactivity independent, TIL culture was later stablished that enabled generation of young-TILs with longer telomeres and higher levels of costimulatory molecules CD27 and CD28, which led to longer *in vivo* persistence (Donia et al., 2012; Powell, Dudley, Robbins, & Rosenberg, 2005; Tran et al., 2008). Administration of these *ex vivo* young-TILs induced tumor regression in 50% of previously lympho-depleted metastatic melanoma patients (Besser et al., 2010; Besser et al., 2009). Among young-TIL products, specificities for shared-tumor antigens, mainly melanoma differentiation antigens followed by cancer/testis antigens and ultimately overexpressed antigens could be identified, but also neoantigen specificities (Kvistborg et al., 2012).

An alternative to the T cell isolation from fresh tumors is the selection of tumor specific lymphocytes from patients' peripheral blood. To do that, peripheral blood lymphocytes are cocultured with tumor antigens expressing antigen-presenting cells and after several

cycles of stimulations tumor-specific T cell clones can be generated. Infusion of tumor-specific T cell clones in combination with low dose of IL-2 led to regression of metastases and stable responses in 8/10 metastatic melanoma patients (Yee et al., 2002).

Genetic engineering has also been used to introduce tumor specific TCRs into T cells of any specificity and thus bypass tumor-specific T cell selection process. Natural occurring tumor specific TCRs but also TCRs with enhanced affinity for the tumor antigen have been applied reaching 80% of clinical response with the latest strategy in multiple myeloma patients (Rapoport et al., 2015). Another strategy to confer tumor antigen recognition is the transduction of chimeric antigen receptors (CARs) which couple antibody-mediated tumor recognition to TCR signaling domains (Gross, Waks, & Eshhar, 1989). CARs carry a B cell receptor derived single chain variable fragment on the extracellular domain and CD3ζ alone (first generation CAR) or in combination with one (second generation) or more (third generation) intracellular costimulatory domains. In addition of having around 10^5 increased affinity for the ligand ($K_D=10^{-9}$M) (Arcangeli et al., 2017) compared to self-tumor antigen specific TCRs ($K_D=10^{-4}$-10^{-5} M) (Cole et al., 2007), CARs bypass the weak MHC dependent antigen presentation in tumors. Thus, enabling HLA haplotype independent treatment of patients. CAR therapy has shown impressive results in hematopoietic malignancies, reaching up to 80-90% complete responses. Therefore, the FDA approved anti-CD19 CARs Tisagenlecleucel (Kymriah, Novartis) for pediatric and young adult patients with relapsed and/or refractory B-cell precursor acute lymphoblastic leukaemia (B-ALL) and Axicabtagene ciloleucel (Yescarta, Kite Pharma) for patients with refractory large B-cell lymphoma in 2017. Both CARs are second generation CARs containing 4-1BB and CD28 costimulatory molecules respectively (Maude et al., 2014; Neelapu et al., 2017). However, despite the high clinical efficacy of anti-CD19 CARs, toxicity is still an issue to overcome (Shank et al., 2017).

Unlike hematological malignancies, solid tumors often remain refractory to CAR therapy (Gauthier & Yakoub-Agha, 2017). Poor T cell trafficking and immunosuppressive tumor microenvironment are the main factors that contribute to the low efficacy of CAR therapy, and ACT in general, in solid tumors. Impaired T cell trafficking is linked to the lack of homing chemokine receptors expression in T cells (Chen et al., 2016) and abnormal tumor vasculature (Peske, Woods, & Engelhard, 2015; Sapoznik et al., 2012). Thus, engineered T cells expressing homing chemokine receptors such as CXCR1 and normalization of the tumor vasculature with antiangiogenic agents have shown improvement of ACT effectiveness (Sapoznik et al., 2012; Shrimali et al., 2010). Nonetheless, even if T cells

reach the tumor, they encounter a highly immunosuppressive environment that impairs their functionality. Immune cells such as $CD4^+$ Tregs, myeloid-derived suppressor cells (MDSCs) and immunosuppressive macrophages contribute to the suppression by producing TGF-β and IL-10 immunosuppressive cytokines, releasing oxygen and nitrogen radicals and/or several enzymatic activities, e.g indoleamine 2,3-dioxygenase (IDO) and arginase that deplete tryptophan and L-arginine supplies respectively (Chen et al., 2016; T. Lu et al., 2011; Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). Moreover, the tumor cells can directly inhibit T cells by secreting immunosuppressive cytokines (Coffey, Shipley, & Moses, 1986; J. Kim et al., 1995; Salomon, Ciardiello, Valverius, Saeki, & Kim, 1989) or expressing PD-L1 which binds to the TCR signaling negative regulator PD-1 receptor expressed in T cells (Blank, Gajewski, & Mackensen, 2005). Together with PD-1, many other negative regulators expressed by tumor infiltrating T cells that contribute to their reduced functionality have been described in the past years, leading to the onset of the immune checkpoint blockade (ICB) therapy field.

Immune checkpoint blockade therapy

ICB therapy aims to block negative regulators or activate stimulatory pathways to unleash full functionality of tumor-infiltrating T cells. Ipilimumab, an antibody against the CTLA-4 negative regulator of T cell responses that binds to B7 molecules (Krummel & Allison, 1995; Leach, Krummel, & Allison, 1996; Walunas et al., 1994), was the first ICB therapy brought to the clinics after successful preclinical testing (Leach et al., 1996). Phase I/II trials showed clinical response in patients with melanoma (J. S. Weber et al., 2008), renal cell carcinoma (Yang et al., 2007), prostate cancer (van den Eertwegh et al., 2012), urothelial carcinoma (Carthon et al., 2010) and ovarian cancer (Hodi et al., 2008). Moreover, two ipilimumab phase III trials showed improved overall survival in metastatic melanoma patients (Hodi et al., 2010; Robert et al., 2011).

The discovery of the PD-1/PD-L1 inhibitory pathway (Freeman et al., 2000; Ishida, Agata, Shibahara, & Honjo, 1992) led to the generation of PD-L1 and PD-1 blocking antibodies. PD-L1 phase I clinical trials induced tumor regression in melanoma, renal cell carcinoma, non-small cell lung cancer and bladder cancer patients (Brahmer et al., 2012; Powles et al., 2014). Moreover, phase III clinical trials of PD-1 blocking monoclonal antibodies showed impressive clinical response in metastatic melanoma (Robert et al., 2015) and non-small cell lung cancer patients (Borghaei et al., 2015). Thus, FDA

approved Nivolumab (OPDIVO, Bristol-Myers Squibb Company), PD-1 blocking antibody, in 2014 for the treatment of unresectable or metastatic melanoma, in 2015 for non-small cell lung cancer and advanced renal carcinoma, in 2016 for classical Hodgkin lymphoma and in 2017 for advanced or metastatic urothelial carcinoma, metastatic colorectal cancer and hepatocellular carcinoma. Pembrolizumab (KEYTRUDA, Merck & Co.), another PD-1 blocking antibody, was also approved in 2016 for recurrent or metastatic head and neck squamous cell carcinoma and in 2017 for the treatment of pediatric patients with refractory classical Hodgkin lymphoma and locally advanced or metastatic, gastric or gastroesophageal junction adenocarcinoma (https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs) . However, not all cancer patients respond to ICB therapy. Many studies are ongoing to identify predictive biomarkers. In the second part of this thesis we address the predictive value of $CD8^+$ T cells miR-155 expression levels. However, the complexity of the tumor and immune response makes it arduous. Presence of $CD8^+$ T cells and PD-L1 expression in tumor biopsies correlates with anti-PD-1 response rates (Daud et al., 2016; Eroglu et al., 2018; Tumeh et al., 2014), suggesting the need of a pre-existing adaptive immune response ("hot tumors") to profit from ICB therapy. Moreover, high mutational load is associated with increased clinical benefit (Daud et al., 2016; Eroglu et al., 2018; Hugo et al., 2016; Le et al., 2015; Rizvi et al., 2015; Tumeh et al., 2014), suggesting that anti-PD-1 treatment enhances reactivity of potentially high affinity neoantigen-specific T cells. However, some tumors with high mutational are still refractory to anti-PD-1 treatment, indicating the presence of additional intrinsic resistance mechanism (Eroglu et al., 2018). Mutations in beta-2-microglobulin (B2M) and JAK1/2 leading to the loss of MHCI expression and inability to respond to IFNγ respectively have been associated to intrinsic (Sade-Feldman et al., 2017; Shin et al., 2017) but also acquired resistance to anti-PD-1 treatment due to the selection of resistant cancer clones (Zaretsky et al., 2016). Therefore, combination of blocking antibodies against PD-1, CTLA-4 and other inhibitory receptors (TIM-3, LAG-3, VISTA and BTLA-4) as well as administration of stimulatory pathways agonists (ICOS, OX40 and 41BB) are currently been developed to bypass monotherapies limitations (Sharma & Allison, 2015). Combination of PD-1 and CTLA-4 blocking antibodies is particularly interesting due to their nonoverlapping mechanisms of action: while CTLA-4 blockade enhances T cell costimulation during T cell priming in secondary lymphoid organs, anti-PD-1 unleashes full functionality of T cells during their effector phase (Pardoll, 2012). Indeed, combination therapy showed increased clinical efficacy

compared to single treatments in patients with advance melanoma (Wolchok et al., 2013) and was approved by the FDA for the treatment of unresectable or metastatic melanoma in 2015. Nonetheless, adverse events are still an issue to overcome that scaled up in some combination therapy trials (Hassel et al., 2017).

3.5. Malignant melanoma

Human malignant melanoma is a type of skin cancer, which generates upon malignant transformation of skin melanocytes, a cell type that comprises 1-2% of epidermal skin cells and resides in the basal layer of the skin. Malignant melanoma is not the most frequent among skin cancers; however, it represents by far the most aggressive one and its incidence has raised up to 5-fold in Caucasian populations in the past three decades due to the increased exposure to ultraviolet upon sunlight exposure. In Switzerland, 30 new cases per 100,000 inhabitants were detected per year between 2008-2012 (https://www.bfs.admin.ch) and despite prevention campaigns, 40-50/100,000 inhabitants/year are expected to declare a malignant melanoma in the following decades in Europe (Leiter, Eigentler, & Garbe, 2014). Surgical resection is only applicable in melanoma when diagnosed early. Once the lesions are larger and have started to spread to distant metastatic sites, therapeutic options such as chemotherapy have only shown low efficacy. Intratumoral administration of the oncolytic virus Talimogene Laherparepvec (IMLYGIC, Amgen), a genetically modified herpes simplex virus type 1 designed to locally produce GM-CSF and selectively replicate and lyse tumor cells, elicited durable responses in unresectable melanoma patients and was approved by the FDA and EMA in 2015. Unfortunately, however, it did not prolonged overall survival of patients (Andtbacka et al., 2015). In 2011, Vemurafenib, a small-tyrosine kinase inhibitor against the mutated B-Raf protein, was FDA approved for the treatment of unresectable or metastatic melanoma patients carrying the V600E B-raf mutation, which occurs in about 50% of cutaneous melanoma (https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs). Despite early achievement of high objective response rates and improvement of overall and progression-free survival, most of the patients unfortunately developed resistance mechanisms to B-raf inhibitors and tumor progression resumed rapidly after a median of 5,3 months (Chapman et al., 2011). A number of different acquired resistance mechanisms have been described leading to reactivation of the MAPK pathway and/or upregulation of the PI3K pathway (Sullivan & Flaherty, 2013). Combination treatments

of B-raf inhibitors with other agents have been used to circumvent or delay resistance. For instance, combination of vemurafenib with the MEK inhibitor cobimetinib, approved in 2015 by the FDA, prolonged progression free survival (PFS) and overall survival compared to vemurafenib alone, yet induced increased toxicity (Larkin et al., 2014). Moreover, not all melanoma patients carry mutated B-raf and therefore are not eligible for such inhibitor treatments. Luckily, immunotherapy appeared as an alternative. Already in 1988 the first metastatic melanoma patients were treated with *ex vivo* expanded autologous TILs that could mediate objective regression of cancer (Rosenberg et al., 1988). Since then, many clinical trials of *ex vivo* expanded autologous TILs transfer after lymphodepletion have been conducted and showed objective response (OR) rates of 40-55% (Rosenberg & Restifo, 2015). In addition, circulating lymphocytes transduced with MART-1 melanoma-melanocyte antigen specific TCR have also been used and mediated tumor regression. However, the OR rate (40%) was not higher than in those trials using bulk *ex vivo* expanded TILs (R. A. Morgan et al., 2006) and toxicities from healthy melanocyte targeting were observed (Johnson et al., 2009). Among the target antigens identified in autologous TILs, tumor associated antigens have been found but also nonsynonymous mutations containing antigens (Y. C. Lu et al., 2014; Robbins et al., 2013). The concept that cancer regression after immunotherapy is due to targeting of mutated antigens could explain tumor regression without developing autoimmunity. In fact, recent therapeutic cancer vaccines using RNA or peptide neoantigens have also shown development of specific T cell responses and prolonged progression-free survival in melanoma patients (Ott et al., 2017; Sahin et al., 2017).

ICB therapy was also introduced for the treatment of metastatic melanoma shortly after targeted therapy and has shown impressive clinical results. CTLA-4 blocking antibody, ipilimumab (YERVOY, Bristol Myers Squibb Company), was the first to be approved by the FDA, in 2011, after achieving an overall response rate (ORR) of 11% and prolonged by 4 months the overall survival of metastatic melanoma patients in a randomized, double-blind clinical trial (Hodi et al., 2010). Later in 2014, PD-1 blocking antibodies were approved, which demonstrated even higher ORR in previously untreated metastatic melanoma patients (ORR, 40%) (Robert et al., 2015) as well as in patients who progressed after ipilimumab or BRAF inhibitor treatment (ORR, 30%) (Weber et al., 2015), leading to prolonged overall survival (Larkin et al., 2017). In addition, combination of PD-1 and CTLA-4 blocking antibodies for the treatment of previously untreated metastatic melanoma patients led to an even greater ORR (60%) and a 58% survival rate after 3-

years compared to the 52% and 34% survival rate in the nivolumab and ipilimumab single therapies respectively (Wolchok et al., 2017). However, ICB related toxicities are still an issue to overcome and a fraction of patients remain refractory. Thus, a better understanding of the anti-tumor immunity of melanoma tumors is necessary to improve the treatments against this aggressive type of human cancer. Preclinical studies are essential for that purpose. The B16F10 cell line (Fidler, 1973) is one of the most frequently used syngeneic mouse melanoma tumor model which was recently shown to contain immunogenic nonsynonymous somatic mutations, as described in melanoma patients (Castle et al., 2012). It is the melanoma tumor model that was used in this thesis. However, other models such as genetically engineered mouse melanoma tumor models have also been employed and are particularly interesting to unravel the role of specific molecular pathways (Perez-Guijarro, Day, Merlino, & Zaidi, 2017).

AIM OF MY THESIS

Immunotherapies have shown clinical benefit in some but not all cancer patients. Thus, my thesis project aimed at a better understanding of the key parameters governing the complex relationship between $CD8^+$ T-cells and tumors in order to improve patients' responsiveness to immunotherapies.

My thesis project has addressed the role of two $CD8^+$ T cell intrinsic parameters. In the first part, the strength of the interaction between the TCR and its tumor antigen was studied with regard to $CD8^+$ T cell differentiation and response to cancer vaccines and PD-1 checkpoint blockade. In the second part, the role of miR-155 levels on the $CD8^+$ T cell antitumor response was studied, on one hand in mouse tumor models and, on the other hand, in $CD8^+$ T cell samples from malignant melanoma patients, with an emphasis on the potential use of miR-155 levels as a positive prognostic factor.

The results described in this thesis were translated into two research articles. The first one is under review in The Journal of Immunology and the second is ready to be submitted.

PART 1: ROLE OF T CELL AFFINITY ON CD8$^+$ T CELL ANTI-TUMOR RESPONSES

Introduction

TCR-pMHC recognition is essential for T-cell activation and differentiation. In fact, TCR affinity affects T-cell-APC interaction (Moreau et al., 2012; Ozga et al., 2016) and survival (Wensveen et al., 2010) and directly correlates with the amplitude of T-cell expansion in bacterial infections without impacting phenotypic differentiation (Zehn et al., 2009). Interestingly however, low-affinity stimulation, in the LCMV clone-13 chronic viral infection model, led to decreased PD-1 expression and increased frequencies of IFNγ and TNFα-producing CD8$^+$ T-cells (Utzschneider, Alfei, et al., 2016), suggesting that in case of antigen-persistence phenotype and functionality of CD8$^+$ T-cells are impacted by the TCR-pMHC interaction strength.

Previous work has shown that tumor-specific CD8$^+$ T-cells with increased affinity for the ligand exhibit enhanced intracellular signaling, proliferation and target cell lysis *in vitro* (Dutoit et al., 2001; Schmid et al., 2010). However, unlike viral and bacterial antigens, most tumor-associated antigens (TAAs) are self-antigens. As a consequence, high affinity tumor reactive CD8$^+$ T-cells are eliminated during thymic selection and the remaining tumor-reactive T-cells found in cancer patients recognize the antigen with low avidity (McMahan & Slansky, 2007).

Cancer vaccines using TAAs have been shown to induce TAA-specific T-cell responses in cancer patients in combination or not with other therapies (Jandus, Speiser, & Romero, 2009; Murahashi et al., 2016; Reed, Cresce, Mauldin, Slingluff, & Olson, 2015). However, not all TAAs can elicit specific T-cell responses (Brinckerhoff, Thompson, & Slingluff, 2000; Legat et al., 2016), and even in cases of specific immune responses, clinical trials conferred modest clinical benefit (Lienard et al., 2004; Romero et al., 2016).

Recently, it is becoming increasingly clear that non-synonymous somatic mutations are a source of neo-antigens in cancer patients' tumors. T-cell recognition of these tumor neo-antigens is presumably of high affinity due to the absence of central tolerance. While vaccination with neo-antigens may induce tumor-protective CD8$^+$ T-cell responses (Ott et al., 2017; Sahin et al., 2017), the need to identify and formulate neo-antigen-based

vaccines in a personalized manner imposes a major hurdle and prohibitive costs. Therefore, it remains imperative to understand how T-cells differentiate and respond to vaccination with non-mutated tumor antigens and to engagement in the tumor microenvironment.

Here, we studied the role of TCR-pMHC interaction strength during peripheral priming with a peptide vaccine and in the effector phase within the tumor. To that end, we first compared the anti-tumor responses of $CD8^+$ T-cells carrying TCRs with high or low avidity for the ligand expressed by the tumor after peripheral peptide vaccination with the tumor antigen. Additionally, we also assessed the response of $CD8^+$ T-cells bearing the same TCR when encountering tumors expressing a high or low affinity antigen after high or low affinity peptide vaccination. We observed that the affinity for the antigen in the vaccine formulation and the affinity for the ligand expressed by the tumor determined the expansion and differentiation of $CD8^+$ T-cells, whereby low-affinity stimulation led to decreased tumor control. Of note, however, low affinity stimulation in the tumor led to decreased PD-1 expression and increased IFNγ and TNFα producing $CD8^+$ T-cell frequencies. Moreover, both high and low affinity stimulated $CD8^+$ TILs showed the same re-expansion capacity when rechallenged with a high affinity bacterial infection in a tumor-free host. Importantly, anti-PD-1 treatment enhanced tumor control of either high or low affinity ligand expressing tumors. Thus, TAA-specific low affinity $CD8^+$ T-cells found in cancer patients may also benefit from anti-PD-1 treatment.

Material and methods

Mice

C57BL/6 mice were obtained from Envigo, OT-1 transgenic mice from The Jackson Laboratory and OT-3 transgenic mice from Prof. Zehn (Enouz, Carrie, Merkler, Bevan, & Zehn, 2012). Mice were at least 7 weeks old at the beginning of the experiment and were maintained in conventional facilities of the University of Lausanne. This study was approved by the Veterinary Authority of the Swiss Canton Vaud and performed in accordance with Swiss ethical guidelines**.**

Generation of B16.N4 and B16.T4 cell lines

B16-F10 cell line was transduced with MigR1-N4-GFP or MigR1-T4-GFP retroviral vectors provided by Prof. Zehn. Transduced cells were cloned and cell lines with similar GFP expression were selected.

Melanoma tumor models

10^5 B16.OVA or $2{\cdot}10^5$ B16.N4 and $2{\cdot}10^5$ B16.T4 cells were subcutaneously (s.c.) engrafted on each flank of C57BL/6 mice. After six days, CD45.1 10^5 OT-1 or 10^6 OT-3 T-cells were intravenously (i.v) transferred. One day later mice were s.c. vaccinated with 10µg SIINFEKL (N4) ($OVA_{257-264}$ peptide) or SIITFEKL (T4) peptide (Protein and Peptide Chemistry Facility, UNIL) and 50µg CpG (CpG-ODN 1826, U133-L01A) (Trilink Biotechnologies). Tumors were measured manually with a caliper every two days from day 6 or 8 post-tumor engraftment. Spleens and tumors were harvested 14 and 21 days post-tumor engraftment. Spleens were mashed through a 100µm diameter filter (Falcon) and red blood cells were lysed with RBC lysis buffer (Qiagen). Tumors were dissociated with Tumor Dissociation Kit (Milenyi Biotec) following manufacturer's instructions.

Immune checkpoint blockade

B16.N4 and B16.T4 tumor bearing mice received 200µg of PD-1 blocking monoclonal antibodies (clone RMP-1-14, Rat IgG2a, BioXCell), or 2A3 isotype control (Rat IgG2a, BioXCell) on day 10, 13 and 16 post-tumor engraftment.

Listeria-monocytogenes-N4 infection

5000 flow cytometry sorted naïve OT-1 cells or OT-1 cells sorted from spleen, B16.N4 and B16.T4 tumors 14 and 21 days post-tumor engraftment were intravenously transferred into naïve C57BL/6 mice. Secondary recipients were infected the same day with N4 expressing *Listeria-monocytogenes* (Lm-N4) provided by Prof. Zehn. Eight days-post infection, spleens from infected mice were collected and processed for flow cytometry analysis of OT-1 cells.

T cell proliferation assay by BrdU incorporation

Seven days post-tumor engraftment mice received 1,8mg 5-Bromo-2'-deoxyuridine (BrdU) (B5002, Sigma) intraperitoneally (i.p), and thereafter 0.8mg/mL BrdU in drinking water until the end of the experiment. Intracellular BrdU staining was performed with APC BrdU Flow kit (BD Pharmigen) following manufacturer's instructions.

Ex vivo stimulation of OT-1 cells

Mouse splenocytes or processed tumor samples were stimulated with 10ug/mL SIINFEKL peptide or 10ng/mL Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) + 500ng/mL Ionomycin (Sigma-Aldrich) for 30min at 37°C in 10% fetal calf serum (FCS), 5mg/mL Penicillin (Invitrogen), 5mg/mL Streptomycin (Invitrogen), 10mg/mL Neomycin (Invitrogen), 0.05mM 2ß-mercaptoethanol (Invitrogen), 10mM HEPES (Amimed) in DMEM (cDMEM). BD GolgiPlug and GolgiStop (BD Bioscience) was added to the cells and incubated for another 4h at 37°C.

Surface and intracellular antibody staining for flow cytometry

Surface antibody staining was performed for 20min at 4°C with different combinations of CD3-A700 (cl.17A2, eBioscience), CD8-PE-TexasRed or CD8-A700 (cl.MCD0817, Life technologies and cl.536.7, FACS facility UNIL respectively), CD45.1 FITC (cl.A20.1, FACS facility UNIL), CD45.2 APCeF780 (cl.104, eBioscience), KLRG1-PE-Cy7 (cl.2F1/KLRG1, Biolegend), CD127-PE (cl.A7R34, eBioscience), PD-1-APC (cl29F.1A12, eBioscience), LAG-3-PE (clC9B7W, BD Bioscience) CD44-PacificBlue (cl.IM781, FACS facility UNIL), CD62L-PE-Cy5 (cl.MEL-14, eBioscience), CD39-PE-Cy7 (cl.24DMS1, eBioscience), CD122-PacificBlue (cl.TM-1b, eBioscience), CD25-PerCp-Cy5.5 (cl.PC61.5, eBioscience) and CD4-BV605 (cl.RM4-5, Biolegend) in 2mM EDTA, 2% FBS in PBS (FACS buffer). After surface staining cells were washed with

PBS and stained with LIVE/DEAD Fixable Aqua Dead (L34957, ThermoFisher) for 20 min at 4°C. Then, cells were washed again with FACS buffer. For intracellular staining of cytokines, cells were fixed and permeabilized with Intracellular Staining Permeabilization Wash Buffer (421002, Biolegend) according to manufacturer's instructions and stained for 20 min at 4°C with IFNγ-PerCp-Cy5.5 (cl.XM61.2, eBioscience), TNFα-Pacific Blue (cl. MP6-XT22, Biolegend), IL-2-PE-Cy7 (cl. JES6-SH4, eBioscience), CTLA-PE (cl. UC19-4F10-11, BD Bioscience) and GranzymeB-PE-TexasRed (GRB17, Molecular probe). Cells were finally washed and resuspended in FACS buffer for flow cytometry analysis with LSRI-II flow cytometer (BD).
For flow cytometry cell sorting, cells were sorted with FACSAria (BD) and collected in cDMEM.

Data analysis and statistics

Flow cytometry data was analysed with FlowJo (TreeStar). Graphs and statistical analysis were made with Prism (GraphPad Software). Specific statistical analyses are described in figure's captions. Overall, normality of data distribution was analysed by Shapiro-Wilk normality test. Comparison between two unpaired groups was performed by parametric Student's t-test or non-parametric Mann-Whitney test. When the two groups were paired, parametric paired t-test or non-parametric Wilcoxon test was used. For multiple comparison, a parametric 1-way ANOVA or non-parametric Krustal-Wallis test was performed followed by Tukey's multiple comparison test or Dunn's multiple comparison test, respectively. Finally, simultaneous analysis of two variables among multiple groups was performed by 2-way ANOVA or 2-way repeated measurements (RM) ANOVA followed by Tukey's multiple comparison test. P-values are coded as *:$p<0,05$; **:$p<0,01$; ***:$p<0,001$ and ****:$p<0,0001$ in figures. All results are representative of at least two independent experiments.

Results

Reduced T-cell expansion of low avidity OT-3 cells leads to weak tumor control.

To assess the role of TCR avidity in $CD8^+$ T-cell mediated anti-tumor responses, we compared naive OT-1 and OT-3 cells. TCR-transgenic OT-3 cells are ovalbumin ($OVA_{257-264}$) specific $CD8^+$ T-cells expressing a TCR which responds to OVA with lower functional avidity than OT-1 cells (Enouz et al., 2012). As we expected that OT-3 cells would expand less than OT-1 cells, we transferred 10^6 OT-3 cells or 10^5 OT-1 cells six days after B16.OVA tumor engraftment in C57BL/6 mice followed by subcutaneous vaccination with SIINFEKL OVA (N4) peptide and CpG-ODN one day later (Perret et al., 2013) (Figure 6A).

While mice receiving OT-1 cells could control B16.OVA tumor growth, those receiving OT-3 cells showed similar tumor growth to those receiving vaccination alone (Figure 6B). Despite transferring 10 times more naïve OT-3 than OT-1 cells, ~10 times lower OT-3 cell numbers were detected in spleens, tumor draining lymph nodes (dLN) and tumors 14 days post-tumor engraftment. One week later, OT-1 numbers decreased in spleen and dLNs while they remained in higher numbers in the tumor as compared to OT-3 cells (Figure 6C and 6D). Thus, curtailed peripheral expansion of low avidity OT-3 cells may largely explain the reduced tumor-specific T-cell numbers in the tumor associated with impaired tumor control.

At the peak of the response to the peptide vaccine, 14 days post-tumor engraftment, increased naïve ($CD44^{low}$ $CD62L^+$) and decreased effector ($CD44^{high}$ $CD62L^-$) T-cell frequencies were found in OT-3 compared to OT-1 cells in dLNs (Figure 6E). In addition, OT-3 cells displayed reduced PD-1 expression in dLNs (Figure 6F). In contrast, most T-cells in the tumor were $CD62L^-$ differentiated T-cells with no differences in CM ($CD44^{high}$ $CD62L^+$) and effector T-cell frequencies (Figure 6E), as well as PD-1 expression levels between OT-1 and OT-3 cells (Figure 6F). Thus, while differentiation status differs between high and low avidity T-cells in the periphery 7 days post-vaccination, no differences are found in the tumor, where mainly differentiated $PD\text{-}1^{high}$ T-cells are present.

In addition, CD127^{low} KLRG1^{high} TE frequencies were higher among intratumoral OT-3 compared to OT-1 cells in contrast with the lower MPECs/TEs ratio in low compared to high avidity T-cells at this time point in lymphoid organs and tumor (Figure 6G).

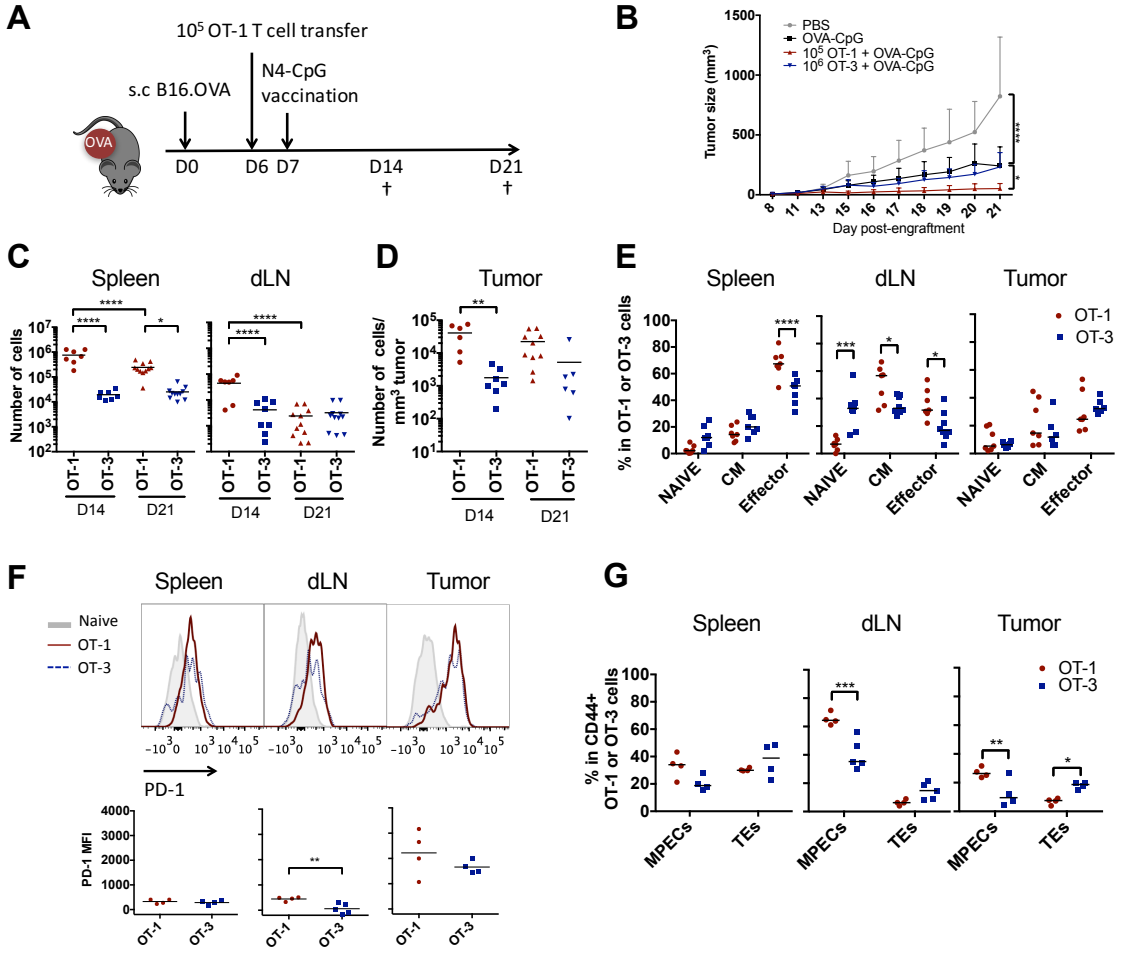

Figure 6. Low avidity OT-3 cells show reduced expansion and tumor control after peptide-CpG vaccination. **A.** Scheme of the experimental design. 10^5 B16.OVA cells were subcutaneously (s.c.) engrafted on the flank of C57BL/6J mice. After six days, 10^5 naive OT-1 or 10^6 naive OT-3 cells were transferred and mice were s.c. vaccinated with N4-CpG one day later. **B.** Tumor growth curve. Dots represent the mean tumor volume in mm^3 ± SD (N=10/group). **C.** Total OT-1 or OT-3 cell numbers in spleen and dLNs 14 and 21 days post-tumor engraftment. **D.** Numbers of OT-1 or OT-3 cells per mm^3 of tumor. **E.** Percentage of naive (CD44^{low} CD62L^{+}), CM (CD44^{high} CD62L^{+}) and effector (CD44^{high} CD62L^{-}) cells in OT-1 or OT-3 cells in spleen, dLN and tumor at day 14. **F.** Representative histograms and PD-1 mean fluorescence intensity (MFI) of endogenous naive CD8^{+} cells (grey, filled), OT-1 (red continuous) and OT-3 (blue, dotted) cells from spleen, dLNs and tumors at day 14. **G.** Percentage of MPECs (CD127^{high} KLRG1^{low}) and TEs (CD127^{low} KLRG1^{high}) in CD44^{high} OT-1 or OT-3 cells in spleen, dLNs and tumor at day 14. Dots represent individual mice and the bar the mean. OT-1 cells are represented in red and OT-3 cells in blue. A 2-way ANOVA followed by Tukey's multiple comparison test were performed in panel B, a 2-way ANOVA followed by Sidak's multiple comparison test in panels C and D, a 2-way RM ANOVA followed by Sidak's multiple comparison test in panel E and G and an unpaired t-test in panel F.

T-cell affinity during peripheral priming is critical for successful CD8$^+$ T-cell response and tumor control

To exclude the influence of cell intrinsic differences between OT-1 and OT-3 T-cells on the results, we compared OT-1 responses to high or low affinity peptide vaccines. In addition, to discriminate the impact of T-cell avidity on the CD8$^+$ T-cell response occurring in the periphery versus in the tumor, mice were engrafted with high and low affinity antigen-expressing tumors. To address the latter, we generated B16 melanoma cell lines expressing SIINFEKL (N4) wild-type OT-1 ligand or its 10 times lower affinity SIITFEKL (T4) variant (Krummey et al., 2016; Zehn et al., 2009) by transduction of B16 cells with N4-GFP and T4-GFP constructs expressing retrovirus. It was previously published that the functional avidity of OT-1 cells in response to the T4 altered peptide ligand is similar to that of OT-3 T-cells responding to the N4 peptide (Enouz et al., 2012). To normalize antigen expression levels of our newly generated B16.N4 and B16.T4 cell lines, stable clones with similar GFP expression levels were selected (Figure 7A). We also confirmed that the functional avidity of OT-1 cells to B16.T4 was lower than to B16.N4 cell line by analysing IFNγ secretion after *in vitro* restimulation of OT-1 cells with the cell lines and by an *in vitro* killing assay (Figure 7B-D).

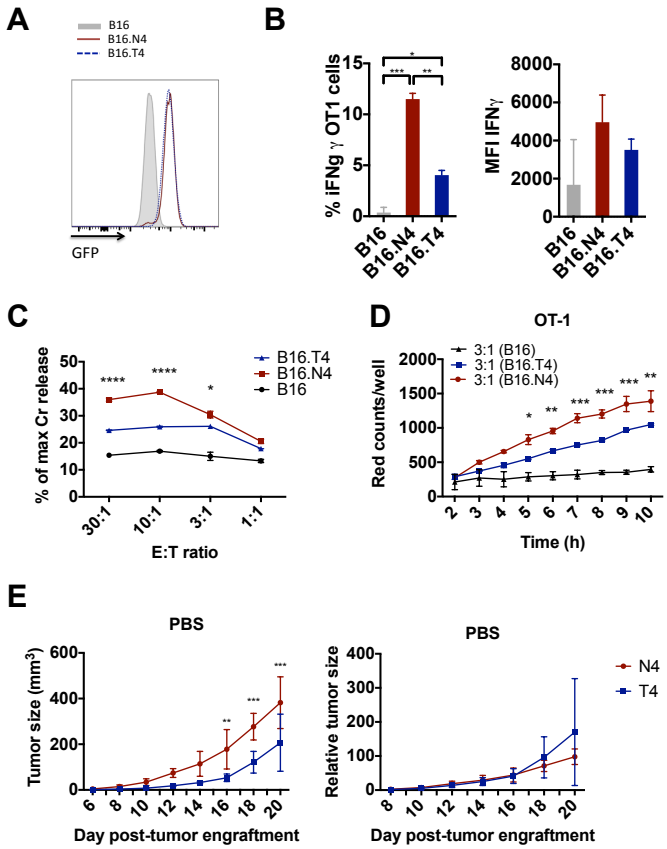

Figure 7. OT-1 cells respond to B16.T4 with lower avidity than to B16.N4 cells. A. Analysis of GFP expression by flow cytometry of B16.N4 and B16.T4 cell lines. **B.** Percentage of IFNγ$^+$ OT-1 cells and IFNγ MFI of OT-1 cells after *in vitro* stimulation of OT-1 cells with B16.N4 or B16.T4 cell lines. Bars represent the mean ± SD (n=2). **C.** Analysis of OT-1-mediated B16.N4 and B16.T4 target cell lysis by chromium release assay. Dots represent the mean ± SD of the percentage of maximum Cr^{51} release (n=2). **D.** Analysis of OT-1-mediated B16.N4 and B16.T4 target cell lysis by live imaging of co-cultures in the presence of Incucyte annexin V Red reagent. A 3:1 effector:target ratio was used. Dots represent the number of dead target cells (red counts) per well ± SD (n=2). **E.** Tumor size in mm^3 (left panel) and relative tumor size to tumor volume on day 6 post-tumor engraftment (right panel) of mice with B16.N4 or B16.T4 tumors on either flank. Dots represent the mean ± SD (n=4). A 1-way ANOVA followed by Tukey's multiple comparison test was performed in panel B, a 2-way ANOVA followed by Tukey's multiple comparison test in panel C and D and a 2-way ANOVA followed by Sidak's multiple comparison test in panel E.

To assess tumor control of low and high affinity ligand expressing tumors after high or low affinity peripheral priming, mice were engrafted with B16.N4 and B16.T4 tumors on either flank, and vaccinated with N4-CpG or T4-CpG seven days post-tumor engraftment and one day after 10^5 OT-1 T-cell transfer (Figure 8A). Since B16.N4 and B16.T4 cell lines showed different *in vivo* engraftment capacity in C57BL/6 mice (Figure 7E), values were expressed as relative tumor size to the volume at day 6, before OT-1 cell transfer.

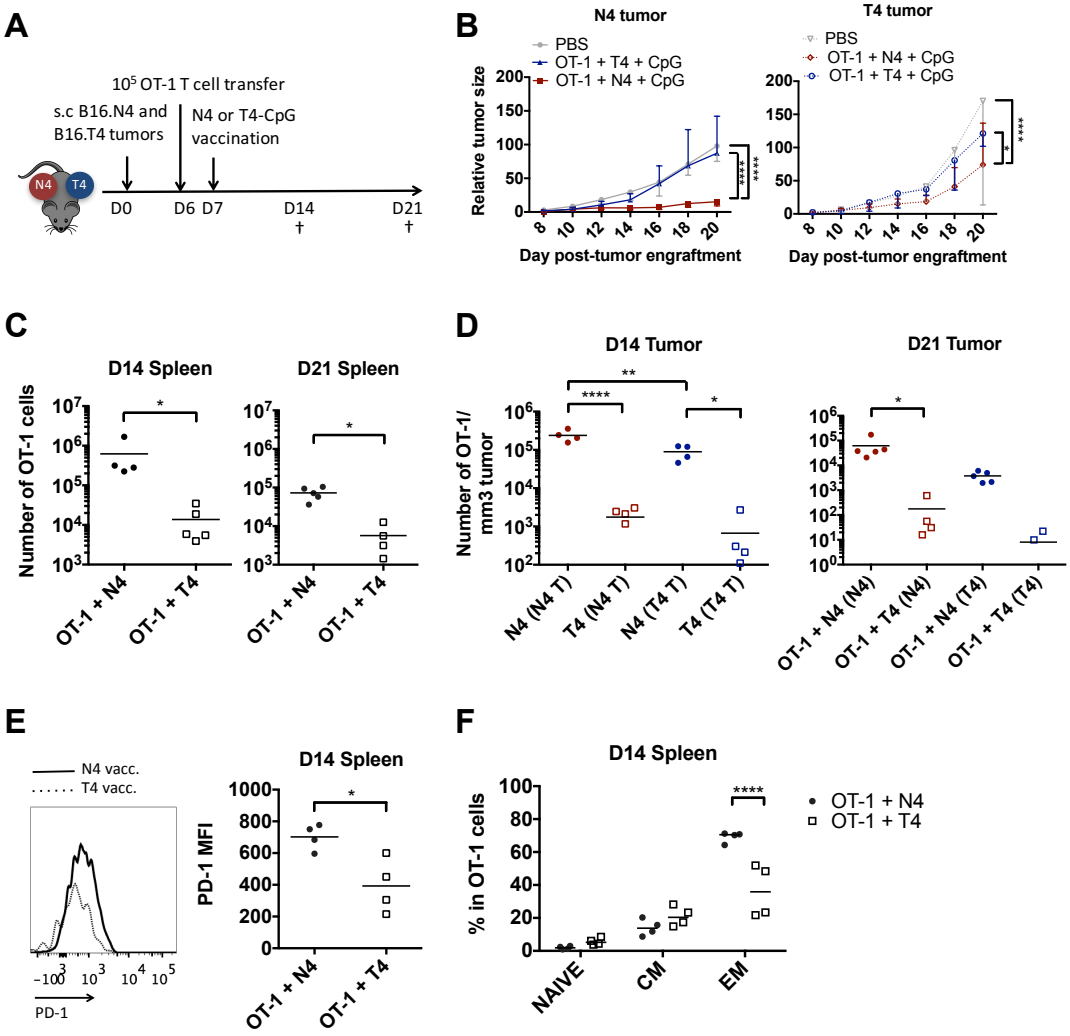

Figure 8. Low affinity peripheral priming leads to decreased OT-1 cell activation, effector differentiation, expansion and tumor control. A. Scheme of the experimental design. $2{\cdot}10^5$ B16.N4 and B16.T4 cells were s.c. engrafted on each flank of C57BL/6J mice. After six days 10^5 naive OT-1 cells were transferred and mice were s.c. vaccinated with N4 or T4 peptide in combination with CpG one day later. **B**. Tumor growth curves represented as tumor size relative to tumor volume at day 6. Dots represent the mean ± SD. n=9 in N4 vacc, n=10 in T4 vacc and n=5 in PBS group. **C.** Number of OT-1 cells in spleen of N4 or T4 vaccinated mice 14 and 21 days post-tumor engraftment. **D.** Number of OT-1 cells per mm^3 of tumor in OT-1 + N4 or OT-1 + T4 vaccinated mice in N4 (red) or T4 (blue) tumors. **E.** Representative histogram and PD-1 MFI in OT-1 cells at day 14 in spleen of mice vaccinated with N4 or T4 peptide. **F.** Percentage of naive, CM and effector cells in OT-1 cells in spleen at day 14. Dots represent individual mice and the bar the mean. A 2-way ANOVA followed by Tukey's multiple comparison test was performed in panel B, an unpaired t-test or Mann-Whitney test after Shapiro-Wilk normality test in panels C and E, a 2-way ANOVA followed by Sidak's multiple comparison test in panel D and a 2-way RM ANOVA test followed by Sidak's multiple comparison test in panel F.

Mice receiving T4 vaccination could control neither N4 nor T4 expressing B16 tumors (Figure 8B). This was associated with reduced peripheral expansion and OT-1 cell numbers infiltrating the tumor (Figure 8C and 8D). As for the vaccine primed low affinity OT-3 cells, reduced PD-1 expression and effector cell frequencies were observed in OT-1 cells after T4 vaccination compared to N4 vaccination in the spleen 7 days post-vaccination, 14 days post-tumor engraftment (Figure 8E and 8F). To exclude the possibility that the observed phenotypic differences between high compared to low affinity stimulated OT-1 cells were due to differences in the response kinetics as observed in a *Listeria monocytogenes* (Lm) infection model (Zehn et al., 2009), we followed the response of OT-1 cells after N4 or T4 peptide vaccination in peripheral blood for 21 days (Figure 9A). We observed an earlier peak of expansion upon low compared to high affinity vaccination, yet the expansion magnitude was around 10 times smaller, like in the Lm infection model (Figure 9B) as previously described (Zehn et al., 2009). Nonetheless, while complete $CD8^+$ T-cell effector differentiation had been reported upon Lm-T4 infection, we observed sustained reduced effector cell frequencies after low affinity vaccination (Figure 9C). In addition, low affinity stimulation in both vaccination and infection settings abrogated PD-1 upregulation (Figure 9D). Altogether, the affinity for the peptide vaccine impacted on $CD8^+$ T-cell activation, effector differentiation and expansion and consequently on the magnitude of tumor control.

<u>$CD8^+$ T-cell accumulation in the tumor is T-cell affinity dependent.</u>

Reduced numbers of $CD8^+$ T-cells in the tumor may explain the lack of tumor control during low affinity TCR/pMHC interaction, such as for OT-3 after N4 vaccination or OT-1 after T4 vaccination. Therefore, we compared OT-1 responses to B16.N4 or B16.T4 tumors after high affinity N4 vaccination (Figure 10A). Importantly, in contrast to low affinity vaccination, N4-primed OT-1 cells could partially control B16.T4 tumors (Figure 10B), demonstrating that high affinity T-cell priming is essential to allow a significant tumor control in case of low affinity tumor antigen recognition. Nevertheless, antigen affinity recognition in the tumor still impacts on tumor control, as OT-1 cells still controlled better N4 than T4 expressing tumors (Figure 10B).

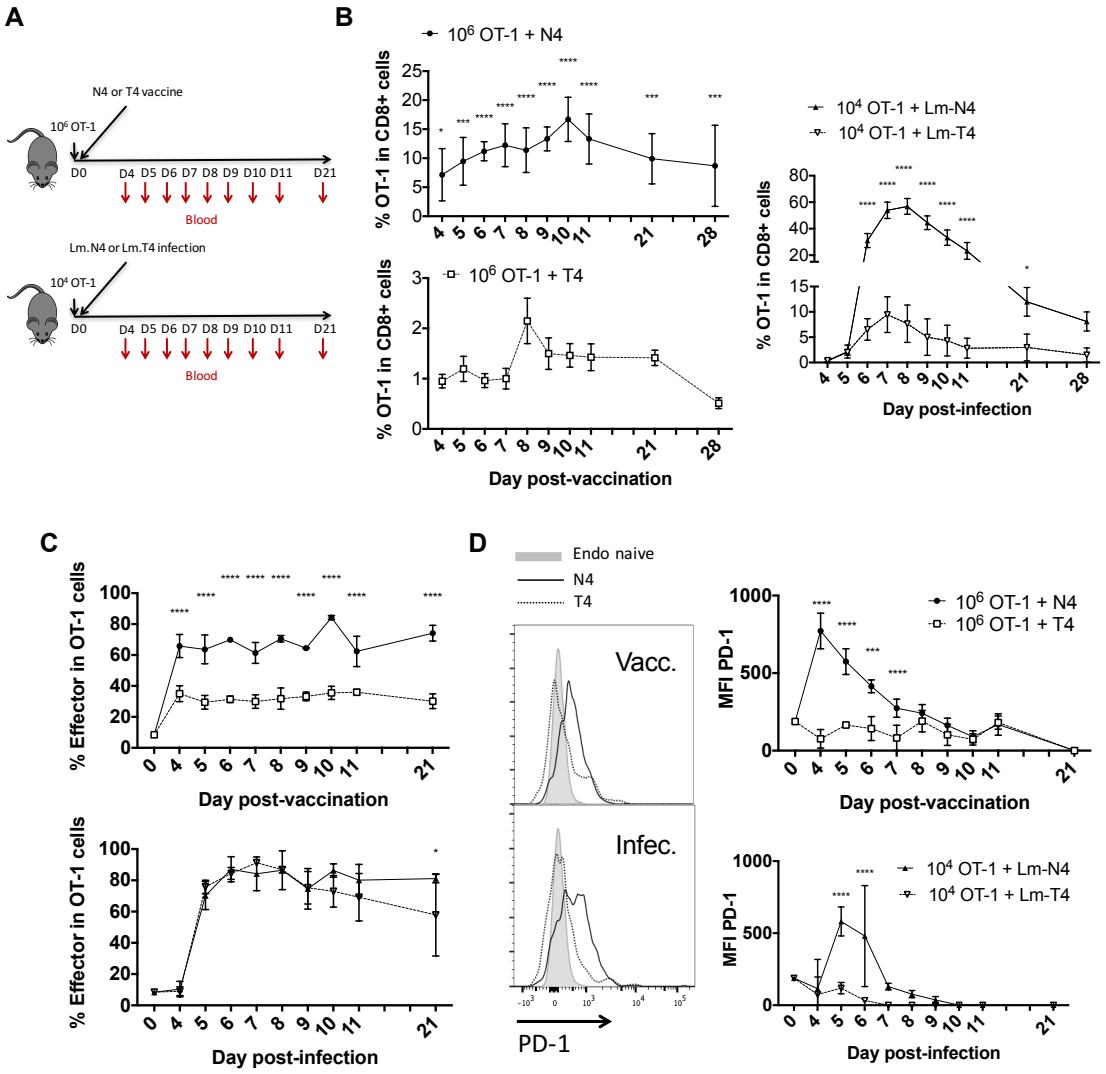

Figure 9. Low affinity CD8$^+$ T cell priming leads to earlier but curtailed expansion. **A**. Experimental design. 10^6 or 10^4 OT-1 cells were i.v. transferred to C57BL/6 mice. The same day mice were s.c. vaccinated with N4 or T4 peptide in combination with CpG or infected with 2000 CFU Lm.N4 or Lm.T4 i.v. Blood was harvested every day (every second day from each mouse) during 11 days and on day 21. **B.** Percentage of OT-1 cells in total CD8$^+$ T-cells, n=5/group. **C.** Percentage of effector (CD44^{high} CD62L$^-$) cells in total OT-1 cells. **D.** Representative histogram and quantification of PD-1 MFI of OT-1 cells. A 2-way ANOVA followed by Sidak's multiple comparison test was performed.

In agreement with the better tumor control, increased OT-1 cell numbers were found in B16.N4 compared to B16.T4 tumors 14 and 21 days post-tumor engraftment (Figure 10C), but no differences were seen in B16.N4 compared to B16.T4 tumor dLN (Figure 10D). Moreover, higher BrdU$^+$ OT-1 cell frequencies, with higher BrdU incorporation per cell, were found in B16.N4 tumors at day 21 suggesting increased OT-1 cell proliferation in B16.N4 tumors. However, at day 14, although OT-1 numbers were higher in B16.N4 tumors (Figure 10C), there was no difference in BrdU incorporation (Figure 10E, 10F and 10G), suggesting that T-cell affinity may also affect T-cell survival in the tumor, as previously shown in acute infections (Wensveen et al., 2010). To test that, we

measured expression levels of CD127 and CD25 that were shown to be important for $CD8^+$ T-cell survival (Schluns, Kieper, Jameson, & Lefrancois, 2000; Wensveen et al., 2010). Indeed, we observed increased $CD127^{high}$ MPEC frequencies (Figure 10H), as well as increased CD25 (IL-2Rα) and CD122 (IL-2Rβ) expression in OT-1 cells from B16.N4

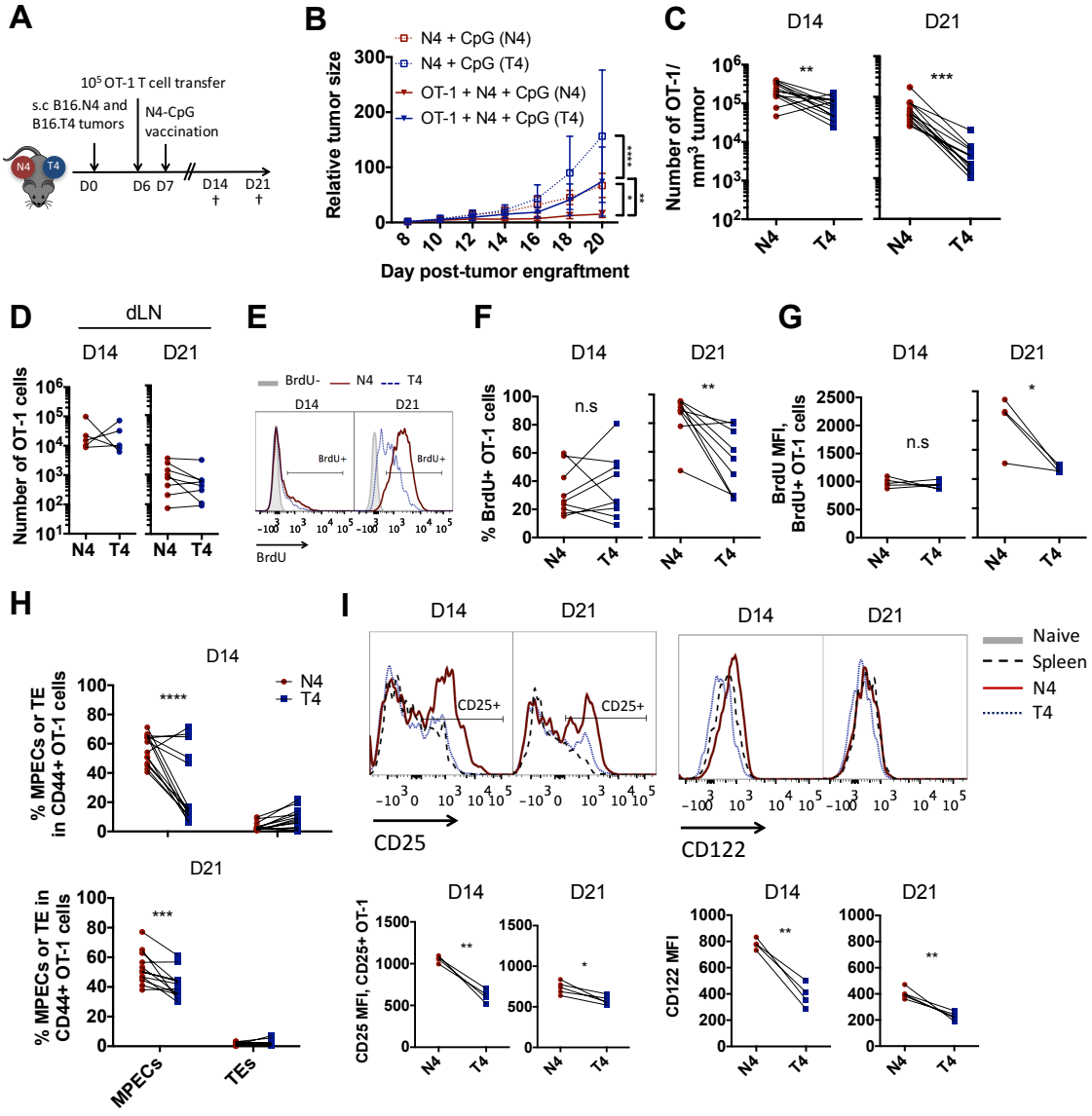

Figure 10. OT-1 cells control better high affinity antigen expressing tumors. A. Scheme of the experimental design. $2{\cdot}10^5$ B16.N4 and B16.T4 cells were s.c. engrafted on each side of C57BL/6J mice. After 6 days, 10^5 naïve OT-1 cells were transferred and s.c. vaccination with N4-CpG was performed one day later. **B.** Tumor growth curves represented as tumor size relative to tumor volume at day 6. Dots represent the mean ± SD. n=9 in OT-1 + N4 vacc group and n=5 in mice with only vaccination. **C.** Number of OT-1 cells per mm^3 14 and 21 days post-tumor engraftment. **D.** Number of total OT-1 cells in B16.N4 and B16.T4 inguinal tumor dLN. **E.** Representative histograms of intracellular BrdU staining of OT-1 cells from B16.N4 (red) and B16.T4 (blue) tumors. Endogenous $CD8^+$ from a mouse not receiving BrdU is represented as control (grey). **F.** Percentage of $BrdU^+$ cells in OT-1 cells in B16.N4 and B16.T4 tumors. **G.** BrdU MFI of $BrdU^+$ OT-1 cells in tumors. **H.** Percentage of MPECs and TEs in total $CD44^{high}$ OT-1 cells in B16.N4 and B16.T4 tumors. **I.** Representative histograms of CD25 and CD122 in OT-1 cells from spleen (black, dashed), B16.N4 (red, continuous) and B16.T4 (blue, dotted) tumors on day 14 and 21. Lower panels show quantification of CD25 MFI on $CD25^+$ OT-1 cells and CD122 MFI on total OT-1 cells. Dots represent individual mice and lines connect the two tumors of each mouse. A 2-way ANOVA followed by Tukey's multiple comparison test was performed in panel B, a paired t-test or Wilcoxon matched-pairs signed rank test after Shapiro-Wilk normality test in panels C, D, F, G and I and a 2-way RM ANOVA followed by Sidak's multiple comparison test in panel H.

tumors compared to B16.T4 tumors (Figure 10I). Thus, increased tumor control of B16.N4 tumors was probably associated with increased proliferation and responsiveness to homeostatic cytokines upon high affinity antigen recognition.

Low affinity antigen recognition leads to decreased and slower acquisition of activation markers including inhibitory receptors.

Tumor infiltrating OT-1 cells expressed increased CD44 compared to those found in the spleen. However, OT-1 cells in B16.T4 tumors had lower CD44 expression levels than in B16.N4 tumors, both at 14 and 21 days post-tumor engraftment (Figure 11A). Therefore, within the tumor, OT-1 cells upregulate CD44 in a T-cell affinity dependent manner.

The expression level of CD45RB, a CD45 isoform highly expressed in naïve cells but downregulated in effector and memory T-cells (Hermiston, Xu, & Weiss, 2003), was also modulated in a T cell affinity dependent, although in the opposite way of CD44 (Figure 11B).

Similar to the observations made when comparing OT-1 and OT-3 cells, decreased MPEC frequencies were observed in OT-1 cells from low affinity B16.T4 compared to high affinity B16.N4 tumors (Figure 10H). Thus, T-cell affinity impacts MPEC $CD8^+$ T-cell formation both upon peripheral priming and in the tumor.

As previously described for melanoma patients TILs (Ahmadzadeh et al., 2009), PD-1 expression was higher in tumor-infiltrating OT-1 cells compared to those from the periphery (Figure 8E and Figure 11C). However, PD-1 expression was lower in B16.T4 compared to B16.N4 tumors at day 14 and 21 (Figure 11C). Similarly, expression levels of CTLA-4 and CD39 T-cell exhaustion markers were strongly decreased in OT-1 cells from B16.T4 tumors compared to B16.N4 tumors (Figure 11D and 11E).

Altogether, T-cell affinity affects $CD8^+$ T-cell differentiation in the tumor. Low affinity antigen recognition leads to decreased acquisition of activation and differentiation markers including inhibitory receptors. Thus, despite numeric differences, low affinity T-cells are interesting for therapeutic purposes as they may be less susceptible to negative regulators despite their decreased tumor control.

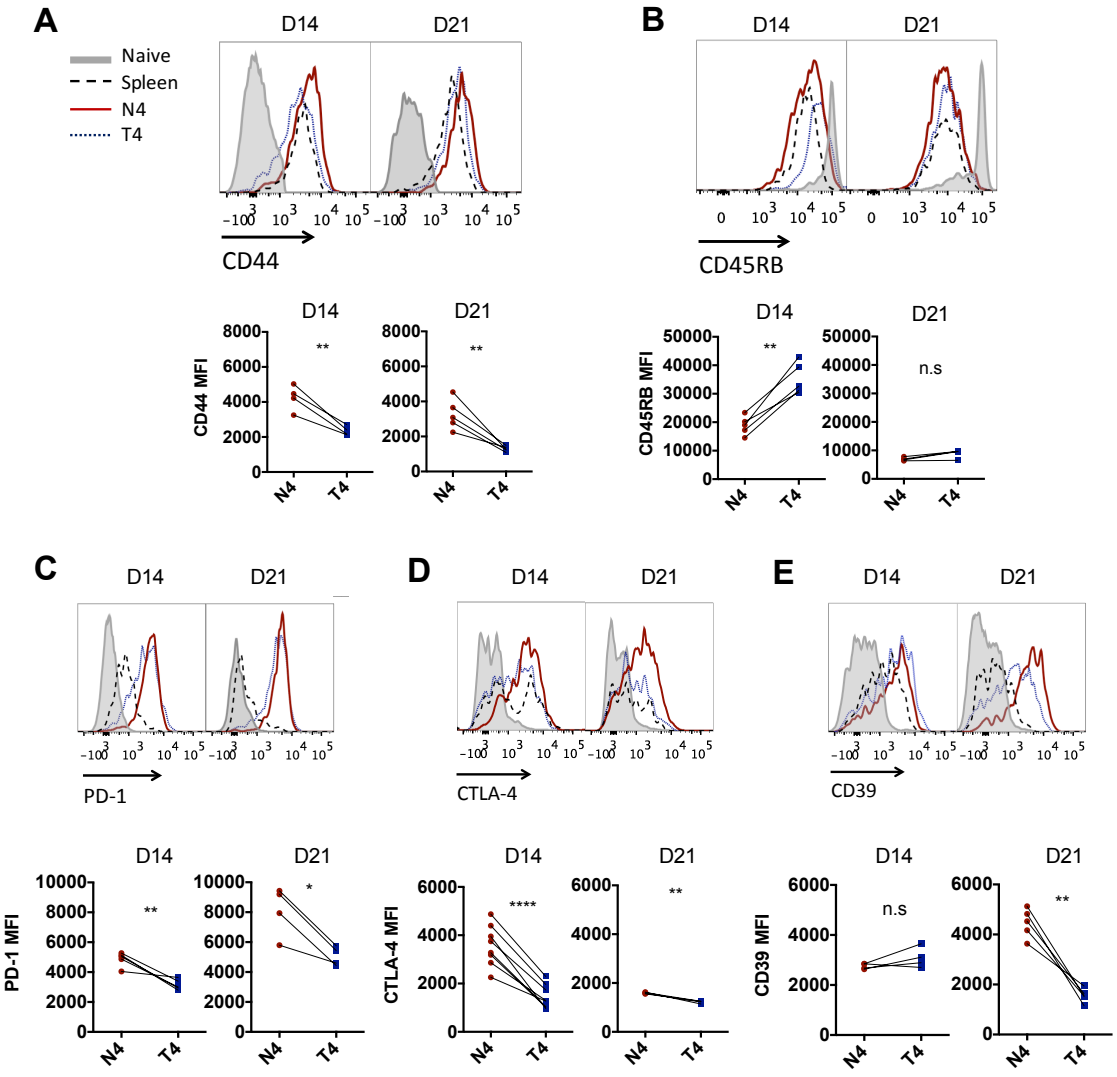

Figure 11. Decreased expression of activation markers upon low affinity antigen recognition in the tumor. **A-E.** Representative histograms of CD44, CD45RB, PD-1, CTLA-4 and CD39 in OT-1 cells from spleen (black, dashed), B16.N4 (red, continuous) and B16.T4 (blue, dotted) tumors and endogenous naïve $CD8^+$ T-cells (filled grey) 14 and 21 days post-tumor engraftment. Bellow quantification of each marker's MFI can be found. A paired t-test or Wilcoxon matched-pairs signed rank test was performed after Shapiro-Wilk normality test.

Intratumoral low affinity antigen recognition leads to decreased Granzyme B production but preserves cytokine production capacity

Decreased expression of inhibitory receptors by OT-1s in B16.T4 versus B16.N4 tumors suggests decreased susceptibility to T-cell suppression. Therefore, we assessed at day 14 the ability of OT-1 cells from spleen and B16.N4 or B16.T4 tumors to produce cytokines and express granzyme B after *in vitro* restimulation with N4 peptide. As expected, IFNγ$^+$ and TNFα$^+$ OT-1 cell frequencies were lower in tumors than in the spleen (Figure 12A and 12B), although the cytokine levels on per cell basis appeared similar in all locations (Figure 12C). Interestingly, when comparing B16.N4 and B16.T4 tumors, higher frequencies of IFNγ-producing OT-1 T-cells were found in T4 tumors (Figure 12A and 12B). Similar results were obtained when measuring TNFα (Figure 12D and 12E). However, unlike cytokine production, both the frequencies and expression levels of granzyme B in OT-1 cells were instead decreased in T4 compared N4 tumors (Figure 12F

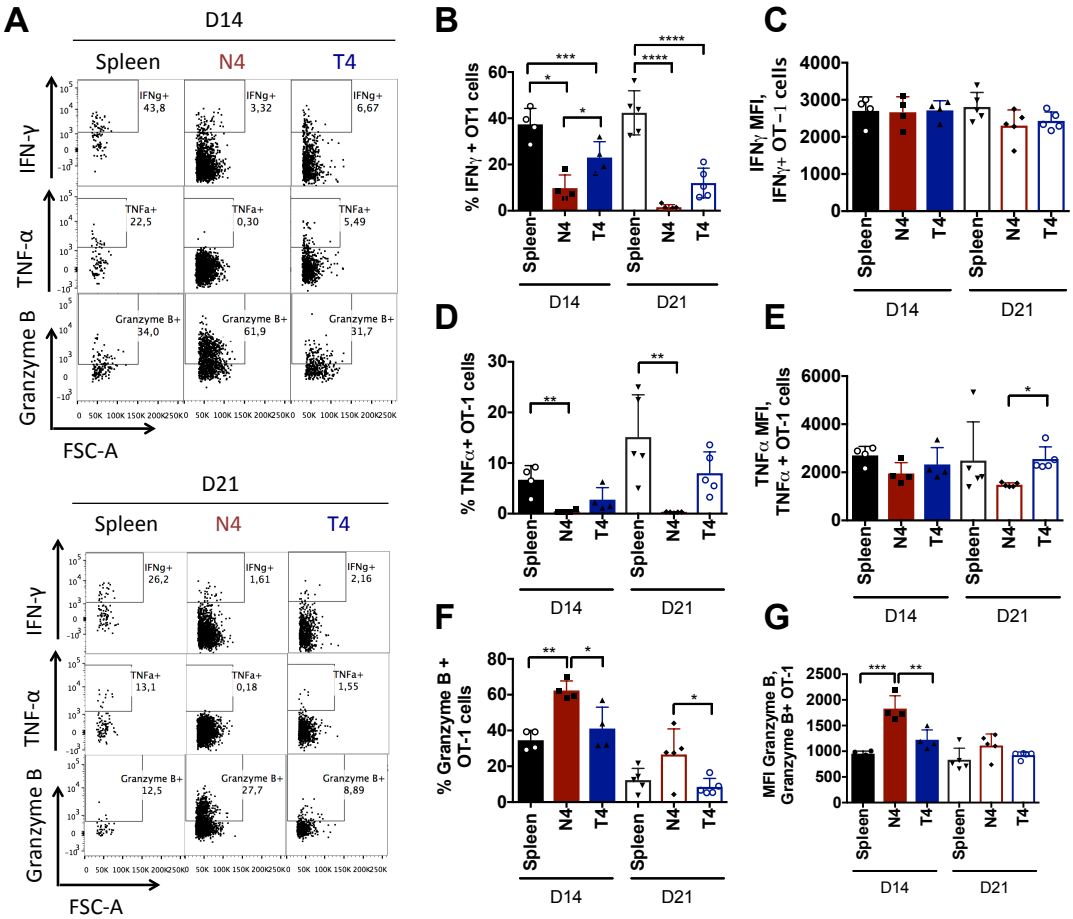

Figure 12. Increased cytokine producing OT-1 cells but reduced Granzyme B+ cells in B16.T4 compared to B16.N4 tumors. A. Representative dot plots of OT-1 cells from spleen, B16.N4 and B16.T4 tumors *in vitro* restimulated with N4 peptide 14 and 21 days post-tumor engraftment. **B, D** and **F**. Percentage of IFNγ$^+$, TNFα+ and Granzyme B$^+$ cells in OT-1 cells. **C, E** and **G**. IFNγ, TNFα and Granzyme B MFI in IFNγ$^+$, TNFα$^+$ and Granzyme B$^+$ OT-1 cells respectively. Dots represent individual mice and bars the mean ± SD (n=5). A 1 way-ANOVA or Kruskal-Wallis test followed by Tukey's multiple comparison or Dunn's multiple comparison test were performed respectively after Shapiro-Wilk normality test.

and 12G), suggesting that cytotoxic activity might be less affected by immunosuppression via PD-1 signaling, which correlates with higher tumor control of N4 tumors. Although cytokine production was further decreased when restimulating day 21 tumor infiltrating OT-1 cells, higher frequencies of IFNγ^+ and TNFα^+ cells were still found in OT-1 cells from B16.T4 compared to B16.N4 tumors (Figure 12B and 12D). Interestingly, frequencies of granzyme B^+ OT-1 cells remained lower in OT-1 cells from B16.T4 compared to B16.N4 tumors (Figure 12F and 12G).
Therefore, stimulation of $CD8^+$ T cells by low affinity antigen expressed by the tumor led to decreased cytotoxicity but preserved the ability to produce cytokines upon restimulation.

$CD8^+$ T-cells from both high and low affinity ligand expressing tumors show similar re-expansion capacity in recall tumor-free responses

To evaluate whether increased B16.N4 tumor control was due to increased OT-1 cell numbers that could compensate their exhausted profile, we assessed the fitness of B16.N4 or B16.T4 OT-1 TILs by analyzing their re-expansion capacity upon antigen reencounter in a tumor free infection settings. To this end, 5000 OT-1 cells sorted from spleen, B16.N4 or B16.T4 tumors were transferred to naïve C57BL/6 mice which were infected with 2000 CFU of Lm-N4 (Figure 13A). As control, a group of mice received 5000 naïve OT-1 cells and Lm-N4.

Strikingly, OT-1 cells from day 14 or day 21 B16.N4 and B16.T4 tumors re-expanded to a similar extent upon Lm-N4 infection (Figure 13B). When comparing OT-1 cells from day 14 tumors and spleen, we observed that OT-1 cells from tumors expanded three times less than OT-1 cells from spleen of the same tumor bearing mice (Figure 13B). However, such difference may be due to the higher frequency of CM OT-1 cells in the spleen at day 14, as at day 21, when only effector cells remained in spleen and tumors (Figure 13C), OT-1 cells showed the same re-expansion capacity regardless of their tissue origin (Figure 13B). Thus, effector OT-1 cells from the periphery or from high and low affinity tumors show the same re-expansion capacity in secondary tumor-free responses.

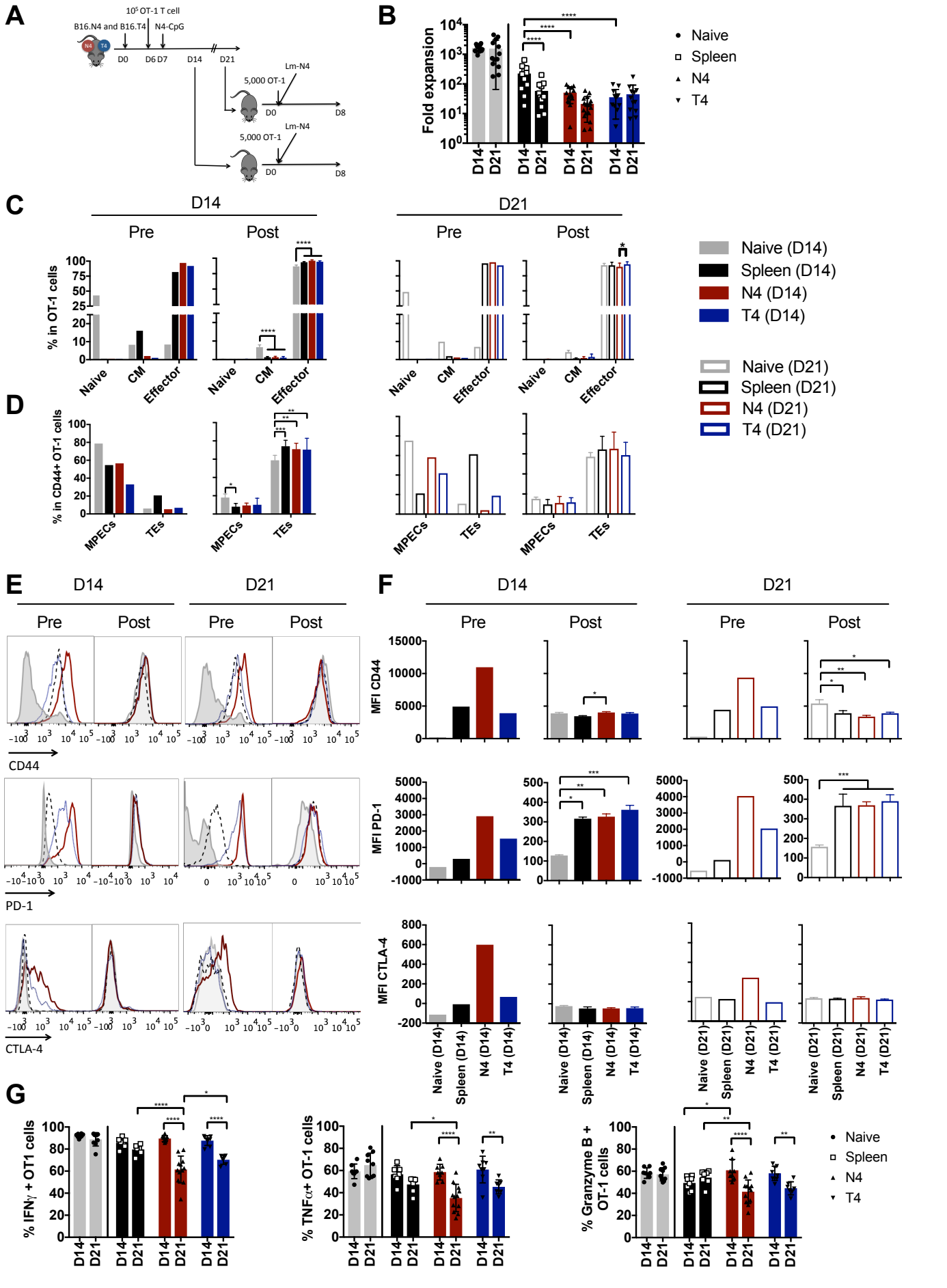
A
10^5 OT-1 T cell
B16.N4 and B16.T4
N4-CpG
D0
D6 D7
D14
D21
Lm-N4
5,000 OT-1
D0
D8
Lm-N4
5,000 OT-1
D0
D8
B
Fold expansion
Naive
Spleen
N4
T4
D14
D21
C
D14
D21
Pre
Post
% in OT-1 cells
Naive
CM
Effector
Naive (D14)
Spleen (D14)
N4 (D14)
T4 (D14)
Naive (D21)
Spleen (D21)
N4 (D21)
T4 (D21)
D
% in CD44+ OT-1 cells
MPECs
TEs
E
CD44
PD-1
CTLA-4
F
MFI CD44
MFI PD-1
MFI CTLA-4
G
% IFNγ + OT1 cells
% TNFα+ OT-1 cells
% Granzyme B + OT-1 cells

Figure 13. Tumor infiltrating OT-1 cells from B16.N4 and B16.T4 tumors show similar re-expansion capacity in secondary tumor-free responses but decreased cytokine production when exposed for a longer time to the tumor microenvironment. A. Scheme of the experimental design. 14 or 21 days post-tumor engraftment 5000 OT-1 cells from spleen, B16.N4 or B16.T4 tumors were transferred into naive C57BL/6 mice. The same day secondary hosts were infected with 2000 CFU of Lm-N4. As controls, a group of mice received 5000 naive OT-1 cells and Lm-N4 infection. **B.** Fold expansion of day 14 or day 21 OT-1 cells in spleen of secondary hosts, 8 days post-infection. **C.** Percentage of naive, CM and effector cells in OT-1 cells 14 and 21 days post-tumor engraftment pre-transfer (Pre) and 8 days post-infection in secondary hosts (Post). **D.** Percentage of MPECs and TEs in $CD44^{high}$ OT-1 cells 14 and 21 days post-tumor engraftment pre-transfer and 8 days post-infection in secondary hosts. **E.** Representative histograms of CD44, PD-1 and CTLA-4 of naive OT-1 cells (grey, filled) or OT-1 cells from spleen (black, dashed), B16.N4 (red, continuous) and B16.T4 (blue, dotted) tumors at day 14 or day 21 pre-transfer (Pre) or 8 days post- infection in secondary hosts (Post). **F.** Quantification of CD44, PD-1 and CTLA-4 MFI. **G.** Percentage of $IFN\gamma^+$, $TNF\alpha^+$ and Granzyme B^+ OT-1 cells in OT-1 cells from spleen of secondary hosts *in vitro* restimulated with N4 peptide. Dots represent individual mice and bars the mean ± SD. n=1 pre-transfer and n=8 post-infection. A 2-way ANOVA followed by Sidak's multiple comparison test was performed in panels B and G, a 2-way RM ANOVA followed by Tukey's multiple comparison test in panels C and D, and a 1 way-ANOVA or Kruskal-Wallis test followed by Tukey's multiple comparison or Dunn's multiple comparison test after Shapiro-Wilk normality test in panel F.

Moreover, 8 days-post infection, the differences in CD44, PD-1 and CTLA-4 expression levels between OT-1 cells from spleen, B16.N4 and B16.T4 tumors in primary hosts (Figure 11) were lost upon re-expansion in the secondary host (Figure 13E and 13F). Remarkably, PD-1 expression levels in secondary hosts receiving OT-1 TILs were much lower than within the tumors during the primary response (Figure 13E). Yet, PD-1 expression levels in secondary hosts receiving OT-1 cells from tumor bearing mice were higher compared to mice receiving naive OT-1 cells. In addition, while increase MPEC OT-1 cell frequencies were found in B16.N4 compared to B16.T4 tumors in primary hosts (Figure 10H), there were no longer differences in MPECs and TE OT-1 cell frequencies in secondary hosts (Figure 13D). TE frequencies were much higher in secondary hosts' spleens compared to primary hosts' day 14 and day 21 tumors (Figure 13D). Thus, upon a high affinity secondary acute infection, there is a major reset of OT-1 cells originally primed in tumor-bearing mice.

While day 14 OT-1 cells from N4 and T4 tumors showed reduced cytokine and increased Granzyme B production upon *in vitro* N4 peptide re-stimulation as compared to those from spleen (Figure 12), similar frequencies of IFNγ, TNFα and Granzyme B-producing OT-1 cells were found upon *Lm*-N4 infection of secondary hosts from all groups (Figure 13G). Nonetheless, like in the *in vitro* re-stimulated OT-1 cells from primary hosts (Figure 12), the frequencies of IFNγ, TNFα and Granzyme B producing OT-1 cells were lower in mice receiving day 21 compared to day 14 tumor-infiltrating OT-1 cells (Figure 13G). Thus, prolonged exposure to the tumor-microenvironment dampens $CD8^+$ T-cells capacity to produce IFNγ, TNFα and Granzyme B even upon rechallenge in a tumor-free environment.

Altogether, prolonged exposure to the tumor microenvironment, regardless of the affinity for the ligand, decreases the capacity of $CD8^+$ T-cells to produce IFNγ, TNFα and Granzyme B. Yet, the re-expansion capacity of tumor infiltrating $CD8^+$ T-cells in secondary tumor-free responses is preserved over time and is similar to the expansion amplitude of effector $CD8^+$ T-cells from the periphery.

Anti-PD-1 treatment enhances tumor control of both high and low affinity antigen expressing tumors.

To address the impact of anti-PD-1 blockade on T-cells activated by high or low affinity tumor antigen, mice were engrafted with B16.N4 and B16.T4 tumors. On day 6 post tumor graft, 10^5 naïve OT-1 or irrelevant P14 cells were transferred and one day later N4-CpG vaccine was administered. Anti-PD-1 or isotype control (ISO) antibodies were given three times, every three days from day 10 post-tumor engraftment (Figure 14A). PD-1 blockade enhanced OT-1 cell mediated tumor control of both B16.N4 and B16.T4 tumors, by 2.4 and 1.6 times compared to isotype control treated mice respectively (Figure 14B). These anti-tumor responses correlated with increased OT-1 numbers 21 days post- tumor engraftment in the spleen (Figure 14C), as well as to a lesser extent in tumors (Figure 14D) of anti-PD-1 compared to isotype control treated mice. In addition, *in situ* proliferation of OT-1 cells was enhanced in B16.N4 tumors of anti-PD-1 treated mice (Figure 14E and 14F), but not T4 tumors. However, when mice were grafted only with B16.T4 tumors, anti-PD-1 treatment further enhanced tumor control (by 2.8) (Figure 15B) and increased OT-1 proliferation was also observed (Figure 15C and 15D), suggesting some preferential anti-PD-1 effect in N4 tumors in B16.N4 and B16.T4 double tumor bearing mice.

At the peak of the response, at day 14 post-tumor engraftment, higher PD-1 expression levels were observed in OT-1 cells from spleen of anti-PD-1 treated mice, indicating an enhanced activation of OT-1 cells during peripheral priming (Figure 14G). At the same timepoint in the tumor, PD-1 levels were ten times higher than in the spleen, as a result from chronic stimulation, but with no difference between anti-PD-1 treated and untreated mice (Figure 14G). Strikingly however, one week later lower PD-1 expression levels were found in tumors of anti-PD-1 treated mice, while there was no more difference in PD-1 expression levels in the spleen (Figure 14G). This lower PD-1 expression in tumor

infiltrating OT-1 cells would suggest a decreased susceptibility to PD-L1/L2 mediated immunosuppression and enhanced functionality of T-cells. Thus, we checked whether cytokine production capacity of OT-1 cells was increased in anti-PD-1 treated mice. Interestingly, selectively increased IFNγ$^+$ frequencies were observed in OT-1 cells from

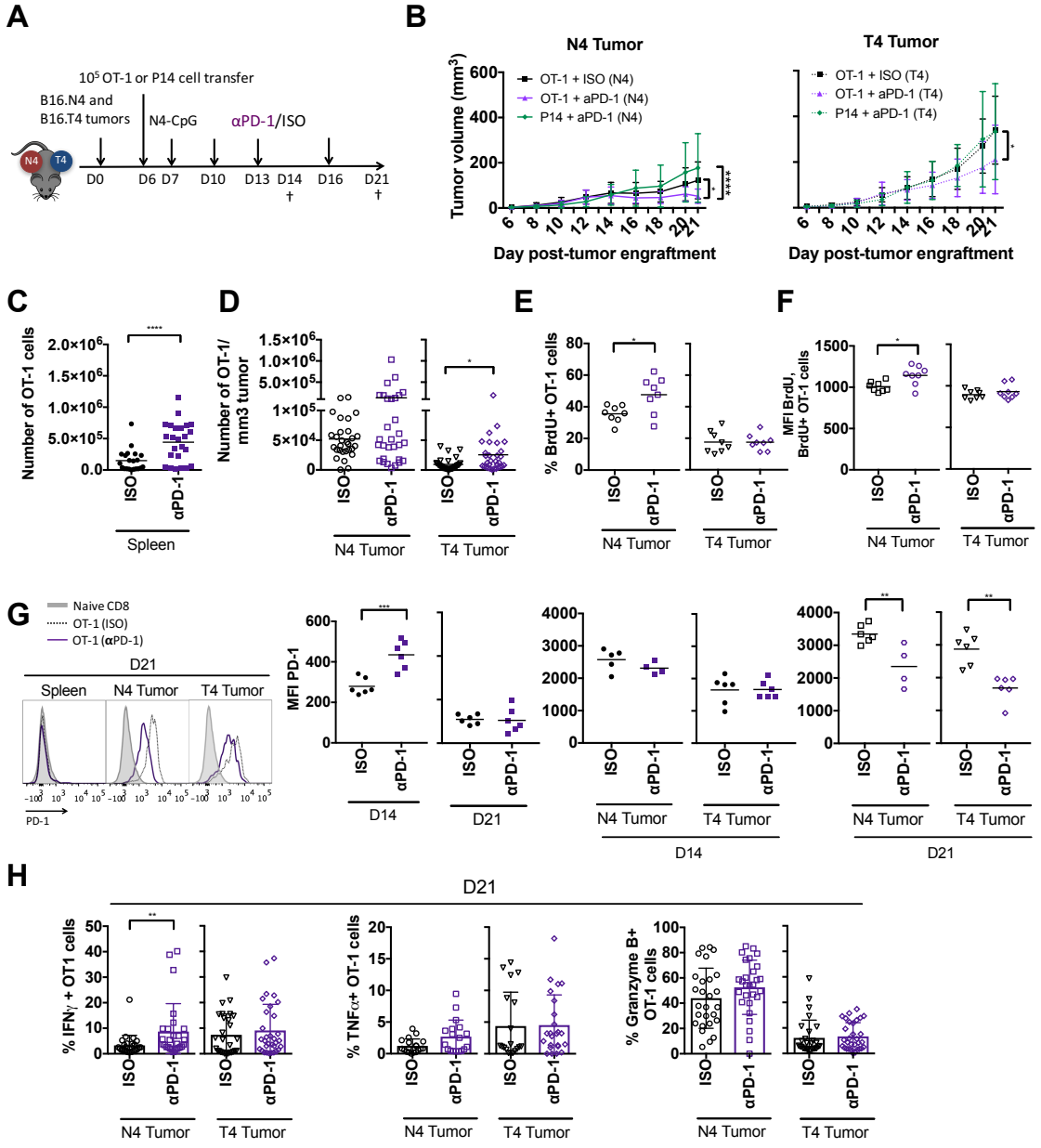

Figure 14. Anti-PD-1 enhances B16.N4 and B16.T4 tumor control. A. Scheme of the experimental design. $2{\cdot}10^5$ B16.N4 and B16.T4 cells were s.c. engrafted on each side of C57BL/6J mice. After 6 days, 10^5 naïve OT-1 or P14 cells were transferred and s.c. vaccination with N4-CpG was performed one day later. On days 10, 13 and 16 anti-PD-1 or isotype control (ISO) Abs were administered i.p. **B.** Tumor growth curves. Dots represent the mean tumor volume in mm^3 ± SD, n=8/group. **C.** Number of OT-1 cells in spleen. **D**. Number of OT-1 cells per mm^3 of tumor in B16.N4 and B16.T4 tumors. **E.** Percentage of BrdU$^+$ cells in OT-1 cells from tumors. **F.** BrdU MFI of BrdU$^+$ OT-1 cells in tumors. **G.** Representative histograms and PD-1 MFI quantification of OT-1 cells from spleen and tumors. **H**. Percentage of IFNγ$^+$, TNFα$^+$ and Granzyme B$^+$ cells in OT-1 cells from B16.N4 or B16.T4 tumors *in vitro* restimulated with N4 peptide 21 days post-tumor engraftment. Dots represent individual mice and the bar the mean ± SD. In black, isotype control antibody (ISO) and in purple anti-PD-1 antibody treated mice. A 2-way RM ANOVA followed by Tukey's multiple comparison test were performed in panel B and an unpaired t-test or Mann-Whitney test after Shapiro-Wilk normality test in panels C-H.

B16.N4 tumors on day 21, while no differences were observed in B16.T4 tumors (Figure 14H). Nonetheless, in single B16.T4 tumor bearing mice increased IFNγ$^+$ frequencies were also observed in anti-PD-1 treated mice (Figure 15E).

Altogether, anti-PD-1 treatment enhanced tumor control of both high and low affinity antigen-expressing tumors by increasing peripheral expansion of OT-1 cells resulting in higher OT-1 numbers in the tumor. Moreover, at a late tumor stage, OT-1 TILs displayed lower PD-1 expression levels and enhanced cell proliferation as well as higher IFNγ$^+$ production.

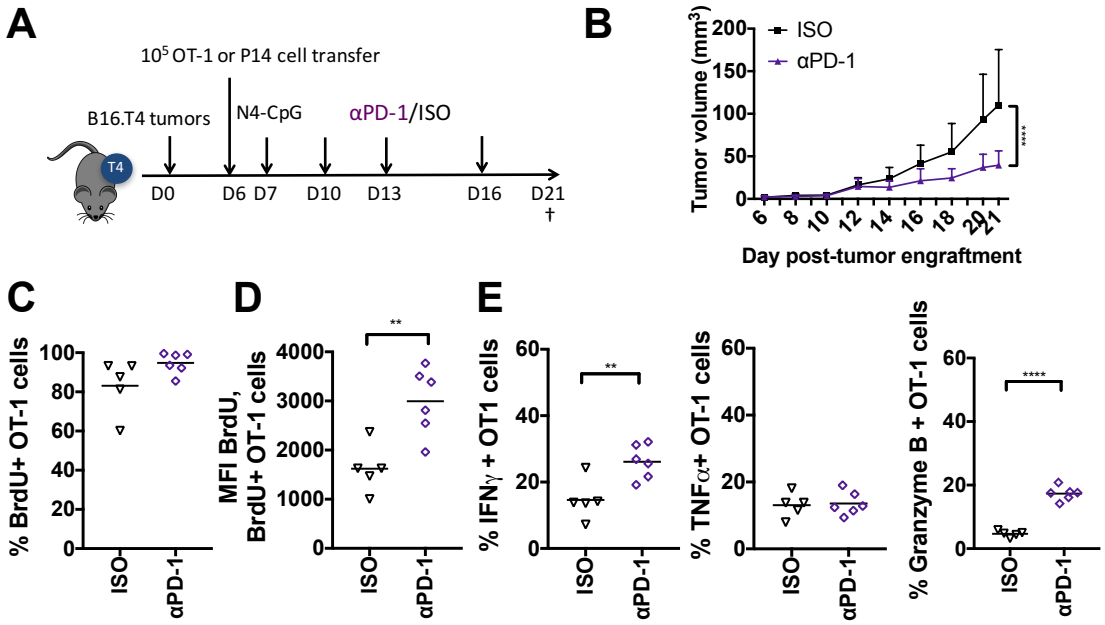

Figure 15. Anti-PD-1 treatment enhances proliferation and cytokine production of OT-1 cells in tumors of B16.T4 tumor bearing mice. **A**. Experimental design. B16.T4 single tumor bearing mice received 10^5 OT-1 cells on day 6 and N4-CpG s.c vaccination the day after. Anti-PD-1 or ISO control antibodies were given on days 10, 13 and 16 i.p. **B.** Tumor growth curves expressed as mean tumor volume ± SD, n=6/group. **C**. Percentage of BrdU$^+$ cells in OT-1 cells from B16.T4 tumors. **D.** BrdU MFI of BrdU$^+$ OT-1 cells. **E.** Percentage of IFNγ$^+$, TNFα$^+$ and Granzyme B$^+$ cells in OT-1 cells from B16.T4 tumors *in vitro* restimulated with N4 peptide. Dots represent individual mice and the bar the mean. In black ISO and in purple anti-PD-1 treated mice. A two way ANOVA followed by Sidak's multiple comparison test was performed in panel B and an unpaired t-test or Mann-Whitney test was performed after Shapiro-Wilk normality test in panels C-E.

Discussion

The weak immunogenicity of cancer cells is one of the hurdles in achieving clinically effective anti-tumor T-cell responses. Indeed, most TAAs are self-proteins and therefore tumor-reactive T-cells found in cancer patients generally recognize their antigen with low avidity (McMahan & Slansky, 2007). The advent of affordable whole exome sequencing has made possible the routine identification of mutant protein antigens (neo-antigen), for which specific high avidity $CD8^+$ T-cells may be found in blood and tumors of cancer patients (Gros et al., 2016) (Yarchoan, Johnson, Lutz, Laheru, & Jaffee, 2017). Thus, high and low avidity TAA-specific T-cells likely contribute to the overall anti-tumor response but their respective relevance is only partially understood. Moreover, the response of high and low affinity $CD8^+$ T-cells to cancer vaccines and ICB therapies has not been studied.

Our comparison of high and low affinity T-cell responses by modulating either the TCR or the antigen affinity revealed that the TCR affinity for the peptide vaccine greatly impacts the magnitude of $CD8^+$ T-cells' peripheral expansion, as previously shown in bacterial infections (Zehn et al., 2009). Curtailed expansion of low affinity primed $CD8^+$ T-cells was accompanied by reduced early PD-1 expression and effector $CD8^+$ T-cell frequencies in dLNs. Moreover, low affinity priming by systemic vaccination hampered effective control of tumors expressing either high or low affinity antigen. In contrast, high affinity priming led to a significant retardation of tumor growth regardless of the antigen recognition strength in the tumor microenvironment. These observations support the use of high affinity altered-peptide ligands to optimize therapeutic cancer vaccines (Jandus et al., 2009; Murahashi et al., 2016; Reed et al., 2015).

Although high affinity peripheral priming allowed tumor control of low affinity ligand expressing tumors, our results clearly show that the magnitude of tumor control is dependent on the affinity of tumor-associated antigen recognition by T cells recruited to the tumor. This was probably due to decreased *in situ* OT-1 cell expansion and Granzyme B expression in low affinity tumors. Yet, OT-1 cells infiltrating the low affinity antigen expressings tumors exhibited lower inhibitory receptor expression levels and higher cytokine producing cell frequencies than those thriving in the high affinity antigen expressing tumors. These results suggest increased functionality of low affinity stimulated tumor infiltrating $CD8^+$ T-cells. Notwithstanding, tumor infiltrating OT-1

cells showed the same re-expansion capacity in secondary tumor free acute responses regardless of the affinity for the antigen ligand expressed by the tumor. This outcome is similar to recent observations made in the model of specific $CD8^+$ T-cell exhaustion in the context of LCMV cl13 chronic infection. Indeed, while low affinity stimulated $CD8^+$ T-cells showed lower PD-1 expression levels in a primary chronic virus infection, high and low affinity stimulated $CD8^+$ T-cells maintained the same re-expansion capacity in a secondary acute viral infection (Utzschneider, Alfei, et al., 2016). Thus, chronic high and low affinity stimulation whether in an infection or in the tumor, do not impair re-expansion capacity of $CD8^+$ T-cells in secondary acute responses.

Interestingly the $CD8^+$ T-cell exhausted phenotype acquired in the tumor microenvironment appears to be reversible upon secondary acute infection. This contrasts with previous observations in the LCMV cl13 chronic infection model where chronically stimulated $CD8^+$ T-cells retained high PD-1 levels in a secondary acute infection (Utzschneider et al., 2013), probably due to permanent demethylation of the PD-1 locus (Youngblood et al., 2011). Therefore, it would be interesting to analyse whether epigenetic regulation of the PD-1 locus may explain the differences observed between the two models. In this regard, a recent report showed that T-cells in mouse spontaneous liver tumors may acquire the exhausted functional state by transitioning through two successive discrete chromatin states. One plastic dysfunctional state that is reversible and a fixed one in which cells become resistant to reprogramming (Philip et al., 2017). Whether this sequence of events occurs in chronically TILs on a regular basis in all types of tumors remain to be elucidated.

In the transplantable tumor model analyzed here, prolonged exposure to the tumor microenvironment led to decreased frequencies of cytokine and Granzyme B-producing OT-1 cells in both high and low affinity ligand expressing tumors. Unfortunately, while early (day 14) tumor infiltrating OT-1 cell transfer to a tumor-free environment restored cytokine and Granzyme B production capacity, a late (day 21) OT-1 cell transfer diminished OT-1 cells functionality in the secondary response. In the chronic LCMV cl13 infection similar observations were made. Albeit similar re-expansion, chronically stimulated $CD8^+$ T-cells maintained reduced cytokine production in secondary acute viral responses compared to $CD8^+$ T-cells from acute infections (Utzschneider et al., 2013). Altogether, chronic antigen stimulation regardless the affinity to the ligand leads to

decreased cytokine production but preserved re-expansion upon re-challenge with the antigen conveyed by acute infection settings.

Signaling through the PD-1 receptor is one of the immunosuppressive signals that $CD8^+$ T-cells encounter in the tumor. Pre-clinical and clinical studies have shown that antibody-mediated PD-1 blockade greatly ameliorates the outcome of cancer patients especially in the case of immunogenic tumors (Hirano et al., 2005; Iwai, Terawaki, & Honjo, 2005; Leach et al., 1996; Topalian et al., 2012; van Elsas, Hurwitz, & Allison, 1999). Our data showed that αPD-1 treatment improves the therapeutic outcome in both high and low affinity antigen expressing melanoma tumors by enhancing $CD8^+$ T-cell numbers and functionality in the tumor. Hence, low affinity TAA-specific $CD8^+$ T-cells may also well contribute to the anti-tumor activity observed in αPD-1 treated metastatic melanoma patients (Topalian et al., 2012).

Altogether, our results suggest that early vaccination with high affinity altered peptide ligands or neo-antigens, in combination with αPD-1 may be a clinically effective strategy to boost endogenous low affinity TAA-specific $CD8^+$ T-cell responses, as well as neo-antigen specific $CD8^+$ T-cell responses. In support of this notion, it is noteworthy that a recent study showed that melanoma patients with tumor recurrences after vaccination with neo-antigens had complete tumor responses following treatment with αPD-1. This was accompanied by broadening of the neo-antigen specific T-cell repertoires (Ott et al., 2017).

PART 2: ROLE AND REGULATION OF MIR-155 ON CD8$^+$ T CELL ANTI-TUMOR RESPONSES

Introduction

High tumor infiltration of memory CD8$^+$ T-cell has been associated with better clinical outcome in different types of human cancer (Fridman, Pages, Sautes-Fridman, & Galon, 2012). Nonetheless, tumors manage to evade immune control through multiple mechanisms (Beatty & Gladney, 2015). Therefore, efforts are being made to better understand the complex relationship between the immune system and the tumor and to develop new therapies. It is particularly crucial for cancers like melanoma, which are immunogenic but generally resistant to radiotherapy and conventional chemotherapy (Trinh, 2008). For instance, re-infusion of autologous tumor reactive T cells expanded *ex vivo* has shown encouraging results in malignant melanoma patients (Dudley et al., 2005; Dudley et al., 2008). However, trafficking, survival and persistence of re-infused T cells need to be optimized, and genetic engineering of T cells is a novel approach currently explored to improve T cell qualities. In particular, genetic manipulation of specific microRNAs may allow improvement of T cell fitness (Ji, Hocker, & Gattinoni, 2016).

Micro-RNAs are ~22nt long non-coding RNAs found in multicellular organisms that regulate protein expression at post-transcriptional level. miRNAs can target hundreds of mRNAs and regulate different biological processes including immune cell differentiation and function, thereby playing a role in the outcome of immune responses (Baltimore, Boldin, O'Connell, Rao, & Taganov, 2008). Recent work from our group showed that miRNAs and in particular miR-155 are regulated during human CD8$^+$ T cell differentiation and thus may play an important role on CD8$^+$ T cell function (Salaun et al., 2011).

miR-155 resides in the non-coding B-cell integration cluster (BIC), which was first characterized as a proto-oncogene associated with lymphoma development (Tam et al., 1997). MiR-155 upregulation is also observed in solid tumors, including breast, lung, pancreatic and thyroid cancers (Greither et al., 2010; Iorio et al., 2005; Nikiforova, Tseng, Steward, Diorio, & Nikiforov, 2008; Yanaihara et al., 2006). However, it also plays an essential role in the immune system (Rodriguez et al., 2007). It is upregulated upon antigen receptor stimulation in B and T cells (Haasch et al., 2002; Rodriguez et al., 2007),

as well as upon stimulation of Toll-like receptors in macrophages and DCs (Lind et al., 2015; O'Connell et al., 2007), via activation of NF-κB and AP-1 transcription factors (Kluiver et al., 2007; Yin et al., 2008). We and others have shown in miR-155 deficient mice that miR-155 is required for effector $CD8^+$ T cell responses against viruses and tumors (Dudda et al., 2013; Gracias et al., 2013). Furthermore, overexpression of miR-155 enhanced murine $CD8^+$ T cell anti-tumor responses (Dudda et al., 2013), suggesting that miR-155 is important for tumor-infiltrating $CD8^+$ T cells fitness. However, little is known about the parameters that regulate miR-155 expression levels in $CD8^+$ T cells of cancer patients.

In this study, we measured miR-155 expression levels in tumor-infiltrating $CD8^+$ T cells isolated from melanoma patients and murine tumors and compared them to miR-155 levels in $CD8^+$ T cells from peripheral blood or murine lymphoid tissues. We observed that sustained antigen recognition leads to affinity-dependent miR-155 upregulation within the tumor, which correlates with increased $CD8^+$ T cell tumor infiltration.

Material and methods

Mice

C57BL/6 mice were obtained from Envigo, OT-1 transgenic mice from The Jackson Laboratory (Stock no 003831), OT-3 transgenic mice from Prof. Zehn (Enouz et al., 2012) and P14 transgenic mice from Prof. Oxenius. Mice were at least 7 weeks old at the beginning of the experiment and were maintained in conventional facilities of the University of Lausanne. This study was approved by the Veterinary Authorities of the Swiss Canton Vaud (authorization n°1850) and performed in accordance with Swiss ethical guidelines.

Melanoma tumor models

10^5 B16.OVA or $2 \cdot 10^5$ B16.N4-GFP and $2 \cdot 10^5$ B16.T4-GFP cells (described in previous part) were subcutaneously (s.c) engrafted on each flank of C57BL/6 mice. After six days, CD45.1 10^5 OT-1 or 10^6 OT-3 T cells were intravenously (i.v) transferred. One day later mice were s.c. vaccinated with 10µg SIINFEKL (N4) peptide (Protein and Peptide Chemistry Facility, UNIL) and 50µg CpG (ODN 1826, U133-L01A) (Trilink Biotechnologies). Spleens and tumors were harvested 14 and 21 days post-tumor engraftment. Spleens were mashed through a 100µm diameter filter (Falcon) and red blood cells were lysed with RBC lysis buffer (Qiagen). Tumors were dissociated with Tumor Dissociation Kit (Milenyi Biotec) following manufacturer's instructions.

Immune checkpoint blockade

B16.N4 and B16.T4 tumor bearing mice received 200µg of αPD-1 (clone RMP-1-14, Rat IgG2a, BioXCell) or 2A3 isotype control (Rat IgG2a, BioXCell) on day 10, 13 and 16 post-tumor engraftment.

MiR-155 overexpression in P14 cells

P14 cells were isolated from spleens of P14 transgenic mice with EasySep Mouse T cell isolation kit (Stem cell, 19851A) and activated with 1:1 αCD3/CD28 Dynabeads (Thermo Fisher, 11452D) in 10% fetal calf serum (FCS, Dominique Dutscher, S1810-500), 50IU/mL/50ug/mL Penicilin/Streptomycin (Gibco, 15070-063), 0.05mM 2ß-mercaptoethanol (Gibco, 31350-010), 10mM HEPES (Amimed, 5-31F00-H), 2mM L-Glutamine (Amimed, 5-10K100-H), 1mM Sodium Pyruvate (Gibco, 11360-039) and 1x

Non-essential amino acids (Thermo Fisher, 11140-035) in RPMI 1640 with Glutamax (Gibco, 61870-010) (mouse T cell media) with 50U/mL rhIL-2 (Glaxo-IMB). Two days post-activation, P14 cells were transduced with MDHI-SCR-GFP or MDHI-miR-155-GFP retroviral vectors (provided by Dr. Gattinoni). One day later, media was replaced by 10U/mL IL-2, 10ng/mL r-human-IL-7 (Reprotech, 200-07) and 10ng/mL r-human-IL-15 (Reprotech, 200-15) containing mouse T cell media. Five days post-activation, media with 10ng/mL IL-7/IL15 was added and two days later, GFP^+ P14-SCR/miR-155 cells were FACS-sorted and transferred into B6 mice.

LCMV infection

10^3 naïve CD45.1 P14 T cells, $5 \cdot 10^3$ P14-SCR-GFP^+ or $5 \cdot 10^3$ P14 miR-155-GFP^+ cells were intravenously (i.v) transferred into CD45.2 B6 mice. One day later, mice were i.v infected with $2x10^6$ pfu of wt LCMV cl13 or 1:3 mixture of wt cl13 and A3 cl13 strains (mix infection). Both LCMV strains were provided by Prof. Dietmar Zehn.

LCMV virus titration

50uL of blood were harvested 6 and 20 days post-infection and frozen at -80°C. Serial dilutions of thawed blood were made with 10% FCS (Dominique Dutscher, S1810-500), 50IU/mL/50ug/mL Penicilin/Streptomycin (Gibco, 15070-063), 0.05mM 2ß-mercaptoethanol (Gibco, 31350-010), 10mM HEPES (Amimed, 5-31F00-H), in DMEM with Glutamax (Gibco, 31966-021) (cDMEM) in duplicates and 200uL of each dilution was mixed with 200uL of $1.6 \cdot 10^5$ Vero cells. After 3h incubation at 37°C, 400uL of 2x DMEM methylcellulose was overlaid and cells were further cultured at 37°C. 72h later, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 1x Triton, washed with 5% FCS in PBS and stained with Rat anti-LCMV nucleoprotein (clone VLA-4) (BE0106, BioXcell) for 1h at RT. After two washes with 2% FCS in PBS, Goat-anti-Rat-HRP secondary antibody was added to the cells (112-035-167, Jackson ImmunoResearch) and incubated for 1h at RT. Cells were then washed twice with 2% FCS in PBS and o-Phenylenediamine dihydrochloride (OPD) (P9187, Sigma-Aldrich) was added for 15' at RT. Finally, cells were washed twice with PBS and number of plaques were counted.

Ex vivo stimulation of P14 cells

Mouse splenocytes were stimulated with 5ug/mL $gp33_{33-41}$ (KAVYNFATM) peptide or 10ng/mL Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) + 500ng/mL Ionomycin (Sigma-Aldrich) for 30min at 37°C in cDMEM. BD GolgiPlug and GolgiStop (BD Bioscience) was added to the cells and incubated for another 4h at 37°C before cell staining.

Human samples

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor (HD) blood (*Service régional vaudois de transfusion sanguine de la Croix-Rouge Suisse*) by density gradient centrifugation on Lymphoprep (Axis-Shield PoC AS). Total $CD8^+$ T cells from HDs PB leucocytes were obtained by magnetic bead enrichment with Human $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec) or CD8 Dynabeads (Life technologies) following manufacturer's instructions.

Liquid nitrogen stored peripheral blood (PB) lymphocytes, non-infiltrated lymph nodes (NLNs), tumor infiltrated lymph nodes (TILNs) and tumors of malignant melanoma patients were thawed in 10µg/mL DNase I (SIGMA-ALDRICH), 10% fetal calf serum (FCS), antibiotic mixture (5mg/mL Penicillin, 5mg/mL Streptomycin, 10mg/mL Neomycin (Invitrogen)), 0.05mM 2ß-mercaptoethanol (Invitrogen) in RPMI medium (Invitrogen). Melanoma patients' clinical data is found in Table 1.

In vitro miR-155 expression kinetic in αCD3/αCD28 beads activated naive $CD8^+$ T cells

130000 sorted naive $CD8^+$ T cells from HDs were placed in a round-bottom 96 well plate in 50ng/mL IL-2, 8% human serum, 100U/mL Penicillin (Gibco), 100µg/mL Streptomycin (Gibco), 0.1mg/mL Kanamycin (Gibco), 2mM L-glutamine (Gibco), 1% in volume non-essential amino acids (Gibco), 1mM Na Pyruvate (Gibco), 0.05mM 2ß-mercaptoethanol (Sigma) in RPMI medium (Gibco) (human T cell media). Cells were activated with 1:2 αhCD3/αhCD28 coated Dynabeads (Life technologies) and collected in RNAlater (AM7020, Lifetechnologies) every 24h during 5 days for RNA extraction and qPCR analysis.

Multimer stimulation and T cell avidity measurement of Melan-A_{26-35} $CD8^+$-T cells by NTAmers

50000 HLA-A2/Melan-A $^{MART-1}_{26-35}$ (EAAGIGILTV) specific $CD8^+$ T cell clones from melanoma patient LAU986 were *in vitro* stimulated with 1µg/mL HLA-A2/Melan-$A^{MART1}_{26-35\ (A27L)}$ (ELAGIGILTV) multimers in human T cell media in 96-well plates for 24h before RNA extraction and miR-155 qPCR.

T cell avidity of Melan-A^{MART1}_{26-35} $CD8^+$ T cell clones was measured by NTAmer staining as previously described (Hebeisen et al., 2015). Briefly, $CD8^+$ T cell clones were stained in 50 µL of 0.2% BSA, 5 mM EDTA in PBS for 45 minutes at 4°C with HLA-A*0201 NTAmers loaded with Melan-A $^{MART-1}_{26-35\ (A27L)}$ peptide containing Cy5-labeled and biotinylated HLA-monomers multimerized through streptavidin-PE. After washing at 4°C, cells were resuspended in FACS buffer and dissociation kinetics was assessed at 4°C using LSR-II flow cytometer (BD Biosciences). Upon 30 seconds of acquisition (baseline), imidazole (100mM) was added and Cy5 fluorescence was measured during 5 to 10 minutes.

Surface and intracellular antibody staining for flow cytometry

Human HDs peripheral blood $CD8^+$ T cells and melanoma patients lymphocytes from PB, NLNs, TILNs and tumors were stained with anti-human(αh)-CD3-AlexaFluor700 (cl.UCHT1, Biolegend) αhCD4-PE (cl.RPA-T4, BD Biosciences), αhCD8-APC-Alexa750 (cl.B9.11, Beckman Coulter), αhCCR7-BrilliantViolet421 (cl.G043H7, Biolegend) and αhCD45RA-ECD (cl.2H4LDH11LDB9, Beckman Coulter).

Mouse splenocytes and dissociated tumor samples were stained with CD3-A700 (cl.17A2, eBioscience), CD8-PE-TexasRed (cl.MCD0817, Life technologies), CD45.1 FITC (cl.A20.1, FACS facility UNIL), CD45.2 APCeF780 (cl.104, eBioscience), PD-1-APC (cl29F.1A12, eBioscience), CD44-PacificBlue (cl.IM781, FACS facility UNIL) and CD62L-PE-Cy5 (cl.MEL-14, eBioscience) after H2-D^b/LCMV gp276-286 (SGVENPGGYCL) multimer staining when necessary. For intracellular staining of cytokines, cells were fixed with Fixation Buffer (420801, Biolegend) and permeabilized with Intracellular Staining Permeabilization Wash Buffer (421002, Biolegend) according to manufacturer's instructions. Cells were then stained with IFNγ-PerCp-Cy5.5 (cl.XM61.2, eBioscience), TNFα-Pacific Blue (cl. MP6-XT22, Biolegend), IL-2-PE-Cy7 (cl. JES6-SH4, eBioscience), and GranzymeB-PE-TexasRed (GRB17, Molecular probe).

For dead cell discrimination, cells were stained with LIVE/DEAD® Aqua fluorescent reactive dye (Vivid) (Invitrogen) after surface antibody staining and before fixation for intracellular staining. All stainings were performed in darkness at 4°C for 20' in 2% FCS, 2mM EDTA in PBS (sorting buffer), except the staining with Vivid, which was performed in PBS and the intracellular staining which was done in Intracellular Staining Permeabilization Wash Buffer (421002, Biolegend). Multimer staining was performed in darkness at 4°C for 60' in sorting buffer. Before and between each staining step, cells were washed with sorting buffer and finally, cells were resuspended in sorting buffer for flow cytometry analysis with LSRI-II flow cytometer (BD) or flow cytometry cell sorting (FACS) with Aria III (BD). Sorted cells were collected in RNA later (AM7020, Life Technologies).

Quantitative PCR (qPCR)

Total RNA was extracted with mirVana kit (Ambion). Mature human miRNA-155 (hsa-miR-155) and RNU44 small nucleolar RNA or mature mouse miR-155 (mmu-miR-155) and snoRNA202 were individually reverse transcribed (30 min at 16°C, 30 min at 42°C, 5 min at 85°C) with TaqMan RT MicroRNA Kit (Applied Biosystems) following manufacturer's instructions. cDNA was amplified using Universal PCR Master Mix, No AmpErase® UNG (Roche) and hsa-miR-155, RNU44, mmu-miR-155, and snoRNA202 specific TaqMan primers (Applied Biosystems) in MicroAmp® Fast Optical 96-Well Reaction Plate (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems) (40 cycles of 15s at 95°C and 60s at 60°C). Single specific qPCRs were performed in duplicates. RNU44 Ct of human samples and snoRNA202 Ct of mouse samples was subtracted to hsa-miR-155 Ct and mmu-miR155 Ct respectively to calculate relative expression (ΔCt). Expression fold change relative to a reference sample was calculated using the following formula:

$$fold\ change\ relative\ to\ a\ reference = 2^{-(\Delta Ct_{sample} - \Delta Ct_{reference})}.$$

MiR-155 *in situ* hybridization (ISH)

Multiplex ISH assay for miR-155 and U6 RNAs was performed as described (Sempere, 2014; Sempere et al., 2010). Briefly, tissue sections were incubated for 90 min at 45°C in standard hybridization solution with 100 nM of dual terminal tag miR-155 (5'FAM-tT+TA+AT+GCT+AAT+CGT+GAT+AG+GG+GTt-3'FAM) and U6(5'-biotin-tCGTGTATCCTTGCGCAGGGGCCATGCTAATCTTCTCTGTt-3'biotin) probes and

washed three times with 0.5X Saline Sodium Citrate buffer (SSC) for 10 min at hybridization temperature. Probe signal was revealed by consecutive rounds of horseradish peroxidase-mediated deposition of tyramide-conjugated fluorescein (miR-155 probe) and tyramide-conjugated rhodamine (U6 probe) using standard protocol on an automated Dako Autostainer Link 48 (Dako North America, Inc, Carpinteria, CA). Multiplex IHC assay for CD8 and Ki-67 was performed on consecutive tissues to ISH assay as described (Ensink et al., 2015). Briefly, slides were pre-treated with standard program with citrate buffer pH=6 for heat-induced epitope retrieval on Dako PT Link. Primary antibody signal (1:200 dilution of mouse anti-CD8 ([4B11], BioRad, MCA1817); 1:400 dilution of rabbit anti-Ki-67 ([SP6], Spring Bioscience, M3064)) was revealed with appropriate combination of HRP-conjugated anti-host species goat secondary antibodies and tyramide-conjugated dyes as above. All tissue slides were counterstained with DAPI. Images were acquired and multispectrally separated using the Vectra Automated Quantitative Pathology Imaging System (PerkinElmer, Waltham, MA). PerkinElmer InForm software package was used for DAPI-based cell segmentation, for quantitating expression of miR-155 with H-score function, and for enumerating Ki-67^+ and $CD8^+$ cells with cell phenotyping algorithm.

Statistical analysis

Flow cytometry data were analyzed with FlowJo (TreeStar). Graphs and statistical analysis were made with Prism (GraphPad Software). Specific statistical analyses are described in figure's captions. Overall, normality of data distribution was analyzed by Shapiro-Wilk normality test. Comparison between two unpaired groups was performed by parametric Student's t-test or non-parametric Mann-Whitney test. For multiple comparison, a parametric 1-way ANOVA or non-parametric Krustal-Wallis test was performed followed by Tukey's multiple comparison test or Dunn's multiple comparison test respectively. Simultaneous analysis of two variables among multiple groups was performed by 2-way ANOVA or 2-way repeated measurements (RM) ANOVA followed by Tukey's multiple comparison test. Long-rank Mantel-Cox test was applied for survival curves and Pearson's correlation coefficients were calculated for correlation analysis. P-values are coded as *p:<0,05; **:p<0,01; ***:p<0,001 and ****:p<0,0001 in figures.

Table 1. Clinical data of melanoma patients. LAU: code number of patients. Sex, F: female and M: male. Age: age at sampling. PB: peripheral blood, NLNs: non-infiltrated lymph nodes, TILNs: tumor-infiltrated lymph nodes and tumor sampling date. Survival in months from date of sampling, A: alive, D: dead. TNM: classification of malignant tumors. T: size or direct extent of the primary tumor. N: degree of spread to regional lymph nodes. M: presence of distant metastasis. Xx: X parameter cannot be assessed, X0: absence of X parameter. X1, X2, X3 size or extension of X parameter. U.K: Unknown.

LAU	Sex	Age	PB	NLNs	TILNs	Tumor	Treatment	Survival (months)	TNM at sampling
108	F	40	20.07.94	20.07.94	20.07.94	-	09.94, IFNα. 08.95, adoptive T cell transfer.	267 (A)	pTx N3 M0, IIIC
18	F	72	15.05.96	19.06.96	19.06.96	-	-	10 (D)	?
115	F	45	13.10.94	14.09.94	14.09.94	-	15.09.94 ILP (TNFα, Melphalan, IFNγ), 22.02.1995 chemotherapy (DTIC + tamoxifen)	77 (A)	T3b N3 Mx
162	F	55	15.05.96	03.05.95	03.05.95	-	02.96 chemotherapy (cisplatin, IL-2, IFNα)	32 (A)	pT2 N0 M0
211	M	80	29.05.97	14.08.96	14.08.96	-	14.08.1996 (TNFα, Melphalan)	29 (D)	T? N2 M0
147	F	38	10.02.05	08.02.95	08.02.95	-	01.11.01, peptide-based immunotherapy, 21.08.2002 vaccination, 21.10.2002 chemotherapy	266 (A).	T3a N2 M0
42	M	51	24.04.96	-	25.01.95	07.11.96	11.98 peptide-based immunotherapy	210 (D).	T4a N1 M0 (25.01.1996) then T4a N1 M0 (07.11.1996)
993	M	57	06.10.04	-	-	06.10.04	U.K	14 (D)	pT1a pN3 M0
820	M	76	15.12.04	-	-	16.12.04	U.K	58 (A)	T2a N1 M0
1299	M	53	24.10.08	-	24.10.08	-	U.K	14 (A)	Tx N1b M0
1318	F	81	21.01.09	-	21.01.09	-	2010 Temodal	36 (D)	pT4b pN3 pM1a
99	F	32	10.09.96	-	-	16.07.97	DTIC/fotemustine, IFN, Temodal	26 (D)	pT4 N0 M0
1256	F	36	15.07.08 and 12.11.07	-	19.02.09	-	Chemotherapy	1 (D)	pTx N3 M0
372	M	59	3.11.99	-	03.11.99	-	Radiotherapy, chemotherapy (DTIC/CDDP) and IFNα	9 (D)	T4a N3 M0
1259	F	46	03.10.07	-	03.10.07	-	Radiotherapy	14 (D)	pTx N2 M0
392	F	33	24.07.00	-	01.03.00	-	27.09.04- 13.04.05 peptide-based immunotherapy	66 (D)	pT3a N3 M0
991	M	60	15.09.04	-	15.09.04	-	Radiotherapy	4 (D)	pT4a pN2a
380	F	63	22.12.99	-	22.12.99	-	None	206 (A)	Tx pN1b M0
161	F	65	-	22.03.95	22.03.95	-	peptide-based immunotherapy and chemotherapy	15 (D)	pT2b pN3 M0
1359	M	76	02.10.09	-	28.07.10	-	None	38 (D)	pT1 pN2a M0
1255	M	87	08.08.07	-	08.08.07	-	None	31 (A)	pT2b pN2 M0
576	M	57	25.04.01	-	25.04.01	-	U.K	60 (D)	pT1 N1 M0

Results

Tumor-specific effector CD8$^+$ T cells retain high miR-155 expression levels in B16 melanoma tumors

We previously published that miR-155 overexpression in tumor-specific CD8$^+$ T cells enhances their ability to control tumor growth (Dudda et al., 2013), suggesting that miR-155 expression is important for CD8$^+$ T cell mediated anti-tumor responses. Therefore, we analyzed miR-155 expression levels in tumor-infiltrating CD8$^+$ T cells upon peptide-CpG vaccination. C57BL/6 (B6) mice were engrafted with B16.OVA tumors and after 6 days, OT-1 cells were transferred. One day later, N4 peptide and CpG were administered subcutaneously (s.c) (Figure 16A). Seven days post-vaccination (day 14), qPCR analysis of *ex vivo* endogenous effector CD8$^+$ T cells and effector OT-1 cells showed higher miR-155 expression levels in the tumor than in the spleen (Figure 16B). Similar high miR-155 expression levels were detected in TILs one week later (Figure 16B). Thus, in the tumor microenvironment, effector CD8$^+$ T-cells upregulate and maintain high miR-155 expression levels.

miR-155, a marker of antigen recognition

A major difference between the spleen and the tumor is the presence of specific antigen. OT-1 cells and tumor-specific CD8$^+$ T cells within the endogenous tumour-infiltrating CD8$^+$ T cell population are chronically exposed to antigens expressed by tumors. To assess whether chronic antigen exposure is the cause of increased miR-155 expression in tumor-infiltrating CD8$^+$ T cells we assessed miR-155 expression in CD8$^+$ T cells from a chronic viral infection. For that we used the clone-13 (cl13) lymphocytic choriomeningitis virus (LCMV) chronic viral infection mouse model. Gp33 LCMV antigen specific naïve CD8$^+$ T cells (P14) were transferred into B6 mice and 7 and 21 days after infection, miR-155 expression levels were analyzed in *ex vivo* sorted P14 and endogenous gp276-specific CD8$^+$ T cells from the spleen (Figure 16C). Already at day 7, P14 and gp276-specific CD8$^+$ T cells showed respectively 20 and 15 times higher miR-155 expression levels than naïve CD8$^+$ T cells (Figure 16D), similarly to our observations in tumor infiltrating CD8$^+$ T cells (Figure 16B). Such high miR-155 expression levels were also observed three weeks post-infection, indicating that chronic antigen exposure leads to increased miR-155 expression. Of note, P14 cells tend to have higher miR-155 expression levels than gp276 specific CD8$^+$ T cells. Such difference could be due to T

cell affinity differences: while P14 cells are high affinity monoclonal cells, gp276tet$^+$ cells are polyclonal cells with probably a range of different affinities. Antigen presentation differences could also explain the difference in miR-155 expression levels as the affinity of gp33 peptide to MHC class I molecules is higher than the affinity of gp276 peptide and is reflected in epitope immunodominance (van der Most et al., 1998; Wherry et al., 2003).

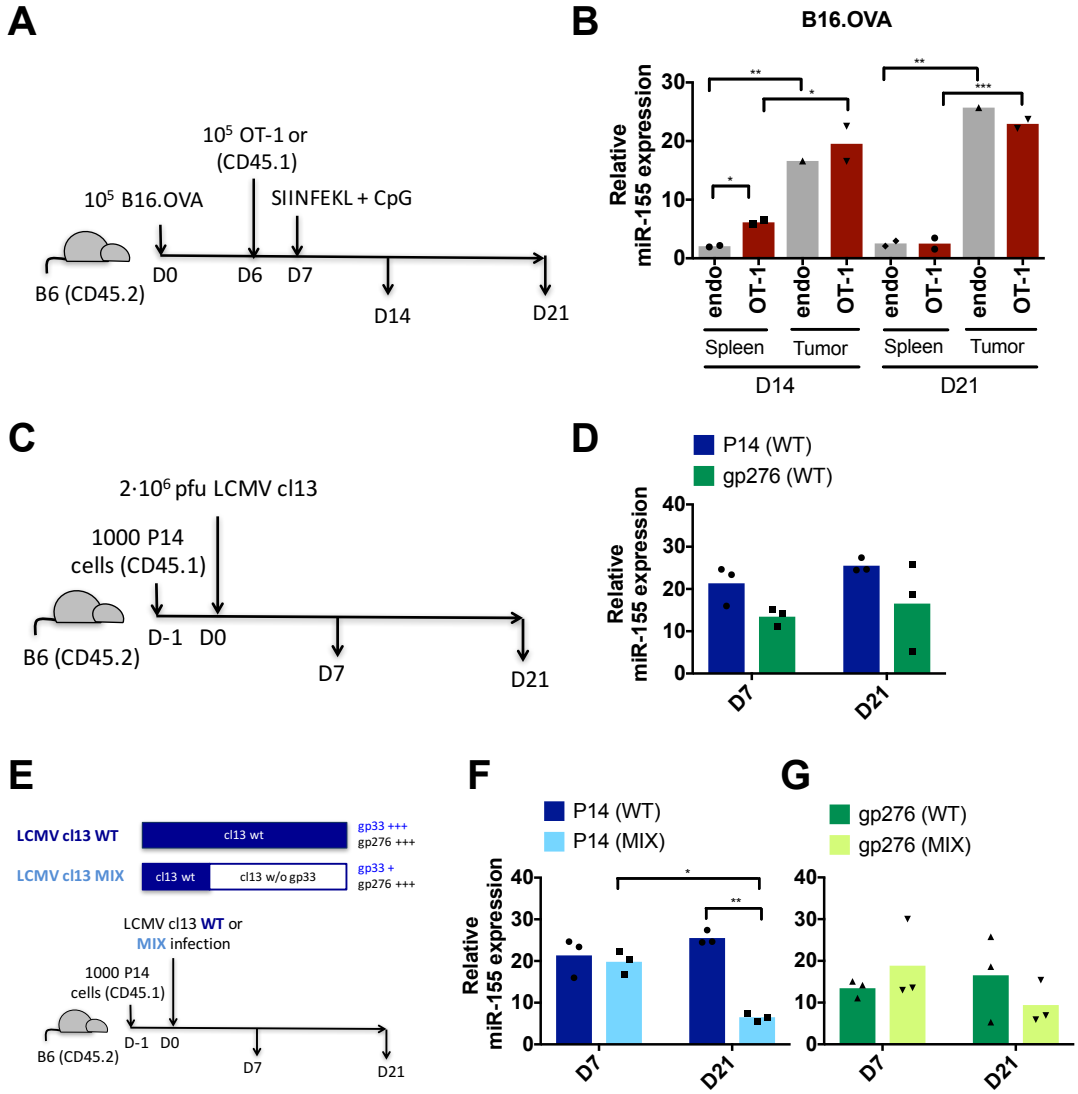

Figure 16. miR-155 expression levels reflect antigen exposure. A. Scheme of the experimental design. Six days after B16.OVA tumor engraftment, 10^5 CD45.1 OT-1 were transferred into CD45.2 B6 mice. One day later mice were s.c. vaccinated with N4-CpG. **B.** miR-155 expression levels in endogenous CD8$^+$ effector and effector OT-1 cells from spleen and B16.OVA tumors 14 and 21 days post-tumor engraftment. **C.** Scheme of the experimental design. CD45.2 B6 mice were i.v infected with 2x10^6 pfu LCMV cl13 one day after 10^3 CD45.1 P14 T cell transfer. **D**. miR-155 expression levels in P14 and endogenous gp276$^+$ CD8$^+$ T cells from the spleen 7 and 21 days after wt cl13 LCMV infection. **E.** Scheme of the experimental design. CD45.2 B6 mice were infected with 2x10^6 pfu of wt LCMV cl13 or 1:3 mixture of wt cl13 and A3 cl13 strains (mix infection) one day after 10^3 CD45.1 P14 T cell transfer. **F**. miR-155 expression levels in P14 cells 7 and 21 days after wt or mix LCMV cl13 infection. **G.** miR-155 expression levels in endogenous gp276$^+$ CD8$^+$ T cells after wt or mix LCMV cl13 infection. Bars represent mean of miR-155 expression as fold change relative to levels in host naïve CD8$^+$ T cells from the spleen. In panel B, each dot represents miR-155 expression levels of pooled cells from 5 mice. In panel D, F and G, each dot represents cells of individual mice. A 2-way ANOVA followed by Sidak's multiple comparison test was performed on non-normalized ΔCt data.

To confirm the role of antigen exposure on the regulation of miR-155 expression, P14 cells exposed to different gp33 antigen doses but same inflammatory environment were analyzed. Mice were infected with wild-type (wt) cl13 LCMV or a 1:3 mixture of wt cl13 LCMV and cl13 A3 LCMV strain, which lacks the gp33 epitope (Figure 16E). Although miR-155 expression levels at day 7 were similar in effector P14 cells from wt and mix infection, at day 21, effector P14 cells from the mix infection showed reduced miR-155 expression (Figure 16F). gp276 specific $CD8^+$ T cells, on the contrary, showed same miR-155 expression levels in wt and mix infection (Figure 16G). To confirm that only gp33 antigen load and not total viral load differ between wt and mix infection, total LCMV cl13 virus titers were measured in blood of wt and mix infected mice. Indeed, similar virus titers were observed in wt and mix infected mice (Figure 17A). Therefore, the use of the mix LCMV cl13 model allowed us confirming that in the same inflammatory environment, the amount and frequency of antigen exposure determines $CD8^+$ T cells miR-155 expression levels.

<u>miR-155-overexpression enhances $CD8^+$ T cells expansion and cytotoxicity in LCMV cl13 chronic infection</u>

$CD8^+$ T cells from tumors and chronic viral infections display an exhaustion profile characterized by increased expression of inhibitory receptors such as PD-1 and reduced cytokine production after *in vitro* restimulation (Baitsch et al., 2011; Moskophidis, Lechner, Pircher, & Zinkernagel, 1993; Wherry et al., 2007; Zippelius et al., 2004) (Figure 11 and 12). Chronic antigen stimulation has been proposed as one of the main T cell exhaustion drivers (Wherry et al., 2003). Indeed, using the LCMV mix infection model, it was previously shown that in the same inflammatory environment, antigen exposure plays an important role in the acquisition of an exhausted phenotype by $CD8^+$ T cells (Utzschneider, Alfei, et al., 2016). Along these results, we also observed that P14 cells from the mix infection showed reduced PD-1 expression (Figure 17B and 17C) and increased cytokine production upon *in vitro* peptide restimulation compared to P14 cells from wt infection (Figure 17D and 17E). Thus, increased miR-155 expression levels were accompanied by decreased P14 cell functionality 21 days after wt infection. To further determine whether high miR-155 expression levels are detrimental and linked to the exhausted phenotype observed in the P14 cells after wt LCMV cl13 infection we analyzed the functionality of miR-155-overexpressing P14 cells after wt or mix LCMV infection. To this aim, naïve P14 cells were *in vitro* activated and transduced with MDHI-miR-155-

GFP or MDHI-SCR-GFP retroviral vectors. Seven days post-transduction, 5000 sorted P14 GFP+ cells were transferred to naïve B6 mice and the same day, mice were infected with LCMV wt or mix infection.

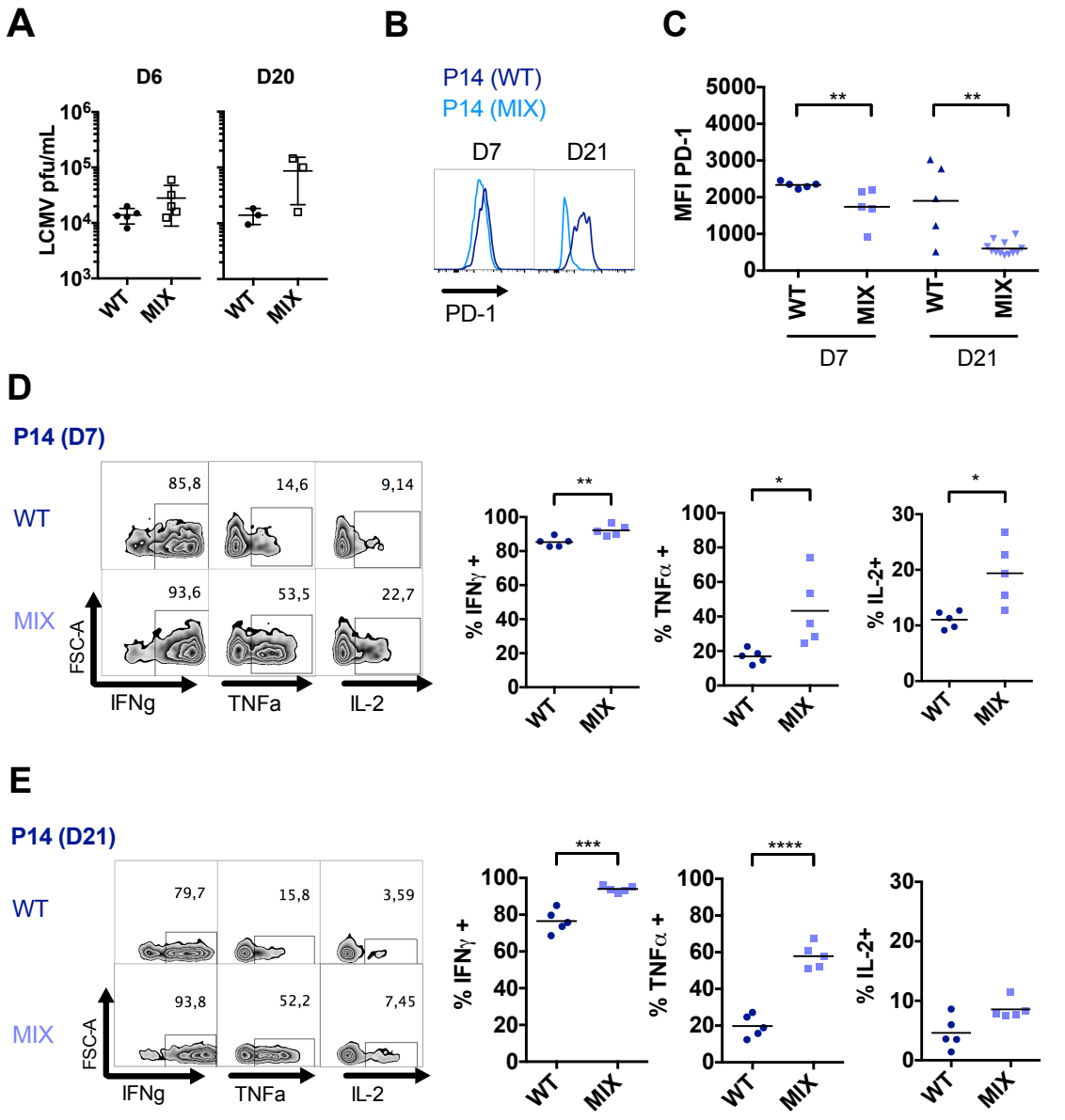

Figure 17. Low antigen dose leads to enhanced functionality of P14 cells upon chronic LMCV cl13 infection. A. LCMV cl13 viral titers expressed as plaque forming units per mL of blood of mix and wt infected mice 6 and 20 days post-infection. **B.** Representative histograms of PD-1 expression of P14 cells from wt or mix infection, seven and 21 days post-infection. **C.** PD-1 MFI quantification. **D** and **E.** Representative histograms and quantification of IFNγ$^+$, TNFα$^+$ and IL-2$^+$ P14 cell percentage in total P14 cells after *in vitro* gp33 peptide re-stimulation of splenocytes, 7 and 21 days after infection respectively. Lines represent the mean and symbols individual mice. An unpaired t-test or Mann-Whitney non-parametric test was performed after Shapiro-Wilk normality test.

QPCR analysis of *ex vivo* sorted P14-GFP$^+$ cells confirmed that P14-miR-155 cells expressed higher miR-155 expression levels than SCR-transduced cells during chronic LCMV infection (Figure 18A). In both infection models, miR-155-overexpressing P14

cells showed increased expansion by day 7 post-infection and were still detectable at day 21 in contrast to P14-SCR cells (Figure 18B), while viral titers were similar in the blood of all mice (Figure 18C). However, on day 7, PD-1 expression levels were higher in miR-155 overexpressing P14 cells (Figure 18D). This could indicate a stronger activation but also increased exhaustion of miR-155 overexpressing P14 cells. Thus, we checked their capacity to produce cytokines upon *ex vivo* peptide restimulation. While IFNγ production was similar, decreased TNFα producing cell frequencies were observed in P14-miR-155 compared to P14 SCR cells. However, this was only observed in the WT LCMV cl13 infection and the amount of TNFα produced per cell was similar (Figure 18E and 18F). Thus, miR-155 overexpression did not lead to striking differences in IFNγ and TNFα production 7 post infection. In contrast, increased granzyme B production was observed in P14-miR-155 compared to P14-SCR cells (Figure 18E and 18F). Therefore, miR-155 overexpression leads to increased cytotoxic capacity, expansion and probably survival of $CD8^+$ T cells in chronic infection regardless of the antigen dose.

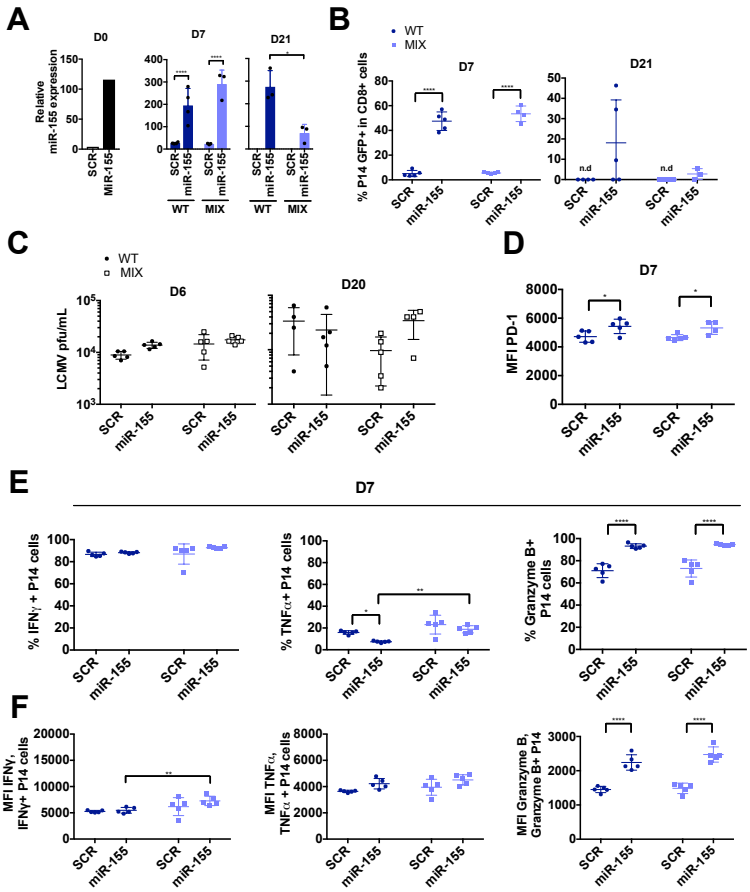

Figure 18. miR-155 overexpression enhances P14 cell expansion and cytotoxic potential in LCMV cl13 wt and mix infection. A. Mir-155 expression levels of P14-miR-155-GFP$^+$ or P14-SCR-GFP$^+$ cells before transfer or 7 and 21 days post-infection represented as fold upregulation relative to levels in naïve P14 cells. **B.** Percentage of P14-SCR or P14-miR-155 cells in total $CD8^+$ T cells. **C**. LCMV cl13 viral titers expressed as pfu per mL of blood of mix and wt infected mice 6 and 20 days post-infection. **D**. PD-1 MFI of P14-miR-155 or P14-SCR cells in the spleen 7 days post-infection. **E.** Percentage of IFNγ$^+$, TNFα$^+$ and Granzyme B$^+$ cells in total P14-SCR or P15-miR-155 cells upon *in vitro* gp33 stimulation of splenocytes 7 days-post infection. **F**. IFNγ, TNFα and Granzyme B MFI in IFNγ$^+$, TNFα$^+$ and Granzyme B$^+$ P14-SCR or P14-miR-155 cells. Dots represent individual mice and the line the mean ± SD. A 2-way ANOVA followed by Sidak's multiple comparison test was performed except in the right panel (D21) of figure A where an unpaired t-test was performed after Shapiro-Wilk normality test. n.d. Non-detected, <0,005% in $CD8^+$cells

MiR-155 expression levels in low affinity tumors positively correlate with tumor control

Most TAAs are self-antigens (Byrne et al., 2011). Therefore, only low avidity specific $CD8^+$ T cells recognizing these antigens overcome thymic selection and are found in cancer patients. Nonetheless, tumor neo-antigens arisen from somatic mutations have recently been described in cancer patients' tumors as well as $CD8^+$ T cells able to recognize them (Gros et al., 2016; Robbins et al., 2013). In contrast to TAA-specific $CD8^+$ T cells, neo-antigen specific $CD8^+$ T cells could potentially recognize their antigen with high affinity due to the absence of central tolerance. Thus, we wondered whether miR-155 expression levels differ between high and low avidity T cells that can likely be found in cancer patients. To address this, naïve high avidity OT-1 or low avidity OT-3 OVA-specific $CD8^+$ T cells (Enouz et al., 2012) were transferred to B16.OVA bearing mice and miR-155 expression levels were analyzed in spleen and tumors after N4-CpG vaccination. While naïve OT-1 and OT-3 cells expressed similar miR-155 expression levels (Figure 19A), effector OT-3 cells expressed lower miR-155 expression levels than OT-1 cells in spleen and tumors at day 14 post tumor engraftment. On day 21, OT-3 cells maintained lower miR-155 expression levels than OT-1 cells in the tumor, whereas there did not differ anymore in the spleen. Nonetheless, like OT-1 cells, OT-3 cells consistently showed higher miR-155 expression levels in tumors than in spleen (Figure 19B).

It was shown that the strength of the TCR-ligand interaction affects the expansion and differentiation of $CD8^+$ T cells upon infection (Knudson, Goplen, Cunningham, Daniels, & Teixeiro, 2013; Zehn et al., 2009). Likewise, we observed in the first part of my thesis a decreased expansion and effector differentiation of OT-3 cells compared to OT-1 cells upon N4-CpG vaccination in B16.OVA tumor-bearing mice (Figure 6C and 6E). Thus, to exclude intrinsic differences between OT-1 and OT-3 cells during peripheral T cell priming which could explain the differences in miR-155 expression, we compared miR-155 expression levels of OT-1 cells isolated from B16.N4 and B16.T4 tumors after N4-CpG s.c vaccination. At day 14 and 21 post-tumor engraftment, sorted OT-1 cells showed increased miR-155 expression in B16.N4 tumors and to a lower extent in B16.T4, compared to miR-155 expression levels in the spleen OT-1 cells (Figure 19C). Moreover, at day 14, OT-1 cells from B16.N4 tumors tent to have higher miR-155 expression levels than OT-1 cells from B16.T4 tumors. The difference was significant at day 21 (Figure 19C), indicating that antigen recognition affinity impacts on miR-155 expression. As a

conclusion, tumor-specific CD8$^+$ T cells upregulate miR-155 in the tumor in a T cell affinity-dependent manner.

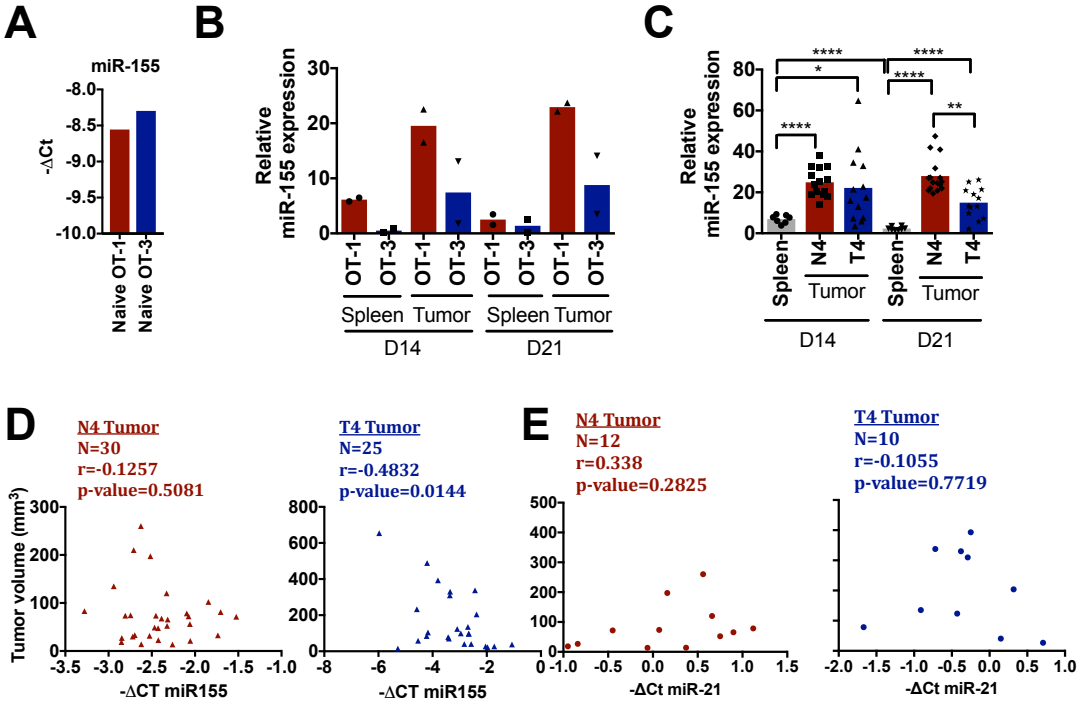

Figure 19. miR-155 expression levels in tumor infiltrating effector OT-1 cells is T cell affinity dependent and positively correlates with tumor control in B16.T4 tumors A. miR-155 expression levels in naive OT-1 and OT-3 cells. **B**. Relative miR-155 expression levels in effector OT-1 and OT-3 cells from spleen and B16.OVA tumors 14 and 21 days post-tumor engraftment. **C.** Relative miR-155 expression levels in effector OT-1 cells from spleen and B16.N4 or B16.T4 tumors. **D** and **E**. Relationship between tumor volume and miR-155 or miR-21 expression levels respectively. Bars represent the mean and symbols individual mice except in Figure B where each dot represents a pool of 5 mice. In red B16.N4 and in blue B16.T4 tumors. miR-155 expression levels are expressed as -ΔCt of miR-155 and snoRNA202. Relative miR-155 expression levels are expressed as fold change relative to levels in host naïve CD8$^+$ T cells from the spleen. An unpaired t-test or Mann-Whitney non-parametric test was performed after Shapiro-Wilk normality test in panels A, a 2-way ANOVA followed by Sidak's multiple comparison test in panel B, a 1-way ANOVA followed by Tukey's multiple comparison test in panel C and a Pearson's correlation in panels D and E. All statistical tests were performed in non-normalized -ΔCt data.

We also noticed that there was low miR-155 expression variability among OT-1 cells from B16.N4 tumors, whereas the variability was high in B16.T4 tumors (SD -ΔCt 0.40 vs 1.18). Interestingly, miR-155 expression levels in those low affinity tumors negatively correlated with tumor volume (Figure 19D). Overall, miR-155 expression levels in tumor infiltrating OT-1 cells likely reflects the activation state of the cells as we previously showed that antigen recognition is one of the main drivers of miR-155 upregulation. However, when we analyzed expression levels of miR-21, another miRNA upregulated upon T cell activation (Salaun et al., 2011; Wu et al., 2007), we did not observe any correlation with tumor volume (Figure 19E), suggesting that miR-155 expression levels

may be an important indicator of CD8$^+$ T cell fitness in low affinity antigen expressing tumors.

Anti-PD-1 treatment enhances tumor-infiltrating CD8$^+$ T cells miR-155 expression levels

Tumor infiltrating CD8$^+$ T cells become dysfunctional due to the highly immunosuppressive tumor microenvironment. Signaling through PD-1 receptor is one of the critical inhibitory signals that CD8$^+$ T cells encounter in the tumor. In fact, it has been shown that PD-1 blockade enhances CD8$^+$ T cell functionality (Wong et al., 2007) leading to impressive clinical responses in metastatic melanoma patients (Topalian et al., 2012). When we measured miR-155 expression levels in tumor infiltrating OT-1 cells of anti-PD-1 treated mice, we observed increased miR-155 expression levels in B16.N4 tumors after anti-PD-1 treatment and a tendency in B16.T4 tumors (Figure 20A). miR-21 levels were however similar despite the treatment (Figure 20B). Anti-PD-1 treated mice showed improved tumor control due to enhanced OT-1 cell infiltration and functionality (Figure 14). Thus, increased miR-155 expression levels in CD8$^+$ T cells from anti-PD-1 treated mice are in line with our hypothesis that miR-155 expression levels correlate with enhanced CD8$^+$ T cell fitness.

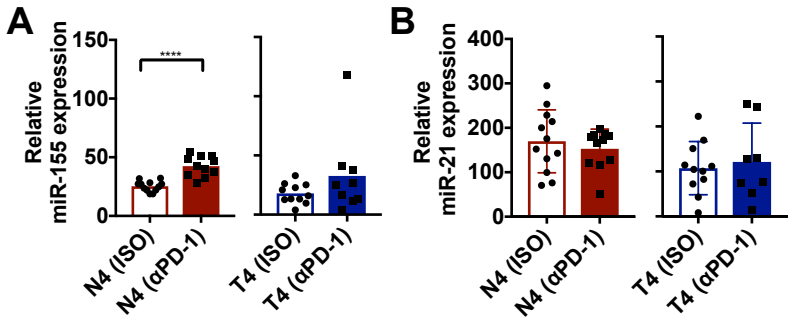

Figure 20. Anti-PD-1 treatment leads to increased miR-155 expression in CD8$^+$ TILs. A and **B.** Relative miR-155 or miR-21 expression levels of effector OT-1 cells from B16.N4 or B16.T4 tumors of mice treated with anti-PD-1 or isotype control Ab. An unpaired t-test or Mann-Whitney non-parametric test was performed after Shapiro-Wilk normality test in panels A, F and G.

miR-155 expression is regulated during human CD8$^+$ T cell activation and differentiation

The role of miR-155 in mouse CD8$^+$ T cells has been largely studied by us and others (Dudda et al., 2013; Gracias et al., 2013; Ji et al., 2015). In contrast, little is known about the modulation of miR-155 in human CD8$^+$ T cells.

We previously reported that miRNA expression profiles differ among the different CD8$^+$ T cells subsets from healthy donors (HDs) (Salaun et al., 2011). miR-155, in particular, was found to be upregulated in EM compared to naïve CD8$^+$ T cells. Here, we sorted naïve, CM, EM and EMRA CD8$^+$ T cells from a different cohort of HDs and confirmed

that peripheral blood (PB) EM and EMRA CD8$^+$ T cells have higher miR-155 expression levels as compared to naïve and CM CD8$^+$ T cells (Figure 21A and 21B). Therefore, HDs PB CD8$^+$ T cells show different miR-155 expression levels depending on differentiation stage, whereby EM and EMRA CD8$^+$ T cells are the ones with the highest expression levels.

This differential miR-155 expression suggests that miR-155 is regulated during human CD8$^+$ T differentiation. Indeed, *in vitro* activation of naïve CD8$^+$ T cells from 4 HDs with αCD3/αCD28 coated beads showed a strong miR-155 upregulation until day 2. Thereafter, miR-155 levels tent to decrease with a big variability between donors, but activated cells retained higher miR-155 expression levels than resting naïve CD8$^+$ T cells (Figure 21C).

Peripheral CD8$^+$ T cell subsets from melanoma patients and HDs show similar miR-155 expression levels

Previous data from the group (Dudda et al., 2013) and my results presented above convincingly demonstrate that miR-155 is important for CD8$^+$ T cell mediated anti-tumor response in the B16 mouse melanoma tumor model. Thus, we aimed to detect a similar association in cancer patients by analyzing miR-155 expression levels in melanoma patients' CD8$^+$ T cells.

miR-155 expression levels in PB CD8$^+$ T cell subsets were similar in melanoma patients and HDs (Figure 21C). Nonetheless, naïve CD8$^+$ T cells miR-155 expression levels were highly variable in HDs and patients with no association with sex or age (Figure 21E and 21F). However, there was a direct correlation between miR-155 expression levels in naïve cells and EM or EMRA subsets from the same donor (Figure 21G), indicating that the higher the basal miR-155 levels, the higher the levels found in EM and EMRA CD8$^+$ T cells. Altogether, miR-155 expression levels are variable among individuals but expression profiles between HDs and melanoma patients polyclonal PB CD8$^+$ T cell subsets are similar.

miR-155 upregulation in melanoma patients Melan-A CD8$^+$ T cells is T cell avidity dependent

miR-155 expression in bulk CD8$^+$ T cells from melanoma patients PB may not reflect miR-155 expression of tumor-specific CD8$^+$ T cells as they are found in relatively low

frequencies in the blood (Pittet et al., 1999). Therefore, we determined miR-155 expression levels in HLA-A2/Melan-A_{26-35} specific $CD8^+$ T cell clones originally isolated from PB of metastatic melanoma patients. Melan-A_{26-35} -specific $CD8^+$ T clones were stimulated with multimers containing the high-affinity ELAGIGLTV (ELA) analog peptide. In parallel, Melan-A $CD8^+$ T cells binding avidity for the peptide/MHC was measured with HLA-A2/ELA NTAmers as previously described (Hebeisen et al., 2015). 24h after multimer stimulation, miR-155 was upregulated in Melan-A_{26-35} specific $CD8^+$ T cell clones and expression levels in each clone correlated with its avidity for the ligand as measured by the NTAmer technology (Figure 21H). Thus, in line with our observations in mouse $CD8^+$ T cells, TCR stimulation of human tumor specific $CD8^+$ T cells leads to avidity dependent miR-155 upregulation.

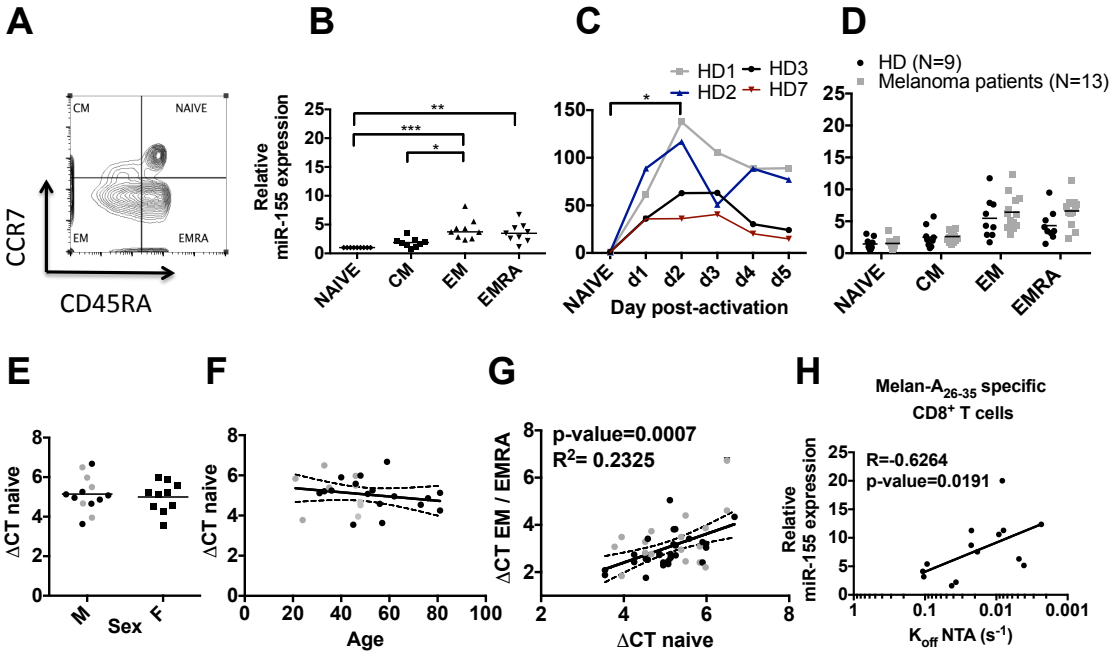

Figure 21. miR-155 is upregulated upon activation and EM differentiation in a T cell avidity dependent manner in human $CD8^+$ T cells. A. Naïve, CM, EM and EMRA $CD8^+$ T cell subset gating strategy based on CCR7 and CD45RA expression**. B.** Relative miR-155 expression levels of PB HDs $CD8^+$ T cell subsets expressed as fold change relatively to levels in naïve cells. **C**. Relative miR-155 expression kinetics in HDs naïve $CD8^+$ T cells activated with 1:2 αCD3/αCD28 coated beads. **D**. miR-155 expression levels in HDs and melanoma patients PB $CD8^+$ T cell subsets. **E.** HDs or patients naïve $CD8^+$ T cell miR-155 expression levels grouped by sex. **F.** Relationship between HDs or patients naïve $CD8^+$ T cell miR-155 expression levels and age (n=26; p=0.1836, n.s.; r=-0.269). **G.** Relationship between miR-155 expression levels in PB naïve $CD8^+$ T cells and PB EM/EMRA $CD8^+$ T cells (n=46; p=0.009; r=0.379). **H.** Correlation between miR-155 expression levels in Melan-A $CD8^+$ T cell clones stimulated with Melan-$A^{MART-1}_{26–35\,(A27L)}$ multimers and T cell avidity measured by NTAmer dissociation kinetics. In black melanoma patients' samples and in grey HDs. Dots represent individual HD or patients except in figure H were dots represent individual clones. Lines in panels D and E represent the mean and the linear regression in panels F, G and H. A Friedman's test followed by Dunn's multiple comparison test was performed in panels B and C, a 2-way ANOVA followed by Sidak's multiple comparison test in panel D, an unpaired t-test in figure E after Shapiro-Wilk normality test and a Spearman's correlation in panels F-H.

miR-155 is upregulated in EM CD8$^+$ T cells isolated from TILNs and melanoma tumors

As tumor specific murine OT-1 cells showed high miR-155 expression levels, we analyzed miR-155 expression levels in *ex vivo* sorted CD8$^+$ T cell subsets from tumors, tumor infiltrated lymph nodes (TILNs) and non-infiltrated lymph nodes (NLNs) from melanoma patients. qPCR analysis revealed that EM CD8$^+$ T cells had higher miR-155 expression levels in TILNs and tumors than in PB. CM CD8$^+$ T cells showed a similar trend but to a lower extent than EM CD8$^+$ T cells (Figure 22A and 22B). Many factors could contribute to the differences in miR-155 expression levels in tumors and TILNs compared to PB CD8$^+$ T cells. However, when we compared miR-155 expression levels in NLNs to levels in TILNs CD8$^+$ T cell subsets, we found that EM CD8$^+$ T cells in TILNs showed higher miR-155 expression levels than in NLNs (Figure 22C). Thus, the presence of cancer cells plays a role in EM CD8$^+$ T cells miR-155 upregulation in melanoma patients. In fact, miR-155 expression levels were very low and similar between PB and NLNs CD8$^+$ T cell subsets (Figure 22D). The only exception was patient LAU117, who showed higher miR-155 expression levels in NLNs EM CD8$^+$ T cells compared to levels in PB EM CD8$^+$ T cells. However, NLNs are LNs from melanoma patients, in which tumor cells are not detectable but often belong to the same LN chain as TILNs, obtained during radical lymphadenectomy of patients positive for tumor invaded sentinel lymph node. Therefore, despite being tumor free, soluble factors from afferent TILNs could have

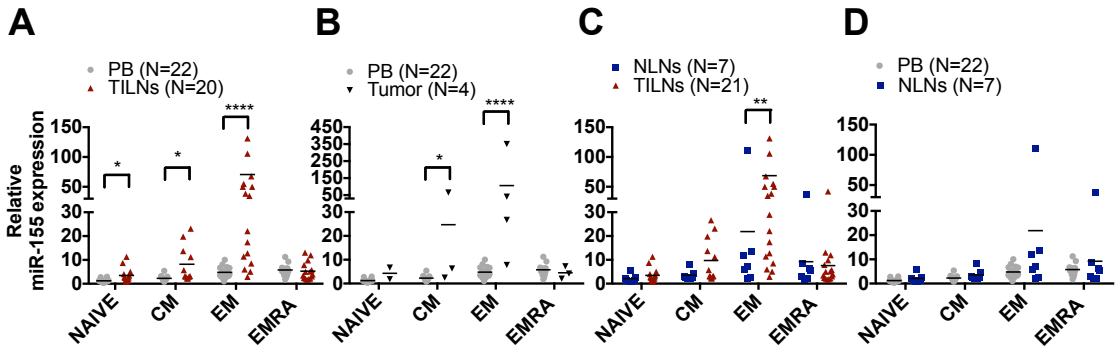

Figure 22. miR-155 expression levels are higher in EM CD8$^+$ T cells of metastatic tumors and TILNs compared to non-infiltrated tissues. Relative miR-155 expression levels in PB, TILNs (**A** and **C**), tumors (**B**) and NLNs (**C** and **D**) CD8$^+$ T cell subsets of melanoma patients. Symbols represent individual patients miR-155 expression levels measured by qPCR and represented as fold change relatively to levels in PB naïve CD8$^+$ T cells. Lines represent the mean. A 2-way ANOVA followed by Tukey's multiple comparison test data was performed on non-normalized ΔCt.

arrived and modified NLNs microenvironment. In addition, it has been shown that T cells can migrate to efferent LNs (Braun et al., 2011). Thus, the possibility that TILNs EM cells with high miR-155 expression levels have migrated from TILNs to NLNs should also be considered. In fact, antigen experienced EM CD8$^+$ T cell frequencies were higher in melanoma patients NLNs compared to healthy donors LNs (Figure 23), indicating that

melanoma patients NLNs differ from healthy LNs, and that the tumor could also be the trigger of the high miR-155 expression levels in NLNs, as observed in the patient LAU117.

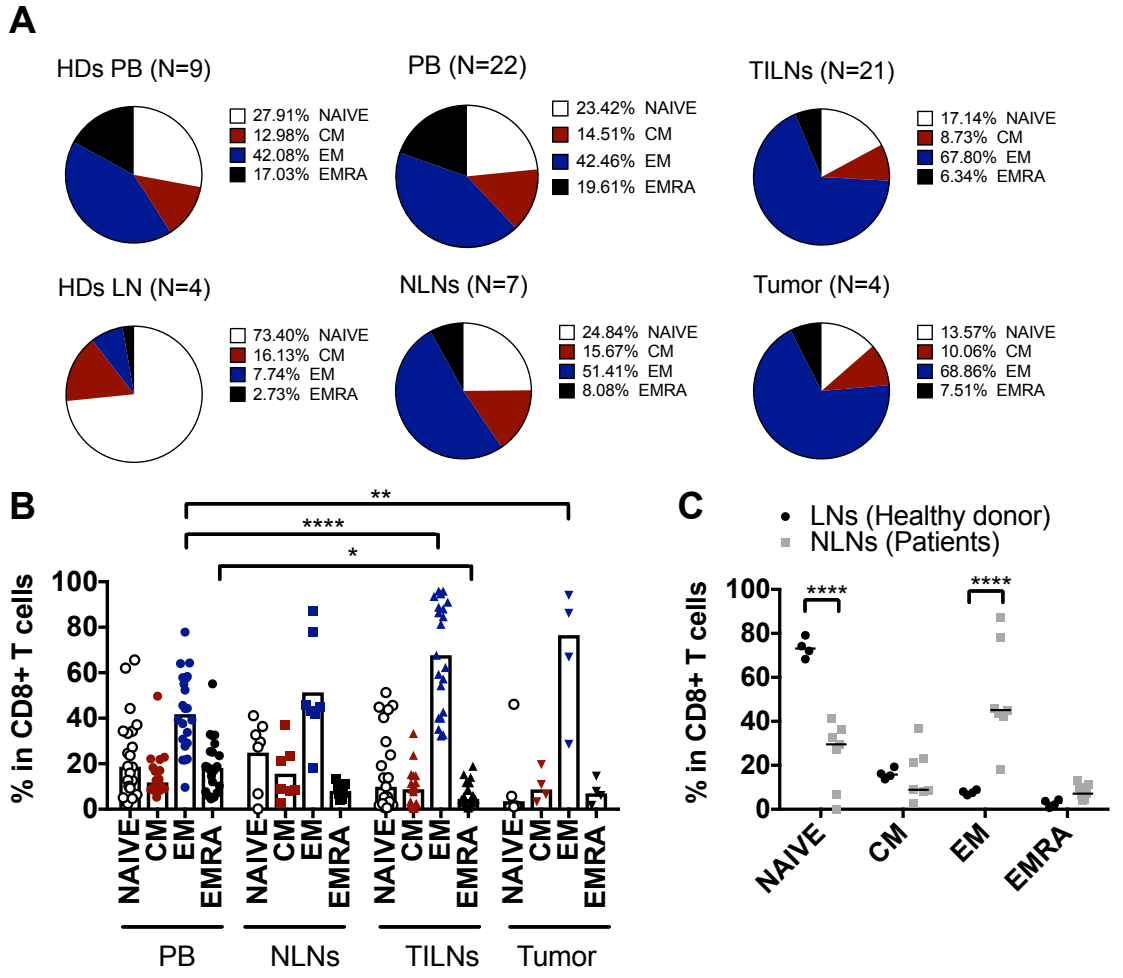

Figure 23. Higher percentages of EM CD8$^+$ T cell subsets are found in tumors and tumor-infiltrated tissues compared to non-infiltrated ones. A. Circles represent CD8$^+$ T cell subsets percentages means in total CD8$^+$ T cells in PB, NLNs, TILNs and tumors expressed as parts of whole. **B.** CD8$^+$ T cell subsets percentages in total CD8$^+$ T cells in PB, NLNs, TILNs and tumors. Dots represent individuals and the bar the mean. **C.** CD8$^+$ T cell subset percentages in total CD8$^+$ T cells in melanoma patients NLNs compared to healthy donors LNs. A 2-way-ANOVA followed by a paired Tukey's multiple comparison test was performed in panel B and a 2-way ANOVA followed by a paired Sidak's multiple comparison test in panel C.

<u>High miR-155 expression levels correlate with increased EM CD8$^+$ T cell frequencies in melanoma patients TILNs</u>

MiR-155 expression levels in EM CD8$^+$ T cells from TILNs and tumors were very variable among patients. Interestingly, we observed a positive correlation between TILNs EM CD8$^+$ T cells miR-155 expression levels and the percentage of EM CD8$^+$ T cells in TILNs (Figure 24A). High EM CD8$^+$ T cells frequencies in TILNs correlated at the same time with better overall survival (OS) (Figure 24B), as it has been shown in other cancer

patients cohorts (Angell & Galon, 2013). These correlations suggest that miR-155 expression levels could be a marker of good prognosis in melanoma patients, although mir-155 expression levels did not directly correlate with prolonged OS (Figure 24C). These observations indicate that other parameters apart from miR-155 expression were involved in the increased TILNs EM CD8$^+$ T cell infiltrates which associated with longer OS in this cohort of melanoma patients.

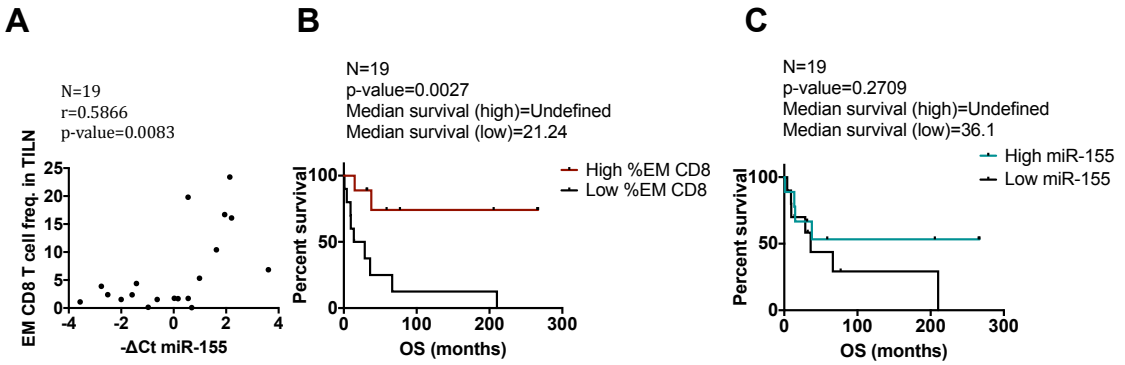

Figure 24. **High EM CD8$^+$ T-cell frequencies characterized by high miR-155 expression levels correlate with prolonged overall survival in melanoma patients. A**. Correlation between EM CD8$^+$ T cell percentage in total live cells in TILNs and EM CD8$^+$ T cells miR-155 expression levels expressed as –ΔCt of miR-155 and RNU44. Symbols represent individual patients. **B** and **C.** Survival curves of melanoma patients segregated according to the median EM CD8$^+$ T cell percentage in TILNs and median of miR-155 expression levels. A Pearson's correlation was performed in panel A and a Long-rank Mantel-Cox test in panels B and C.

MiR-155 expression by immune cells in TILNs and tumor tissue sections of melanoma patients

Fresh or frozen tumor and TILNs samples are rarely available from melanoma patients. Moreover, miR-155 qPCR analysis on sorted EM CD8$^+$ T cells is time-sensitive, resource-intensive and technically-demanding to be applied in standard diagnostic or prognostic assessments. In contrast, formalin-fixed paraffin embedded (FFPE) tumor and TILNs biospecimens are often available as they are obtained for standard diagnosis. Therefore, in collaboration with Prof. Lorenzo Sempere in Van Andel Research Institute (Michigan, USA), we performed an automated miR-155 *in situ* hybridization (ISH) in a collection of FFPE sections from patients' tumors and TILNs, which matched samples already analyzed by qPCR (Figure 22). We observed evidence of miR-155 expression in CD8$^+$ cells, but also in other infiltrating cells of small diameter, which likely corresponded to other lymphocyte subsets. Overall, the small-diameter cells showed the highest miR-155 expression levels, while larger cells such as cancer cells displayed low miR-155 expression levels (Figure 25A and 25D), as seen by qPCR on sorted cells (Figure 25E). Interestingly, most miR-155$^+$ small immune cells were in the peri-tumoral

region, and the miR-155 H-score was variable among different areas of the same section as well as among tumors, as we previously reported by qPCR (Figure 22A and 22B). Of note, we could also detect $CD8^+$ cells in the intra-tumoral regions (Figures 25A, 25B and 25C) with evidence of a small subset of *in situ* proliferating $Ki\text{-}67^+$ $CD8^+$ cells in some tumors (Figure 25A, 25B and 25D). Thus, we were able to provide proof-of-principle for automated miR-155 staining in melanoma tissue sections which could be used for high-

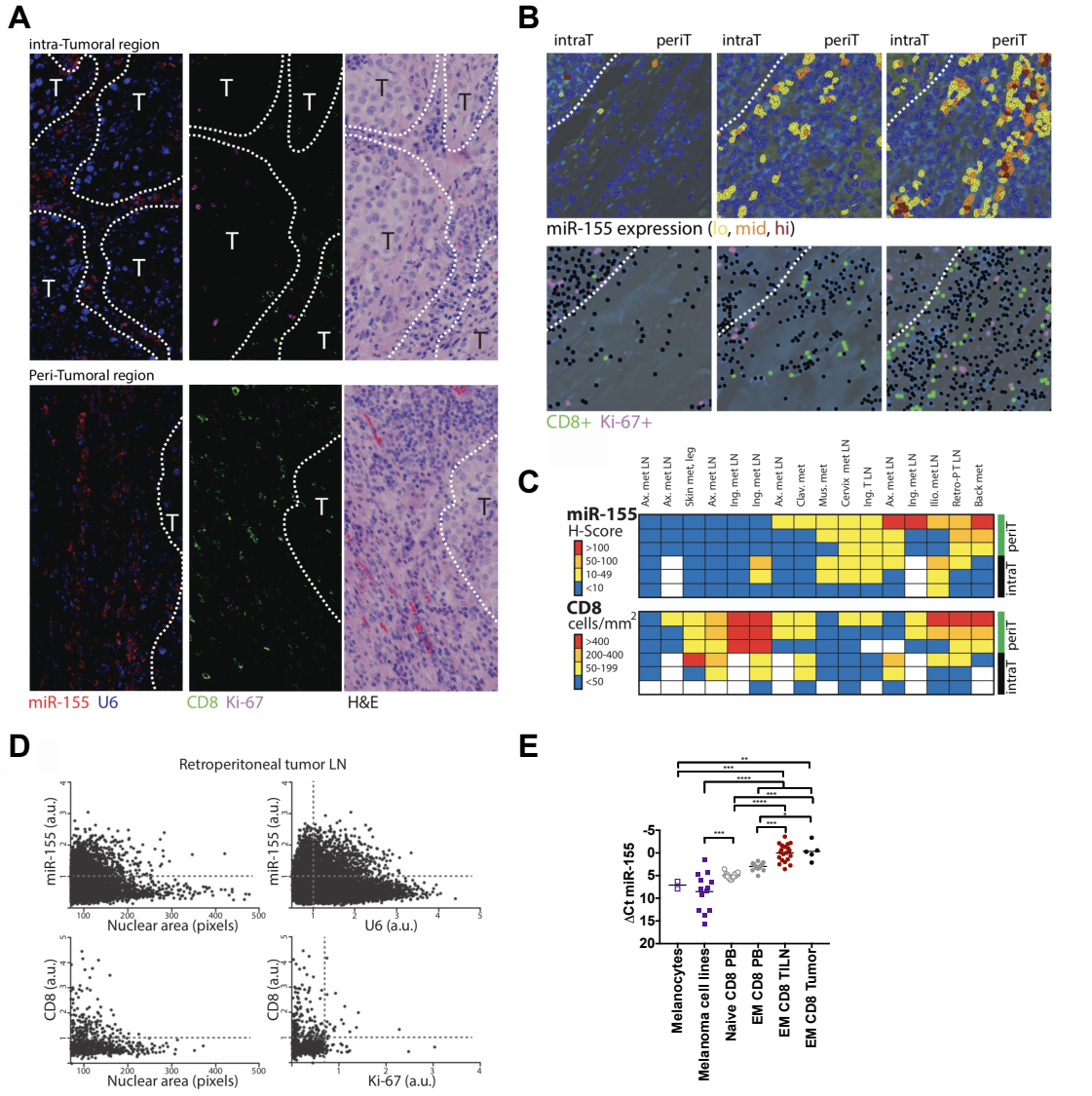

Figure 25. **Mir-155 expression analysis on melanoma patients' FFPE sections. A.** Representative pictures of miR-155 and U6 ISH co-detection, CD8 and Ki-67 co-staining and hematoxylin and eosin (H&E) staining on melanoma patients TILNs/tumor consecutive sections. **B.** mir-155 staining intensity coded as low (yellow), medium (orange) and high (red) per cell on the top panel and $CD8^+$ and $Ki\text{-}67^+$ cells marked in green and purple respectively on the bottom panel. Intra-tumoral (intraT) and peri-tumoral (periT) regions are distinguished; heatmaps in **C** show quantification in each tissue field per region. **D**. miR-155 staining intensity represented in arbitrary units (a.u) vs nucleus size determined by number of pixels (left) or U6 endogenous control staining intensity in a.u (right) at the top and CD8 staining intensity represented in arbitrary units (a.u) vs nucleus size (left) or Ki-67 staining intensity in a.u (right) on the bottom on multiple tissue fields from same TILN specimen. **E**. qPCR miR-155 expression analysis on normal melanocytes, melanoma cell lines or sorted naive EM $CD8^+$ T cells from PB, TILNs and tumors of melanoma patients. A one-way ANOVA followed by Tukey's multiple comparison test was performed in panel **E**.

throughput analysis and patient stratification. Follow up correlative studies in larger patient cohorts will be required to calibrate a miR-155-based score that would integrate tissue heterogeneity as well as subclonal expression variety.

Discussion

We have demonstrated that antigen dose impacts on $CD8^+$ T cells endogenous miR-155 expression levels. Therefore, miR-155 expression levels in $CD8^+$ T cells reflect *in situ* antigen stimulation and could be used as a marker of $CD8^+$ T cell responsiveness. For instance, the high miR-155 expression levels observed in B16.OVA endogenous bulk $CD8^+$ TILs suggest that, in addition to the epitopes of the OVA protein, $CD8^+$ TILs cells recognize probably other endogenous tumor antigens. In addition, we propose that high miR-155 expression levels in melanoma patients TILNs EM $CD8^+$ T cells indicate the presence of antigen responding EM $CD8^+$ T cells.

The fact that miR-155 expression levels reflect antigen-reactive $CD8^+$ T cells may promote the correlation between miR-155 expression levels and $CD8^+$ T cells frequencies in melanoma patients TILNs. In fact, our results are in line with previous observations in HIV infected individuals where miR-155 expression levels in PB $CD8^+$ T cells correlated with increased $CD8^+$ T cell numbers as well as their activation state determined by high PD-1 and CD38 expression (Jin C et al. HIV Medicine 2017).

Increased survival of $CD8^+$ T cells with miR-155 expression levels may also explain the positive correlation between miR-155 expression levels and EM $CD8^+$ T cell infiltration in melanoma patients TILN. Previous experiments in the lab showed that lack of miR-155 impaired $CD8^+$ T cell proliferation and survival in acute viral infections (Dudda et al. 2013, Immunity). Accordingly, it was later shown that enhanced tumor control of miR-155 overexpressing $CD8^+$ T cells was due to increased responsiveness to IL-7 and IL-15 homeostatic cytokines (Ji et al., 2015), which are essential cytokines for survival and homeostatic proliferation of memory T cells (Surh & Sprent, 2008). Moreover, Ji et al. reported that miR-155 overexpressing $CD8^+$ T cells showed increased functionality in tumors. While enhanced survival of miR-155 overexpressing $CD8^+$ T cells was due to increased Stat5 signaling, Akt signaling was responsible for the increased functionality. SOCS1 and PTPN2 Stat5 signaling negative regulators and Ship1 Akt inhibitor were identified as responsible miR-155 targets respectively (Ji et al., 2015).
Nonetheless, miRNA-mRNA targeting effects should be carefully addressed and should not be generalized to all cell contexts. Indeed, it was shown that the effect of miR-155-SOCS1 regulation axis is cell and context dependent (L. F. Lu et al., 2015). While SOCS1

regulation by miR-155 is dispensable for acute antiviral $CD8^+$ T cell responses, it is essential for $CD8^+$ T cells maintenance in chronic viral infections (L. F. Lu et al., 2015), indicating that SOCS1 targeting effect depends on whether $CD8^+$ T cells are chronically exposed to the antigen or not.

As tumor infiltrating $CD8^+$ T cells are chronically exposed to the antigen, increased EM $CD8^+$ T cell infiltrates in melanoma patients with high miR-155 expression levels could involve SOCS1 targeting. In fact, silencing of SOCS1 could recapitulate enhanced tumor control of miR-155 overexpressing $CD8^+$ T cells in B16 tumor bearing mice (Dudda et al., 2013). In addition, we can speculate that the increased persistence of miR-155 overexpressing transduced P14 cells in LCMV cl13 wt and mix infection models also depends on increased survival capacity by SOCS1 targeting.

We did not observe increased IFNγ production of miR-155 overexpressing $CD8^+$ T cells in LCMV cl13 chronic infection as previously shown in tumors and acute infections (Ji Y et al. PNAS 2015, Gracias et al. Nat Immunology 2013). However, increased Granzyme B expression was detected, suggesting that miR-155 enhanced not only cell survival but also cytotoxicity in chronic viral infection.

Moreover, we reported that miR-155 upregulation by tumor-specific $CD8^+$ T cells is T cell affinity dependent. Thus, high miR-155 expression levels in melanoma patients TILNs EM $CD8^+$ T cells could also indicate that those EM $CD8^+$ T cell recognize the antigen with higher affinity than EM $CD8^+$ T cells from patients with low miR-155 expression levels. In fact, we saw in the first part of this thesis that high compared to low affinity stimulation in the tumor leads to increased tumor control due to increased $CD8^+$ T cells expansion/survival and cytotoxicity (Figure 9). Furthermore, miR-155 quantification upon antigen stimulation could be an inexpensive method to compare the affinity of different $CD8^+$ T cell clones, avoiding the technical challenging SPR measurements.

Altogether, as miR-155 expression levels reflect responsiveness and affinity for the antigen as well as survival and effector function potential, it could be used as an overall marker of tumor infiltrating $CD8^+$ T cells fitness. Therefore, it could be included in the recently defined “Immunoscore” term used to classify cancer patients, that may have

superior prognostic value to the American Joint Committee on Cancer/ Union for International Cancer Control TNM-classification (Angell and Galon 2013).

GENERAL CONCLUSIONS AND PERSPECTIVES

We have demonstrated that the interaction strength between the TCR and the pMHC finely controls $CD8^+$ T cells expansion and differentiation by modulating the levels of intracellular molecular regulators such as miR-155. Naïve T cells are constantly stimulated with weak self-pMHC interactions (Malissen & Bongrand, 2015) due to TCRs cross-reactivity (Birnbaum et al., 2014; Mazza et al., 2007). Thus, restraining T cell activation upon low affinity TCR triggering is essential to prevent autoimmunity. However, this regulation is unfavorable for $CD8^+$ T cell anti-tumor responses that mostly rely on low affinity tumor antigen recognition. We have seen in the B16 mouse melanoma model that a way to overcome such limitation is the use of cancer vaccines containing high affinity altered peptide ligands in combination with CpG. This approach enabled sufficient peripheral $CD8^+$ T cell activation and expansion to control B16 tumors expressing a low affinity antigen. Thus, optimizing the affinity of the peptide ligand in the vaccine formulation is our next priority. Currently, *in silico* tools allow prediction of peptide-MHC-I binding affinities. However, it was shown that despite inducing increased Melan-A T cell frequencies, vaccination with a high affinity HLA-A2 binder E*L*AGIGILTV-Melan-A analog peptide led to inferior T cell responses compared to the native, low affinity HLA-A2 binder, EAAGIGILTV Melan-A peptide (Speiser et al., 2008). This was probably due to increased E*L*AGIGILTV analog peptide presentation by DCs and consecutive activation of low avidity T cells (Bullock, Mullins, & Engelhard, 2003). Adjustment of the immunization dose to have limiting amounts of antigen during the *in vivo* T cell priming could be a strategy to prioritize selection of high avidity T cells. Another hypothesis is that the TCR repertoire activated by the analog peptide is not entirely cross-reactive with the native peptide. In fact, it was shown that vaccination with the $gp100_{209\text{-}217(T210M)}$ analog peptide, which has increased binding affinity for HLA-A2 (Parkhurst et al., 1996), elicits high frequencies of specific $CD8^+$ T cells, yet only about half of them react to the native peptide (Rosenberg et al., 2005). Thus, we should emphasize the need of altered peptide ligands with increased affinity for the TCRs of the tumor-specific $CD8^+$ T cells. One approach to identify such peptides could be to test the ability of TAA derived altered peptide ligands to induce T cell affinity dependent responses such as Granzyme B production, proliferation and miR-155 upregulation in tumor infiltrating $CD8^+$ T cells from cancer patients.

Combination of high affinity cancer vaccines with ICB therapies may be particularly necessary for low affinity antigen expressing tumor targeting. Anti-PD-1 treatment enhanced tumor control of low affinity antigen expressing tumors. However, combination of PD-1 blocking antibodies with other checkpoint inhibitors such as anti-CTLA-4, may further enhance tumor control (Curran, Montalvo, Yagita, & Allison, 2010). In fact, anti-PD-1 and anti-CTLA-4 double blockade showed improved treatment outcome without an escalation of adverse events compared to single therapies in melanoma patients (Wolchok et al., 2013).

ICB therapies aim to block inhibitory signals of the tumor microenvironment to unleash full functionality of T cells. In fact, we have reported here that tumor infiltrating $CD8^+$ T cells retain full capacity to reexpand in tumor-free inflammatory environments, proving that functionality of $CD8^+$ T cells is rescuable, particularly at early timepoints, when cytokine production capacity is also recovered. However, it remains to be elucidated whether all tumor-infiltrating $CD8^+$ T cells or a specific subpopulation is the one maintaining the reexpansion and functional capacity. In fact, it was shown in chronic viral infections, a condition in which $CD8^+$ T cells are chronically exposed to the antigen and inflammation as in tumors, that TCF-1 transcription factor expressing virus specific $CD8^+$ T cells are the memory-like $CD8^+$ T cells sustaining the anti-viral response and responding to anti-PD-1 treatment (Utzschneider, Charmoy, et al., 2016). Thus, we now aim to determine whether this memory-like $CD8^+$ T cell population is also present in tumors. In addition, we would like to identify specific surface markers of this population that would enable their selection for adoptive T cell transfer as well as definition of predictive biomarkers for ICB therapies.

Not only the presence of memory-like $CD8^+$ T cells but miR-155 expression levels may also be used as a biomarker of $CD8^+$ T cells fitness and responsiveness to anti-PD-1 treatment. Active $CD8^+$ T produce IFNγ which upregulates PD-L1 expression on cancer cells (Dong et al., 2002) and it has been shown that PD-L1 expression in tumor biopsies correlates with anti-PD-1 response rates in cancer patients (Daud et al., 2016). In addition, it has been suggested that presence of $PD\text{-}1^+$ activated $CD8^+$ T cells is also a predictive biomarker of anti-PD-1 treatment (Huang et al., 2017). Thus, success of anti-PD-1 treatment relies on the presence of reactive $CD8^+$ T cells that we showed to be characterized by high miR-155 expression levels. Nonetheless, a large-scale analysis

needs to be performed to confirm the predictive value of $CD8^+$ T cells miR-155 expression levels in melanoma patients and other cancer patients.

Studies in preclinical models have shown that miR-155 overexpression enhances anti-tumor responses by increasing $CD8^+$ T cells survival and effector functions (Dudda et al., 2013; Ji et al., 2015). We also demonstrated here that miR-155 overexpression enhances persistence of transduced cells in chronic viral infections. Thus, miR-155 overexpression may be beneficial in ACT therapies involving *ex vivo* T cell transduction with tumor-specific TCRs, for instance, to enhance persistence of transferred cells, a hurdle still to overcome in the field of ACT therapy (Dudley & Rosenberg, 2003). Nonetheless, experiments presented in the thesis of my colleague Gwennaëlle Monnot showed that $CD8^+$ T cells do not always equally benefit from miR-155 overexpression, whereby low affinity $CD8^+$ T cells may particularly profit from its overexpression (Monnot et al. manuscript in preparation). Silencing of negative regulators of the TCR signaling such as CD5, cbl-b or SHP-2 could be additionally combined with miR-155 overexpression when engineering T cells for ACT to increase the sensitivity of low affinity tumor specific T cells to the antigen (Hollander, 1984; Hui et al., 2017; Loeser et al., 2007; Presotto et al., 2017; Tabbekh et al., 2011). In addition, safety restraints such as expression of suicide genes may also be considered to prevent off-target related toxicities (Straathof, Spencer, Sutton, & Rooney, 2003).

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