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Harnessing microRNAs for cancer immunotherapy

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Harnessing microRNAs for cancer immunotherapy

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par

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Résumé grand public:

Exploiter les microRNAs pour les immunothérapies contre le cancer

Ces dernières années, l'immunothérapie contre le cancer s'est faite connaître du grand public, notamment grâce au magazine *Science,* qui l'a nommée « découverte de l'année » en 2013.

En effet, cette approche est une révolution dans les traitements contre le cancer, car elle s'attache à réactiver le système immunitaire du patient pour qu'il attaque sa propre tumeur. En l'occurrence, les immunothérapies telles que les inhibiteurs de points de contrôle immunitaire, ont démontré des effets à long terme impressionnants, avec certains patients n'ayant à ce jour pas développé de récurrence de leur cancer.

Il existe plusieurs types d'immunothérapies. L'une d'elle consiste à extraire des cellules du système immunitaire du patient et à les modifier génétiquement pour qu'elles deviennent spécifiques pour la tumeur. Une fois re-transférées dans le patient, ces cellules peuvent attaquer la tumeur et la détruire. Cette technique se nomme le transfert adoptif thérapeutique.

Mon projet s'est attelé à étudier l'importance des microRNAs dans la fonctionnalité des cellules T CD8+ et à utiliser ces connaissances pour améliorer les thérapies de transfert adoptif. Les microRNAs sont des petites molécules qui s'attachent spécifiquement aux molécules d'ARN messager, et qui de cette façon bloquent la traduction de l'ARN messager en protéines. Les microRNAs sont très importants pour les fonctionnements cellulaires, et permettent aux cellules de passer plus rapidement d'un état à un autre.

Durant mon projet de thèse, j'ai tout d'abord observé que la présence du cluster de microRNAs miR-17-92 était cruciale pour la bonne fonctionnalité des cellules CD8+. En particulier, pour leur capacité à répondre à une infection et à produire des cytokines qui sont les molécules effectrices de la réponse immunitaire. Nous avons aussi observé que les cellules CD8+ ne possédant pas de miR-17-92 montraient un phénotype « mémoire ».

Deuxièmement, nous avons étudié la surexpression d'un autre microRNA, miR-155, dans les cellules CD8+, et l'impact de cette surexpression dans la capacité des cellules à induire une régression tumorale. Nous avons observé dans un modèle murin que la surexpression de miR-155 pouvait améliorer la régression tumorale lorsque les tumeurs exprimaient un antigène de basse affinité. Ceci est intéressant pour les thérapies humaines, car les antigènes tumoraux sont souvent de basse affinité puisqu'ils sont des molécules du « soi » contre lesquelles le système immunitaire est tolérant contrairement aux pathogènes.

Finalement, nous avons étudié la surexpression de miR-155 en combinaison avec les thérapies de récepteur d'antigène chimérique (CAR). Dans le modèle murin que nous avons utilisé, la surexpression de miR-155 ne changeait pas l'efficacité du traitement avec des cellules CAR qui probablement était déjà optimale.

De par leur capacité à réguler l'expression de plusieurs protéines à la fois et de façon extrêmement rapide, les microRNAs sont des molécules qui comportent un fort potentiel de manipulation à des fins thérapeutiques. Dans mon travail, j'ai démontré que les microRNAs miR-17-92 ainsi que miR-155 pourraient être des cibles intéressantes pour améliorer les immunothérapies contre le cancer.

Résumé

Ces dernières années, la recherche de thérapies contre le cancer a pris un nouveau tournant. Ceci a eu pour conséquence la nomination de l'immunothérapie contre le cancer en tant que « découverte de l'année 2013 » par le magazine *Science*. En effet, les dernières publications démontrent une efficacité clinique sans précédent pour plusieurs immunothérapies contre le cancer. En particulier, le transfert adoptif de lymphocytes infiltrants de la tumeur ou de lymphocytes génétiquement modifiés pour attaquer la tumeur. Ces technologies ont suscité l'intérêt des immunologistes ainsi que du grand public car ces approches ont montré des résultats extrêmement encourageants. Pourtant, les tumeurs solides restent les plus difficiles à traiter. Ceci démontre la nécessité d'améliorer les fonctions effectrices des lymphocytes transférés.

Pour ce faire, nous avons étudié l'influence des microRNAs sur les fonctions effectrices des lymphocytes T CD8+, ainsi que les façons de moduler l'expression de ceux-ci pour améliorer les immunothérapies contre le cancer.

Dans le premier chapitre, nous avons observé l'importance pour la fonctionnalité des cellules T CD8+ du cluster de microRNAs miR-17-92. L'expression de ce cluster est augmentée dans les cellules T CD8+ après leur activation. Nous avons utilisé un modèle murin de délétion du cluster dans les lymphocytes T, et observé une diminution de la fonctionnalité des cellules T CD8+ en son absence. Sans le cluster miR-17-92, les cellules T CD8+ avaient une capacité diminuée de prolifération et de production de cytokines. De plus, leur différentiation en cellules effectrices était compromise. En effet, les cellules exprimaient en majorité des marqueurs phénotypiques des cellules de la mémoire centrale.

Dans le second chapitre, nous avons étudié comment la surexpression de miR-155 a affecté le potentiel antitumoral des cellules T CD8+ dans un modèle murin de vaccination thérapeutique, ainsi que leur habilité a réagir à des antigènes de basse affinité. Nous avons observé que la surexpression de miR-155 permettait aux cellules T CD8+ d'augmenter leur capacité prolifératives ainsi que leur production de cytokines après activation. De plus, la surexpression de miR-155 a permis d'améliorer le contrôle des tumeurs exprimant un antigène de basse affinité.

Dans le troisième chapitre, nous avons mesuré la fonctionnalité des cellules humaines T CD8+ transduites pour exprimer un récepteur d'antigène chimérique (CAR) ainsi que miR-155. Nous avons pu augmenter légèrement la quantité relative de miR-155 dans ces cellules, et ceci a suffi pour améliorer leur potentiel de prolifération après activation. Malgré tout, la surexpression de miR-155 n'a pas induit d'amélioration dans la régression des tumeurs médiée par les cellules T CAR+ CD8+ dans un modèle de xénogreffe.

Dans sa globalité, ce travail de thèse démontre l'importance de l'expression et de la régulation des microRNAs pour la fonctionnalité des cellules T CD8+ et pour leur activité antitumorale.

Summary

In the last few years, the fight against cancer has reached a turning point, which led to the proclamation of cancer immunotherapy as the “2013 Breakthrough of the year” by Science magazine. Indeed, recent results have shown clinical efficacy of several approaches to specific immunotherapy of cancer, such as, adoptive transfer of tumour infiltrating lymphocytes or lymphocytes genetically modified to attack the tumour. This has not only been sparking the interest of immunologists themselves, but also of the general public. Although these approaches show encouraging results, not all patients can benefit from it, and especially solid tumours have proven to be more difficult to treat.

For this reason, there is an interest in improving the effector functions of the transferred cells. To do so, we have studied the potential of modulating the expression of microRNAs in CD8+ T lymphocytes.

In the first chapter of this thesis, we studied the importance of the microRNA cluster miR-17-92 in the functionality of CD8+ T cells. This cluster was shown to be selectively upregulated after T cell activation, which indicated a potential function of this cluster in CD8+ T cells effector functions. To study this, we used a CD4 conditional knockout of the miR-17-92 cluster and observed a decreased functionality of CD8+ T cells in its the absence. We found that the deletion of miR-17-92 impaired the CD8+ T cells in their proliferative ability upon activation and decreased their production of cytokines. Moreover, we observed that CD8+ T cells were impaired in their effector cells differentiation and showed a phenotype of central memory cells.

In the second chapter, we studied how overexpression of miR-155 affected the CD8+ T cells antitumour efficiency upon vaccination. Moreover, we observed the influence of miR-155 in modulating the functional avidity of CD8+ T cells. We found that CD8+ T cells overexpressing miR-155 had an increased proliferative capacity and cytokine production upon activation. We also observed that overexpression of miR-155 improved tumour control when tumours expressed a low affinity ligand. Our findings indicate that miR-155 overexpression rescues CD8+ T cells that have been suboptimally activated or restimulated by a low affinity ligand.

In the third chapter, we assessed miR-155 overexpression in human CD8+ T cells expressing a chimeric antigen receptor (CAR) against a prostate surface antigen (PSMA). We managed to induce a small increase of miR-155 levels in those cells. This increase was sufficient to improve the ability of CAR+ CD8+ T cells to proliferate *in vitro* upon activation. However, miR-155 expression did not improve the efficiency of the CAR+ CD8+ T cells in a mouse xenograft model.

As a whole, this thesis work demonstrates the importance of the expression and regulation of microRNAs for the functionality of CD8+ T cells and their antitumour efficiency.

Abbreviations

APCs	Antigen presenting cells
ADCC	Antibody-dependant cell-mediated cytotoxicity
BrdU	Bromodeoxyuridine
CD	Cluster of differenciation
CFU	Colonie-forming unit
CpG	Unmethylated deoxycytidylate-phosphate-deoxyguanylate
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EGFR	Epidermal growth factor receptor
IFN	Interferon
IL	Interleukin
KO	Knock-out
LCMV	Lymphocytic choriomeningitis
Lm	Listeria Monocytogenes
LPS	Lipopolysaccharides
MCA	3-methylcholanthrene
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NK	Natural killer
NSG	NOD SCID gamma
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cell
PFU	Plaque-forming unit
PRRs	Pattern-recognition receptors
PSMA	Prostate-specific membrane antigen
RNA	Ribonucleic acid
RT	Room temperature
SOCS-1	Suppressor of cytokine signaling 1
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Th	T helper (cell)
TLRs	Toll-like receptors
WT	Wild-type

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General introduction:

1. The immune system:

Since the apparition of life, organisms evolved from very simple microorganisms, to more and more intricate forms of life. As the complexity of organisms increased, their cells started specializing and were assigned more precise functions.

The immune system is defined as the combination of specialized tissues and cells, which defend an organism against external enemies. The core idea of the immune system is the discrimination between "self" and "non-self", and hence to both protect the host and fight pathogens. Nonetheless, during its evolution the immune system has also become able to discriminate and get rid of "altered self", such as malignant cells.

The immune system of mammals is divided in two main parts: the innate immune system, which can immediately respond and fight broad classes of pathogens, and the adaptive immune system, which takes a few days to be ignited, but gives rise to a peptide-specific response. The innate and the adaptive immune system, as well as key characteristics defining them, will be presented in this chapter.

1.1. The innate immune system

The innate immune system is the first line of defence when pathogens manage to cross the physical barriers protecting the inside of the body from the outside – namely internal and external epithelia. To prevent the crossing, epithelial surfaces are able to secrete mucus, that coats bacteria and interferes with their adhesion and invasion, as well as antimicrobial peptides that can directly kill the aforementioned pathogens[1]. If a breach in the barrier occurs and pathogens are able to enter, their first encounter will be with innate cells named **macrophages** that permanently reside in tissues throughout the body. These cells are able to recognize **pathogen-associated molecular patterns** (PAMPs), via receptors recognizing broad classes of molecules expressed at the surface of microbes. There are several classes of pattern recognition receptors (PRRs), and the most extensively studied are the **Toll-Like Receptors** (TLRs). Different receptors will bind to different PAMPs (see Figure 1). For example, TLR-9 binds in the endosome to the unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) DNA, which is found in viruses. TLR-9 is expressed in macrophages as well as plasmacytoid dendritic cells (pDCs) and B cells. Recognition of CpG by TLR-9 induces the expression of type I interferon, and inflammation, which is why it is often used as an adjuvant to start an immune response.

Upon recognition of a PAMP, macrophages can phagocyte the invader, and destroy it internally via acidification and the fusion of the phagosome with another vesicle containing lysing peptides, proteins and enzymes. Once activated in this manner, macrophages are able to produce chemokines, such as CXCL8, to recruit neutrophils[2] and cytokines, such as IL-12 to recruit natural killer (NK) cells.

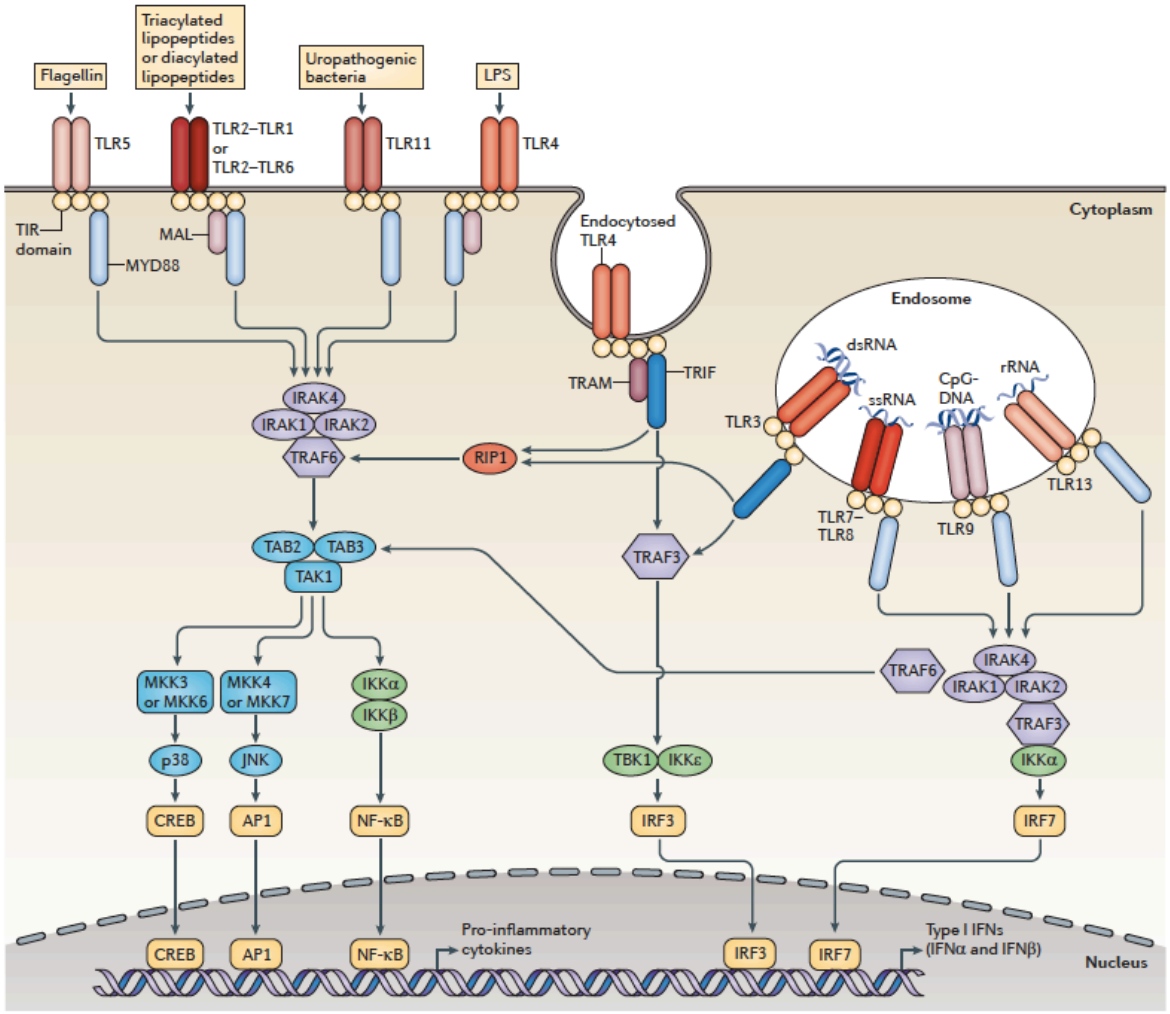

Figure 1: Toll-like receptors, their ligands and signalling pathways. TLR-1, 2, 4, 5, 6 and 11 can bind extracellular PAMPs and induce signalling. TLR3, 7, 8, 9 and 13 are present in the endosome and detect viral nucleic acids[3]

Neutrophils are a second family of phagocytes that are recruited to the tissue upon inflammation. They can recognize and destroy microbes through the generation of reactive oxygen species. Upon activation, they produce inflammatory cytokines and chemokines, which in turn recruit more macrophages and neutrophils to the inflammation site. Moreover, they are able to present peptides to T cells via the expression of a membrane glycoprotein named Major Histocompatibility complex (MHC).

Immature **dendritic cells** (DCs) are a third class of cells that can phagocyte and destroy microbes while also acting as antigen-presenting cells (APCs). Their primary function upon engulfment of the pathogen is to degrade it into peptides and present it to the adaptive immune cells.

Lastly, **natural killer** (NK) cells are able to directly lyse infected or malignant cells. NK cells are activated by interferon-(IFN-) α and β and the interleukin IL-12. These cytokines are produced by activated macrophages and DCs. Once activated, NK cells are able to destroy target cells through the release of cytotoxic granules containing the lytic enzymes perforin and granzyme. NK cells do not recognize ligands that derive directly from a pathogen, but rather react to molecules that are associated with an altered cellular state. For instance,

several viruses as well as tumours have evolved to downregulate MHC class I surface expression in order to escape recognition from the adaptive immune system[4]. In this context, NK cells are useful as they are able to detect changes in MHC class I expression. Indeed, a low level or complete absence of surface MHC-class I renders the target cell susceptible to NK cell killing while normal levels of MHC class I keeps NK cells tolerant[5]. In addition to the absence of MHC class I, an inflammatory context and the expression of activatory ligands are also required to trigger NK cell cytotoxicity. The ones that are best characterized are MICA, MICB and ULBP4, which can bind the receptor NKG2D on the surface of the NK cell. These ligands are not expressed on healthy cells, but are upregulated upon cellular stress[6].

Cells of the innate immune system have the ability to directly attack microbes, and to produce cytokines and chemokines to attract more cells to the site of inflammation. However, this is often not sufficient to control an infection. Thus, a crucial role of the innate immune system is to initiate the adaptive immune response, which is specific and more potent. A key step in this initiation is the presentation of specific peptides, called **antigens,** to the cells of the adaptive immune system.

1.2. Antigen presentation

Antigen presentation by APCs to lymphocytes occurs in 3 step: APC activation and maturation, processing of the antigen for presentation, and finally migration to the lymph node - where APCs and naive lymphocytes can meet.

1.2.1. APCs maturation and activation

Immature APCs have a high turnover of MHC class I and II and low surface expression of these molecules. Moreover, they have a high rate of endo- and phagocytosis. **Maturation** of the APCs reduces their endocytosis rate, and increases the peptide:MHC half life and surface expression. Upon recognition of PAMPs, APCs get **activated**. They upregulate costimulatory markers, such as CD80 and CD86 and start producing cytokines. This costimulation and inflammatory environment is essential to the ignition of a potent immune response. In contrast, when antigens are presented by a mature but non-activated APC, in the absence of an inflammatory context, it creates tolerogenic CD4+ or CD8+ T cells[7]. This mechanism is called **peripheral tolerance**, and ensures that T cells do not react against self-antigens when encountered in the periphery.

1.2.2. Antigen processing and loading on the MHC

APCs can present peptides originating from either their cytosol, or from intracellular vesicles. In the case of intracellular proteins, they are degraded by the proteasome, and processed into peptides. These peptides are then transported into the endoplasmic reticulum (ER) by specialized transporters called TAP1 and TAP2 (Transporter associated with Antigen Presentation)[8]. In the ER, 8-10 amino acid peptides can bind to the MHC class I. The MHC:peptide complex is then exported on the surface of the cell for presentation. The pathway is different for extracellular pathogens. Since they are phagocytosed, they are not

present in the cytosol but in intracellular vesicles. These vesicles are gradually acidified until the proteins are degraded into peptides by proteases. At this point, the peptide-containing vesicle fuses with another vesicle containing the MHC class II. In opposition to MHC class I peptides that are limited to 10 amino acids, MHC class II peptides are of more variable length, but usually 13 to 17 amino acids. Once on the surface of the APC, MHC class II binds the TCR of CD4+ T cells and can trigger T helper cell differentiation, while MHC class I binds to the TCR of CD8+ T cells to turn them into cytotoxic T cells[9]. However, it was shown that a subtype of APCs is able to **cross-present** peptides originating from vesicles onto MHC class I. This is useful when the APC itself is not infected, but ingests debris of another cell infected by an intracellular pathogen. This ability of cross-presentation is a feature of a subclass of DCs, CD8+ DCs, that are able to engulf debris of infected cells, and **cross-present** the antigen coming from the outside of the cell, onto the MHC class I, and hence to trigger the differentiation of specialized CTLs[10,11]. In this case, proteins present in the ER can be transported into the cytoplasm by retrograde translocation. There they can be processed by the proteasome and loaded onto MHC class I. This only seems to occur when CD4+ T cell help is provided, in the form of the T cell molecule CD40L binding to CD40 on the DCs[12]. Indeed, CD4 help could be replaced by a CD40-stimulating monoclonal antibody to induce DCs cross-presentation of an exogenous antigen and thus potent CTL priming[13].

1.2.3. Migration to the lymph node

Maturation of the APC drives a reprogramming of the cell that induces it to travel to the nearest lymph node for antigen presentation to B and T cells. Upregulation of CCR7 on the DC as well as the expression of its ligand, CCL21, on lymphatic endothelial cells, was shown to be necessary for DC migration to the lymph node[14]. DCs could be detected in the lymph node already 1 day after subcutaneous injection, and their accumulation reached a plateau after 3 days.

1.3. The adaptive immune system development

In contrast to the innate immune response, which is immediate upon encounter with a pathogen, the adaptive immune response takes a few days to be launched. There are 2 main types of adaptive immune cells; T cells and B cells. Both are created in the bone marrow from common precursors, but T cells have to travel to the thymus for their maturation. Through a large variety of T cell receptors (TCRs), T lymphocytes are able to recognize specifically a high number of antigens. The variety in the T cell **repertoire** is generated because of the recombination of TCR genes. The TCR is composed of 2 chains, α and β, which are comprised of 1 variable (V), 1 joining (J), and 1 constant (C) segment. These single segments are randomly joined through somatic recombination of the immature T cell TCR genes , which originally comprises 70-80 V segments and 61 J segments (see Figure 2). The main enzymes responsible for this genetic rearrangement are RAG-1 and RAG-2[15]. The genetic recombination gives rise to a potential diversity that has been estimated to be of 10^{15} possible TCRs, which can each recognize several different peptides[16]. However, the human body contains a total of only 10^{12} T cells at a given time. Moreover, the total number

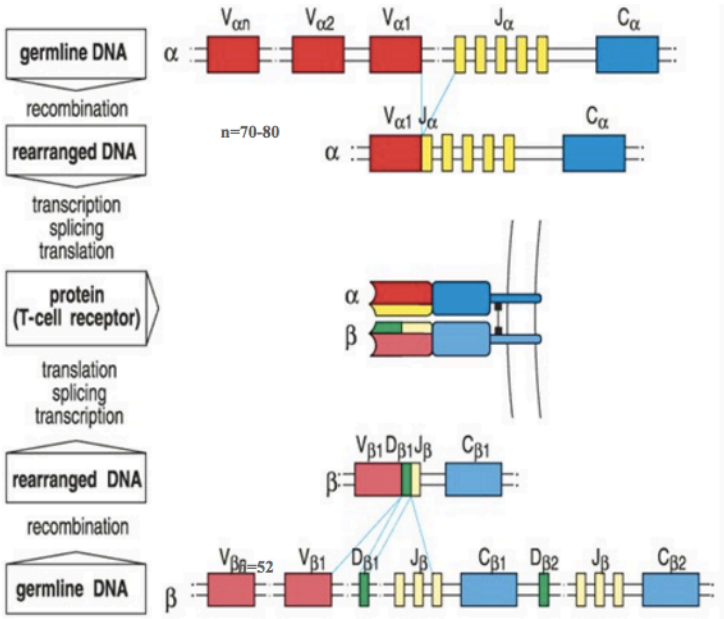

Figure 2: T cell receptor α- and β - chain recombination and surface expression[9]

of different TCRs actually present in a human is probably less than 10^8 [17]. Indeed, many potential TCRs are never expressed because they are deleted in the thymus before they can reach the periphery during a process called **clonal selection**, which occurs in two steps.

Firstly, thymic **positive selection** is mediated via presentation of self-antigens by epithelial cells in the thymic cortex. Only T cells that are able to bind the self MHC-peptide complex receive survival signals and are selected. Next, **negative selection** occurs in the medulla of the thymus, mainly via the presentation of self-antigens by bone marrow derived DCs and macrophages. During this step, T cells that possess a TCR of too high affinity to a self-antigen are deleted[18]. This ensures that T cells that exit the thymus and enter the periphery are not self-reactive and will not cause autoimmunity. If T cells respond to MHC class II, they become CD4 positive. If they get activated by MHC class I, they become CD8 positive.

Single positive T lymphocytes reaching the periphery are mature but have not yet been activated via the encounter of their specific antigen. Hence, they are called **naïve T cells**. They are characterised by the expression of CD62L (or L-selectin), which allows them to adhere to the vascular endothelium of peripheral lymphoid organs.

B cells originate from the same progenitor as T cells and also undergo gene rearrangement to produce clonal diversity. Unlike the T cell receptor that is only expressed as a transmembrane protein, the B cell receptor can be expressed either as a transmembrane receptor (BCR) or as a secreted antibody. This molecule is called **immunoglobulin** (Figure 3). Immunoglobulins are composed of 2 chains, the heavy and light chains. The heavy chain is the first one to be rearranged at the pre-B cell stage, and its successful surface expression induces

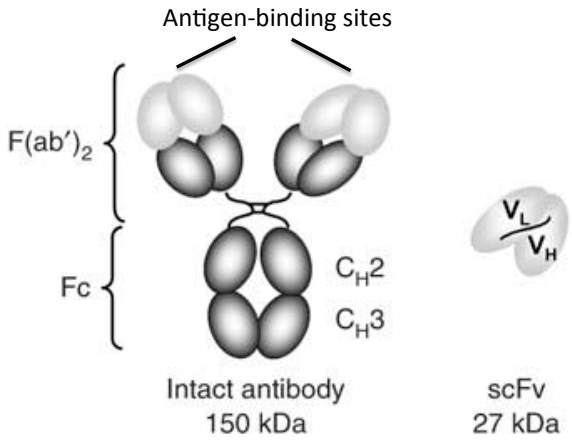

Figure 3: Immunoglobulins are made from a heavy and a light chain connected via disulphide bonds. They can be separated into an Fc portion and two F(ab) portions via cleavage with the enzyme papain. They possess two antibody-binding sites. Single chain antibody fragments (scFv) can be made via joining the variable region from the light chain (V_L) and from the heavy chain (V_H). Adapted from Olafsen *et. al.* 2006 [203]

cell-survival signalling and the start of the light chain gene rearrangement. The expression of a functional light chain produces an immature B cell. There are several subtypes of immunoglobulins that serve different functions. Immunoglobulins M (IgM) are always the first type to be expressed. They are usually of low affinity and expressed in their soluble form as a pentamer. Affinity maturation of the immunoglobulin, as well as isotype switching is possible thanks to CD4+ T follicular helper cells (Tfh). Firstly, upon binding of the BCR to its cognate antigen, the pathogen or virus is internalised and degraded. Afterwards, the peptide is presented in the context of MHC class II on the surface of the B cell to CD4 helper T cells that would have been activated by the same peptide. The activated CD4 T cell can in turn activate the B cell through CD40L:CD40 interaction and induce the production of specific **antibodies** against the pathogen. Different classes of antibodies are specialized in different functions. IgMs, are produced before affinity maturation and hence are of lower affinity. Nonetheless, they can form pentamers and activate the complement system very efficiently. IgG is the most common class in the blood and extracellular fluid. IgGs can also activate the complement system, but mainly act as an inducer of antibody-dependant cell-mediated cytotoxicity (ADCC), via the binding of Fc receptors on innate immune cells. IgE are the mediators of allergic reactions. Finally, IgAs form dimers and can be secreted in the gut and the lungs[9].

1.4. Induction of an adaptive immune response

Naive T cells constantly recirculate between the blood stream and peripheral lymphoid organs, scanning for peptides. They access the lymph nodes by the high endothelial venule, which expresses CD34, the ligand for CD62L. Once there, they can make contact with many dendritic cells and other antigen presenting cells. If none of the presented peptide binds to their TCR, naive T cells exit the lymph node via the efferent lymphatic. If one T cell is specific for a peptide presented by a mature antigen presenting cells, and if the appropriate costimulatory signals are provided, T cell activation and clonal expansion will start. Three consecutive signals are necessary for correct and functional **T cell priming**.

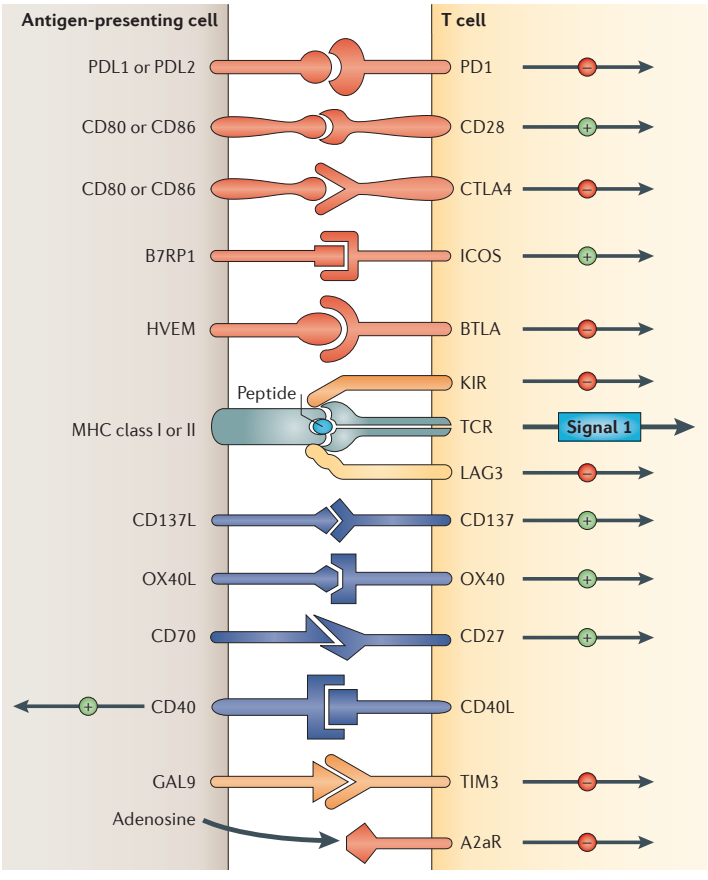

Figure 4: Scheme of known APCs ligands providing either an activatory or an inhibitory second signal and their respective receptors on T cells. Adapted from Pardoll, 2012[204]

The **first signal** is the binding of the MHC and TCR together. MHC class II binds to the TCR of CD4+ T cells, whereas the MHC class I binds to the TCR of CD8+ T cells. CD4 and CD8 both contribute to stabilise the MHC-TCR interaction. The **second signal** comes from costimulatory molecules expressed on the surface of the APC that will bind to surface receptors on the T cell. The best characterized costimulatory molecules are from the B7 family. These ligands can induce either a stimulatory or a suppressive signal 2, as shown on Figure 4, in red. The **third signal** is the secretion of cytokines from the antigen presenting cell (APC), which can direct T cells to differentiate into the appropriate effector phenotype. Several subtypes of CD4 T cells (also called T helper cell) exist. The main ones and their function are summarized in Table 1. The role of CD8 T cells (also called **cytotoxic T cell, CTL**) is also summarized.

Once activated, Th1, Th17, and CTL cells will clonally proliferate and migrate to the site of infection to respectively activate infected macrophages, recruit neutrophils and kill infected cells. However, some T helper cells, and especially Th2 will remain in the lymph node to interact with B cells and induce the proper antibody class switching. T regulatory cells (Tregs) can locally supress an immune response via the expression of IL-10 and TGF-β. They are important to mediate peripheral tolerance and protect against autoimmune disease.

An additional role of Th1 cells is to provide better CD8+ T cell priming when interacting through CD40-CD40L with the same antigen-presenting cell in the lymph node. It was shown that the CD4 T cell help was necessary for CD8+ T cell reexpansion upon a second stimulation[19]. The ability of the immune system to reexpand upon a second antigen encounter is called **immunological memory.**

Name	Induced via	Role	Production of
Th1	IL-12, IFN-γ	• Activation of macrophages • Stimulation of CD8 proliferation	IFN-γ, TNFα
Th2	IL-4	• Promotion of antibody production by B cells • Protection against extracellular parasites	IL-4, IL-5, IL-13
Tfh	IL-6, IL-21	• Antibody class switching	IL-21
Th17	TGF-β, IL-6	• Recruitement of neutrophils • Development of acute inflammation	IL-17, IL-22
Treg	TGF-β	• Immunosuppression	IL-10, TGF-β
CTL	-	• Killing of cells presenting the antigen	IFN-γ, TNFα

Table 1: Induction and function of CD4 (T helper cells) main subtypes and CD8 (cytotoxic T cell)

1.5. The immunological memory

The first observations of the ability of the immune system to "remember" a past infection occurred a long time ago, precisely in Athens in 430 BC during a smallpox outbreak. It is then that the Greek historian Thucydides first noticed that it "never attacked the same person twice; so, at least, as to be mortal"[20]. However, in Europe, it is much later that it was attempted to use this characteristic for protection against infections. In 1796, Edward

Jenner made the observation that people working with cows were less prone to die from smallpox. He then assumed that the infection with the non-lethal cowpox virus could protect the individual against smallpox infection. To test his hypothesis, he inoculated material from a cowpox pustule into a healthy individual. He could then demonstrate that this method was indeed protective against smallpox infection. He named his newfound remedy **vaccine**[21].

Vaccines take advantage of the **immunological memory** – namely, the aptitude of the immune system to remember past infections and to provide a more efficient immune response upon a second encounter with an identical or similar pathogen. The immunological memory is also a defining feature of the adaptive immune system, which is able to give rise to long-lived memory cells, although some innate cells and especially NK cells have been described as having some memory features as well[22]. One key aspect of the immunological memory is that it occurs in the absence of the "remembered" peptide or pathogen[23].

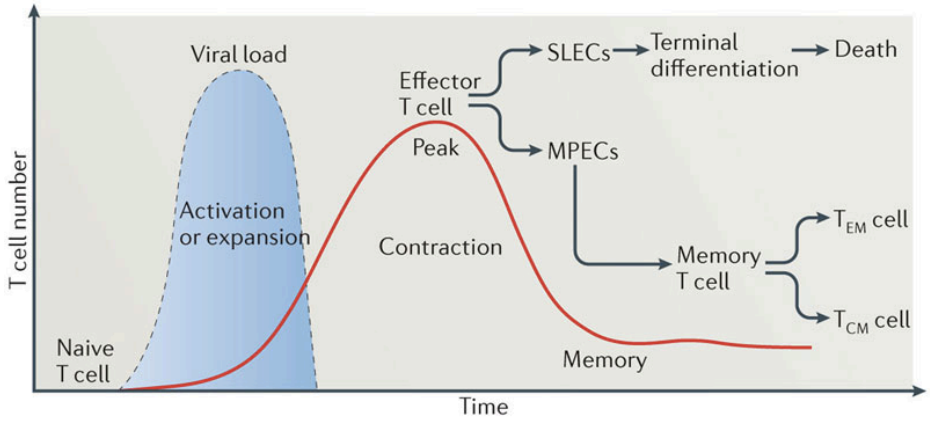

Figure 5: T cell response occurs in three phases: expansion, contraction, memory. Short-lived effector cells (SLECs) differentiation into terminal effectors and disappear after the clearance of the infection. Memory precursors effector cells (MPECs) give rise to the effector memory (T_{EM}) and central memory (T_{CM}) T cells[24]

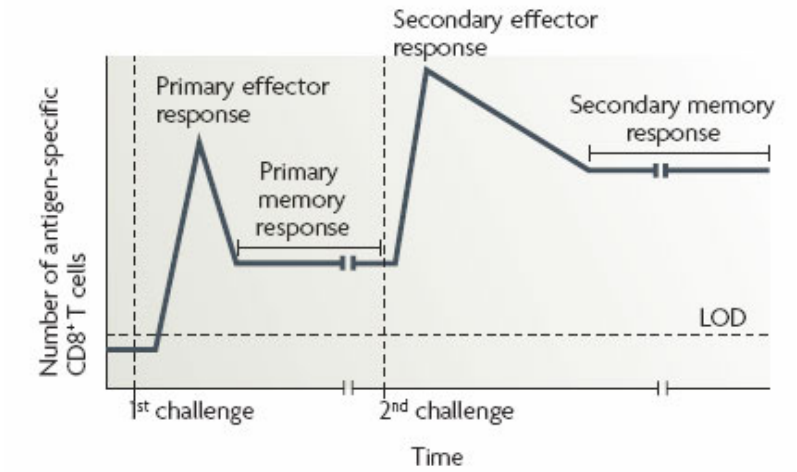

Figure 6: Memory CD8+ T cells are generated after a first infection and can respond faster and with a higher magnitude to a second encounter with the pathogen[25]

The response of the adaptive immune system occurs in three phases: expansion, contraction and memory (see Figure 5). In the contraction phase, the short-lived effector

cells (SLECs) start dying. These cells are characterized by the surface expression KLRG1^{high} and CD127^{low}, and the transcription factor T-bet. The memory T cells are the cells that are left after the contraction phase. They stem from memory precursor effector cells (MPECs) that can already be detected in the effector phase of the CD8+ T cell response via their surface expression of KLRG1^{low} and CD127^{high}, and of the transcription factor eomesodermin (EOMES)[26]. Memory T cells undergo a defined reprogramming after activation and can survive for long periods of time, and respond rapidly, and with greater magnitude, to a second encounter with the same microbe (see Figure 6).

Memory T cells have characteristics that differentiate them from naïve lymphocytes, and that allow them to respond faster to the second encounter with a germ, and hence mediate protection against the pathogenicity.

Different surface markers and cytokine receptors:

Memory CD8+ T cells express specific surface markers and transcription factors

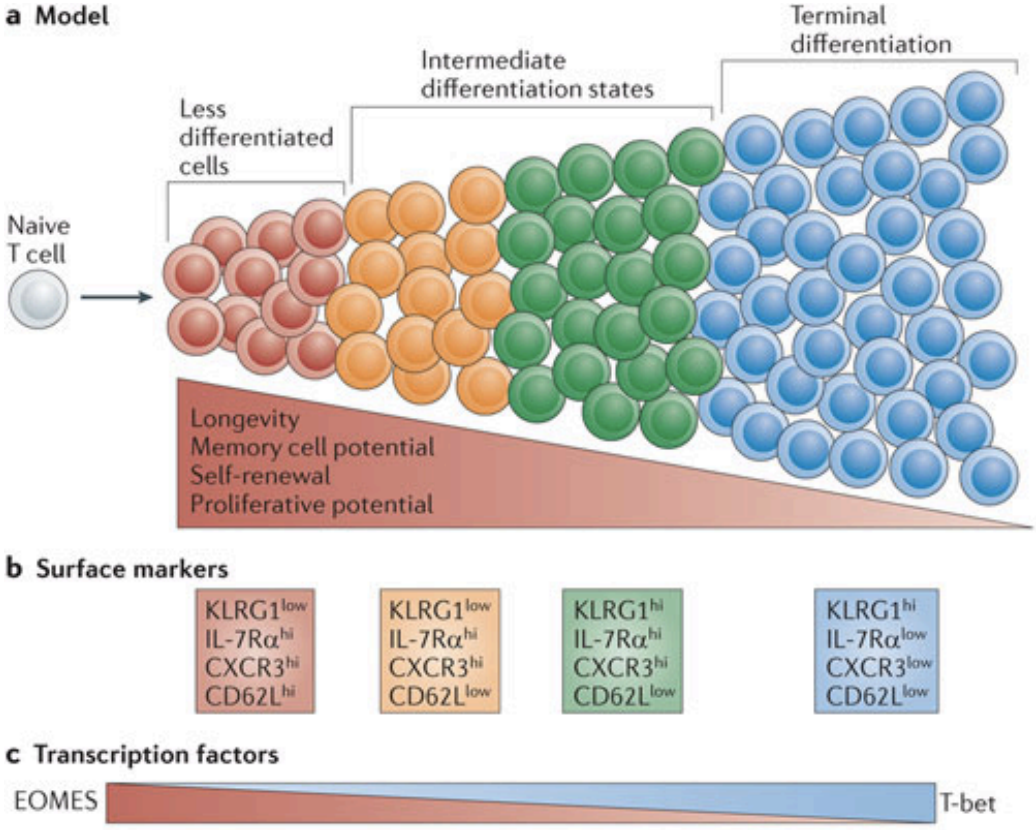

Figure 7: Characteristics of memory CD8+ T cells in terms of function, surface markers, and the expression of the transcription factor EOMES or T-bet. Adapted from Kaech *et. al.* 2012[27]

Higher precursor numbers

The amount of antigen-specific CD8+ T cells available to respond to an infection is greater after a first encounter. Indeed, after the initial clonal expansion and effector phase, comes the contraction phase in which most of the effector cells undergo apoptosis, after the infection is cleared. However, some T cells are able to survive this contraction step and remain in the circulation of long periods of time. These memory cells are more numerous than the original naïve precursor, which gives a mathematical advantage for a secondary

response[28]. For example, the frequency of antigen specific CD4+ T cells following acute LCMV infection was increased 10-fold after the resolution of the infection and was maintained for long periods of time (>300 days)[29].

Lower activation threshold (?)

Logically, it was firstly considered that memory cells had a lower threshold for TCR activation and signalling. Indeed, it was shown in 1997 that CD4+ T cells that had been activated and cultured for 12 days could respond to lower amounts of peptide:MHC complex compared to naive cells[30]. Moreover, the previously activated cells could also respond to peptides of lower affinity compared to naïve cells[31]. However, some contradictory data have been produced recently, which show that memory CD8+ T cells could not respond to low level of antigen, at which naive CD8+ T cells were able to start proliferation[32].

Metabolic reprogramming:

Upon activation, T cells switch their metabolic program from oxidative phosphorylation (OXPHOS) towards aerobic glycolysis. This change in respiration is not necessary for the proliferation and survival of T cells, but it is crucial for their functionality, in particular cytokine secretion. Indeed, it was shown in CD4+ T cells that the switch to aerobic glycolysis mobilised the enzyme GAPDH and allowed its release from the IFN-γ mRNA, thus inducing the cytokine's expression[33]. Memory CD8+ T cells were also shown to have the ability to switch from oxidative phosphorylation to aerobic glycolysis when OXPHOS was pharmacologically inhibited with oligomycin. Moreover, memory CD8+ T cells could upregulate their glycolytic respiration at higher level than naive T cells upon CD3/CD28 activation. Similarly to CD4+ memory T cells, CD8+ T cells' GAPDH's activity was higher and localised in the cytoplasm, which would increase its availability for rapid glycolysis upon activation[34].

In the case of cancer or chronic infection, the antigen is never cleared from the circulation, which prevents the normal contraction phase and memory differentiation of the T cells. In that case, they gradually differentiate into a specific state called **exhaustion.**

1.6. T cell exhaustion

Exhausted T cells arise upon chronic stimulation with a specific antigen, and gradually become functionally impaired. Exhausted T cells exhibit a high surface expression of inhibitory markers and are unable to adequately respond to stimulation. It was firstly described in 1998 that CD8+ T cells, specific for the antigen gp33, could persist after acute or chronic infection with LCMV. But the cells recovered from the chronically infected animals were not able to produce IFN-γ upon PMA ionomycin restimulation[35]. Moreover, this impairment was more pronounced in the absence of CD4 helper cells, and the duration of antigen exposure was positively correlated with the severity of the exhaustion[35]. The usefulness of such an inhibitory mechanism was illustrated by the intracranial infection of mice with the LCMV virus. Mice infected with low dose LCMV die of immunopathology 9

days after the infection. Mice receiving the high dose LCMV, on the other hand, develop a chronic infection, but do not succumb to the disease as their CD8+ T cells become rapidly inactive due to the high amount of antigen exposure[36]. It is in fact the antigen exposure specifically that drives the exhausted phenotype. Indeed, it was shown in a recent study that CD8+ T cells exposed to a chronic inflammation but low dose of antigen could remain functional[37].

A key aspect of the exhausted phenotype is the surface expression of **inhibitory markers.** PD-1 was the first receptor to be linked to the exhausted phenotype. Indeed, blockade of PD-L1 during a chronic infection could restore virus-specific CD8+ T cells' persistence, as well as ability to proliferate and secrete TNF-α and IFN-γ. Moreover, infection of PD-L1$^{-/-}$ mice with the chronic LCMV clone 13 strain induced lethal immunopathology between 6-7 days after the infection[38].

For a while it was thought that memory CD8+ T cells could not form in chronic infections. However, it was demonstrated recently that a subpopulation of exhausted CD8+ T cells, characterized by high PD-1 expression and low production of IFN-γ and TNF-α, could reexpand after transfer into a naive host and subsequent infection. After re-infection, PD-1^{high} CD8+ T cells from a chronic LCMV infection could increase their numbers of 10-fold, compared to 100-fold for PD-1^{low} cells from an acute LCMV infection. Moreover PD-1^{high} cells expressed less cytokines[37]. This subpopulation of PD-1^{high} cells that could reexpand relied on the expression of the transcription factor TCF-1. Indeed, TCF-1$^{-/-}$ CD8+ T cells from chronically infected animals could not reexpand upon transfer into a naive host and infection with acute LCMV. Moreover, when CD8+ T cells were extracted from chronically infected mice and sorted according to their expression of TCF-1, only TCF-1+ CD8+ T cells could reexpand upon a second infection[39]. These TCF-1+ PD-1^{high} CD8+ T cells had a specific transcriptional profile resembling CD4+ Tfh cells and CD8+ memory precursors. They could be identified via the surface expression of CXCR5 and costimulatory molecules, such as ICOS, OX-40 and CD28[40].

Wherry *et. al.* characterised the gene signature of exhausted CD8+ T cells and identified several inhibitory markers that were upregulated in addition to PD-1, such as 2B4, CTLA-4, NKG2A and LAG-3[41]. Exhausted T cells were also observed in human cancers. CD8+ T cells extracted from tumour infiltrated lymph nodes of melanoma patients exhibited an increased expression of the inhibitory markers LAG3, 2B4 and CTLA-4, and a decreased ability to secrete IFN-γ upon restimulation[42].

Finally, exhausted CD8+ T cells also have a different metabolic program than normal memory T cells or effector cells. In humans, hepatitis B virus (HBV) specific CD8+ T cells expressed higher levels of the glucose receptor Glut1 and hence could uptake glucose more efficiently than the cytomegalovirus (CMV) specific CD8+ T cells. Glut1 expression was positively correlated with PD-1 expression and negatively correlated with IFN-γ secretion[43]. Moreover, CD8+ T cells that are activated and forced to use OXPHOS for their respiration (cultured in galactose instead of glucose), upregulate PD-1 and cannot produce cytokines, as GAPDH binds to the IFN-γ mRNA and prevents its expression[33].

2. MicroRNAs

MicroRNAs are small RNA molecules that regulate gene expression by repressing the translation of mRNAs or inducing their degradation[44]. They were first discovered in 1993, when it was observed that *C. Elegans* expresses a small antisense RNA, named lin-4, that is complementary to the 3'UTR sequence of the lin-14 mRNA, and thus induces lin-14 protein levels downregulation[45]. Since lin-4 did not encode for a protein, it was understood that its sole function was as a non-coding microRNA.

Mature microRNAs are single strand RNAs and are usually encoded in intronic regions, although they can exceptionally be encoded in exonic regions as well. They are often transcribed in the form of microRNA clusters. Transcription is classically carried out by RNA pol II, but it was shown by Chen *et. al.* and others, that it is possible to express miRNAs using RNA pol III promoters[46]. They are first expressed in the form of a long (typically over 1kb) pri-miRNA, that contains the stem-loop miRNA sequence (see Figure 8).

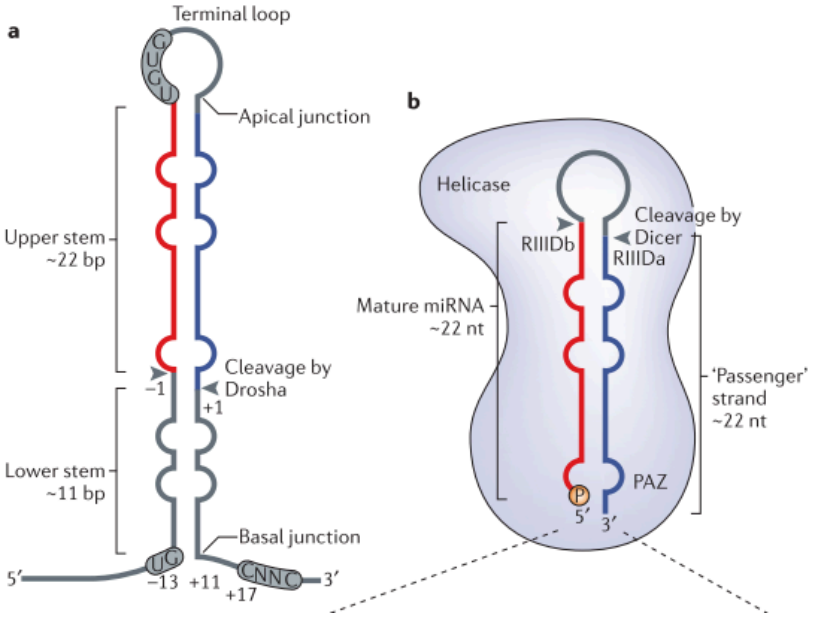

Figure 8: Nuclear processing of the pre-miRNA by Drosha. Cytoplasmic processing of the pri-miRNA by Dicer[205]

The enzyme Drosha cleaves the pri-miRNA on both ends of the stemloop sequence in the nucleus[47]. Then, the pre-miRNA strand, of approximately 70 nucleotides, is exported into the cytoplasm, where it is processed by the enzyme Dicer into a 20-25 nucleotides mature microRNA[48]. The hairpin secondary structure is characteristic of microRNAs and is necessary for their correct processing by Dicer. Indeed, it was shown that disruption of the base-pairing at the beginning of the hairpin stem was deleterious to mature microRNA expression[49]. Finally, it is incorporated into the miRNA-induced silencing complex (RISC) which can bind to the 3'UTR of the target mRNAs and repress translation (Figure 9)[50]. This is done mainly via a 6 nucleotides-long **seed sequence**. The binding of the microRNA seed sequence to its mRNA complementary sequence involves imperfect base-pairing, which allows one microRNA to regulate several mRNAs[51]. The highest complementarity induces

the strongest translation inhibition. Indeed, it was shown that a single mismatch in the seed sequence could still inhibit the expression of the mRNA target, but with a 2-fold reduction in efficiency[49]. Often, microRNAs will affect protein production by blocking translation, without actually affecting the levels of the mRNA[49,52,53]

Based on this complementarity, computational approaches can be used to predict microRNA targets. However, such targets should always be confirmed through biological assays. Reporter sequences, encoding for the GFP protein or the luciferase enzyme, and containing the mRNA seed sequence in their 3' UTR, can be used to confirm a certain microRNA is indeed complementary to an mRNA of interest[54]. Other methods exist to screen for new targets of a microRNA of interest: for example the immunoprecipitation of proteins of the RISC complex, and identification of co-precipitated mRNAs by microarray[55].

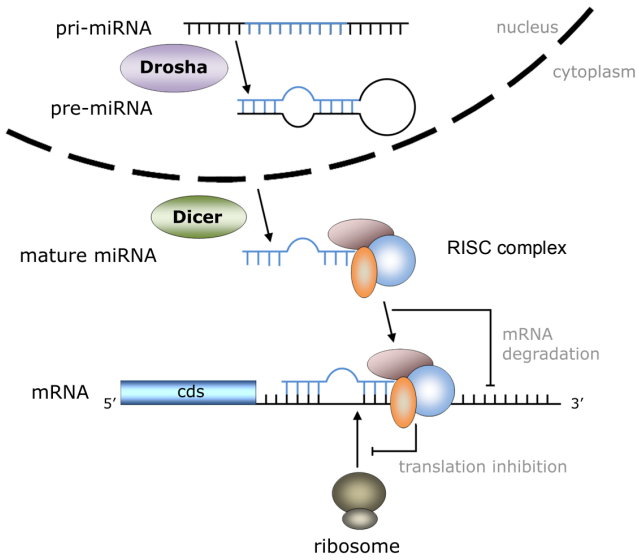

Figure 9: miRNA processing and translational repression[206]

The vital importance of microRNAs was highlighted by experiments deleting the enzyme Dicer, which is responsible for the production of most mature microRNAs, with some exceptions[56]. A complete Dicer knock-out (KO) mouse prevented the formation of viable embryos by blocking the differentiation of embryonic stem cells[57,58]. MicroRNAs are also important for the immune system. Conditional deletion of Dicer in the late stage of the lymphocyte maturation (CD4-CRE x Dicer fl/fl), decreased the number of mature CD4+ and CD8+ cells in the periphery and interfered with their ability to proliferate and resist apoptosis[59]. Moreover, CD4-Cre Dicer deletion drove T helper cells towards the Th1 differentiation program[59], while strongly reducing the numbers of Tregs, inducing immune pathology as a consequence[60]. Experiments knocking-out Dicer in the CD8+ T cell compartment specifically, demonstrated that microRNAs are crucial for lymphocyte effector functions, migration and survival[61]. To identify which microRNAs could mediate such striking CD8 effector defects, Salaun *et. al.* performed microarrays and quantitative PCRs (qPCR) on human CD8+ T cells and identified a few microRNAs that were strongly upregulated upon CD8+ T cell activation. These were namely miR-155, miR-21, miR146a, miR146b and the miR-17-92 cluster[62]. The functions of miR-155 and miR-17-92 will be further detailed in this chapter, as they are the most important microRNAs in the context of this thesis.

2.1. MiR-155

MiR-155 was firstly discovered as the mediator of the oncogenic property of the B cell Integration Cluster (BIC) gene. Indeed, chicken B cell lymphoma induced by avian leukosis virus could develop only with the concomitant insertional activation of c-myc and BIC[63]. The

BIC gene lacks an open reading frame, and the complicated predicted secondary structure suggested an oncogenic function directly through BIC's RNA and not via protein translation[64]. Moreover, BIC homologs were identified in the mouse and human genome, which also did not possess an open reading frame and in which the microRNA secondary structure was conserved[65]. All this evidence pointed towards the functionality of BIC being mediated via the expression, in its third exon, of the microRNA miR-155.

Once miR-155 was identified, its overexpression was associated with many types of cancer in humans, such as B cell lymphoma[66], breast, lung and colon cancer[67]. In breast cancer, miR-155 high expression was associated with poor prognosis and metastasis[68], as well as increased radioresistance[69]. MiR-155 was shown to be responsible for the downregulation of the suppressor of cytokine signalling 1 (SOCS1)[70] and FOXO3a[71], leading to increased cell growth and survival. In malignant- and non-malignant T cell lines and in PBMCs, it was shown that miR-155 is upregulated upon STAT5 signalling but not STAT3[72]. Finally, the transcription factor IRF4 induced BIC expression in EBV-transformed B cells[73].

But miR-155 is not only expressed in cancerous cells. Indeed, its upregulation has been shown to be very important for the functionality of the immune system as well.

MiR-155 has a role in many cells of the innate immune response. Indeed, in human monocyte-derived DCs, miR-155 is upregulated upon stimulation with lipopolysaccharides (LPS) and functions as a negative feedback signal, reducing the production of the inflammatory cytokine IL-1 after the initial inflammatory response, by targeting TAB2 and BACH1[74]. MiR-155 was also upregulated in macrophages after TLR2, 3, 4 or 9 signalling as well as upon IFN-β stimulation[75]. In exhausted NK cells, low expression of miR-155 resulted in impaired IFN-γ secretory ability, as well as high levels of Tim-3 and T-bet. Artificial reconstitution of high miR-155 levels could re-establish some IFN-γ production and decrease the Tim-3 levels[76].

MiR-155 also has a crucial role in the development of the adaptive immunity. In activated CD4+ T cells, miR-155 is upregulated and induces the downregulation of IFN-γRα which bias the CD4+ differentiation into TH1 cells[77]. Moreover, miR-155 is necessary for the induction of experimental autoimmune encephalomyelitis (EAE), as miR-155$^{-/-}$ CD4+ T cells could not differentiate into Th17[78]. Surprisingly, Tregs also need miR-155 upregulation and subsequent suppressor of cytokine signaling 1 (SOCS-1) downregulation for their proper activation and expansion[79], which might indicate that there are additional factors that are critical in the decision of the CD4 differentiation route.

Finally, miR-155 has also proven to be important for CD8+ T cells effector functions. Indeed, experiments using miR-155 KO mice and adoptive transfer of miR-155 KO CD8+ T cells showed a strong impairment in clonal proliferation upon antigen recognition in the absence of this microRNA[80–82]. On the other hand, miR-155 did not seem to be crucial for intrinsic effector functions per se, such as cytokine production, killing and migration. The miR-155 enhanced T-cell fitness seemed to result from both reduced stability of the suppressor of cytokine signaling 1 (SOCS-1), as well as decreased responsiveness to the antiproliferative effect of type I interferon signalling[81,82].

Overexpression of miR-155 in CD8+ T cells increased the IFN-g production[82] and induced a more potent antitumour effect of CD8+ T cells in the pmel model[81]. Moreover, mice transferred with pmel CD8+ T cells overexpressing miR-155 could mediate B16 tumour rejection in the absence of lymphodepletion or injection of cytokines[83].

2.2. MiR-17-92

MiR-17-92 is a polycistronic microRNA cluster that contains 6 hairpin structures, which are ultimately processed into 6 mature microRNAs: miR-17, miR-18a, miR-19a, miR-20, miR-19b-1 and miR-92-1 (see Figure 10). Based on their seed sequence, these microRNAs are classified in 4 different microRNA families[84]. Redundancy for these microRNAs exists in the genome, as homologues to these microRNAs can be found on the chromosome X both in mouse and in human. However, miR-17-92 seems to mediate the strongest effect[85].

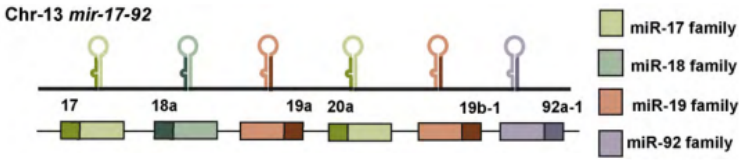

Figure 10: The human miR-17-92 cluster[84]

The differential expression of the miR-17-92 cluster, or *oncomir-1*, has been associated with the development of cancer[86]. The involvement of this cluster in B cell and other haematological malignancies has been shown in human, and miR-17-92 is proposed as a diagnostic tool for large B cell lymphoma[87,88].

In mice, overexpression of the truncated cluster miR-17-19b synergizes with the overexpression of c-myc to induce more aggressive B cells lymphomas. Moreover, the cancerous B cells were less differentiated when the microRNA cluster was overexpressed[89]. Furthermore, *Mu et al.*, showed that different members of the 17-92 cluster have unequal oncogenic potential, as the reconstitution of the miR-19 family alone was able to recapitulate the effect of the whole cluster in miR-17-92 KO lymphoma cells. In addition, they identified PTEN as a miR-19 target, thus partially explaining the increased proliferation of mir-17-92 overexpressing cells[90]. MiR-17-92 has also been proven to be important in other hematopoietic malignancies such as erythroleukemia[91].

In addition to its role in general development and its oncogenic properties, the miR-17-92 cluster is also crucial for the immune system development, response to infections and memory differentiation[92]. In pre-B cells, conditional knock-out of miR-17-92 enhances the apoptosis rate by the loss of regulation of the pro-apoptotic gene *Bim*[85]. In CD4+ T cells, IL-4 downregulates the expression of miR-17-92, and the overexpression of the cluster promotes the Th1 phenotype in differentiating T cells[93]. Furthermore, Jiang *et al*. showed that miR-17-92 regulates the efficacy of the effector Th1 cell response, by controlling IFN-γ production and proliferation, as well as preventing the differentiation into inducible regulatory T cells. In consequence, mice with a CD4-specific deletion of the miR-17-92 cluster are more susceptible to tumour challenge with B16 melanoma cells[94]. Recently, an article from Wu *et al.* showed that conditional granzyme-B-driven deletion of the miR-17-92 cluster impairs the CD8+ T cell effector response, by reducing their proliferative potential and IFN-γ production.

Finally, their results indicate that the overexpression of the cluster promotes a KLRG1^{high} CD127^{low} terminal effector phenotype[95]. This was due to miR-17-92 targeting and downregulation of PTEN, inducing enhanced mTOR signalling as a consequence[96].
Overexpression of miR-17-92 in CD8+ T cells bearing the chimeric antigen receptor (CAR) specific for EGFRvIII increased their ability to produce IFN-γ and to proliferate in contact with EGFRvIII+ U87 cells. CAR+ CD8+ T cells could mediate tumour regression *in vivo* however with no difference whether miR-17-92 was overexpressed or not. But miR-17-92 overexpressing CAR+ CD8+ T cells could mediate long term protection against later (49 days) tumour challenge[97].

These data demonstrate the importance of microRNAs for the functionality of all cells. More specifically, both miR-155 and the miR-17-92 cluster have crucial functions for the adaptive immune response, which explains the interest in targeting those microRNAs for the immunotherapy of cancer.

3. Cancer development:

It is now well understood that the core characteristic of a cancerous cell is its **mutated DNA**. Overexpression of certain pro-tumorigenic proteins, as well as deletion of important proliferation checkpoints is sometimes sufficient to start tumour formation. However, it is usually not a single mutation but rather an accumulation of mutations that can unleash a fully aggressive cancer form.

Indeed a tumoral lesion has to possess several characteristics in order to be able to develop into a full cancer. These characteristics, which were named **hallmarks of cancer**, firstly by Hanahan and Weinberg in 2000[98], encompass many aspects of the tumour. Some hallmarks are features related only to tumour cells themselves and their gene expression. Firstly, tumour cells should express survival and proliferation signals, and activate pathways to resist apoptosis. They should also be able to withstand many rounds of replication by blocking telomere shortening. In contrast, there are other hallmarks that take into account the relationship of the tumour with its environment, such as its ability to resist to the growth suppressors that might be present, as well as to induce the creation of new blood and lymphatic vessels. Finally, another hallmark of cancer is its ability to go through epithelial-mesenchymal transition (EMT) and invade new areas of the body.

Malignant cells also need to control the emergence of an antitumour immune response, and this ability was recently added to the list of characteristics as an "emerging hallmark"[99]. Indeed, although some level of inflammation can be desirable for the tumour formation, a strong immune activation together with infiltration of immune cells is usually fatal to the tumour.

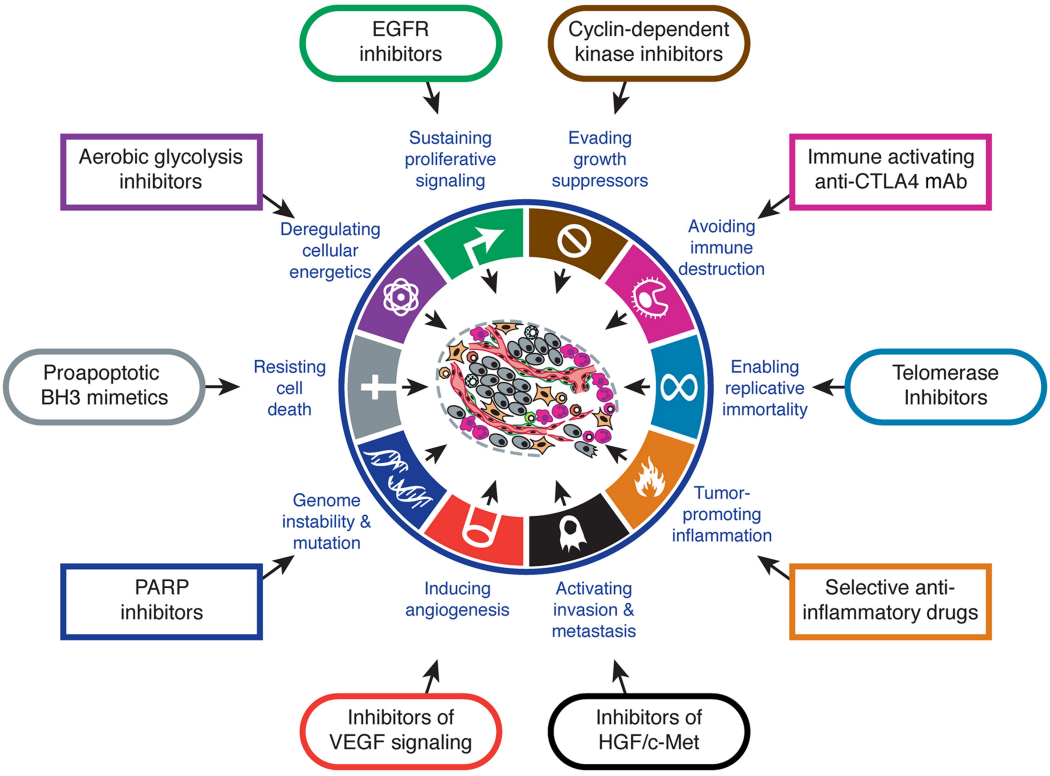

Figure 11: The hallmarks of cancer and drugs interfering with them[99]

3.1. Immunosurveillance:

DNA replication associated with cell division brings the risk of a potentially cancer-causing mutation. Intrinsic safety mechanisms, such as DNA repair and cell apoptosis, cause abnormal cells to die swiftly, before they can cause any harm to the organism. But the immune system is another important player that actively participates in patrolling the human body and eliminating undesirable mutant cells. This ability of the immune system to prevent tumour formation without external intervention is known as **cancer immunosurveillance**.

The first attempts to prove this hypothesis were unfruitful, as athymic nude mice and wildtype controls did not differ in their tumour development rate upon subcutaneous injection of 3-methylcholanthrene (MCA), a highly carcinogenic substance[100]. Both wildtype and nude MCA-treated cohorts developed subcutaneous sarcomas at similar rates - less than 20% - 120 days after treatment. Moreover, lung adenomas were detected in all cohorts, with an increase for mice that had received MCA but no difference relative to the mouse strain. From this study, Stutman and colleagues concluded that tumour immunosurveillance did not occur in their model. However, as it was later discovered, athymic nude mice do retain some immune cells and the ability to mount limited but useful immune responses[101]. Despite their lack of thymus, these mice possess some residual functional T cells, as well as an intact repertoire of many innate immune players such as NK cells. These elements might have been enough to compensate for the absence of thymus-maturated T cells and mask the effect of immunosurveillance in Stutman's aggressive tumour model.

Later on, the ability of the immune system to prevent tumour development was demonstrated using $RAG2^{-/-}$ mice. The recombination-activating gene-2 (RAG2) is necessary for the rearrangement of the T and B cell receptor during the lymphocyte development, and mice deficient in this protein lack T, B and NKT cells. In 2001, Shankaran and colleagues showed that $RAG2^{-/-}$ mice developed tumours earlier and with greater frequency than their wildtype counterparts. They also tested IFN-GR1 and STAT1 knockout (KO) mice strains, which turned out to be more sensitive to MCA treatment compared to wild type animals. Interestingly, double KO for RAG2 and STAT1 did not do worse that single KO in terms of tumour control, which shows a high level of overlap between the adaptive immune system and the IFN-γ response pathway. From this study, they concluded that the immune system, and in particular lymphocytes, were playing an important role in the control of tumour development[102].

But the innate immune system and innate-like T lymphocytes also play a part in tumour immunosurveillance. The TCR Jα281-deficient mice ($B6.J\alpha281^{-/-}$) are deficient in type I invariant NKT cells (iNKTs). This model was used to assess the importance of iNKT cells in the protection against spontaneous tumour induction by MCA treatment. Mice deficient in iNKT cells were more prone to tumour development than their wild type counterpart. Besides, additional depletion of NK cells did not worsen the phenotype, showing that NK cells are not able to compensate the absence of NKT cells[103]. Finally, absence of NK cells alone was

sufficient to aggravate the tumour phenotype, and abrogate the antitumoural effect of IL-12 injection. This shows that NK and NK T cells collaborate in the protection against MCA-induced tumours, and that NK cells are the main mediator of the immune response to the cytokine IL-12[104].

The cytokine IFN-γ was shown multiple times to be a very important immune mediator of protection against tumours, although in some cases it can contribute to tumour progession[105]. Dighe *et al.* demonstrated that the neutralization of IFN-γ by the injection of monoclonal antibodies completely abrogated the LPS-induced rejection of Meth A tumours[106]. IFN-γ can directly increase tumour cells' immunogenicity. Indeed, it was shown in 1988 by Weber and Rosenberg that surface expression of MHC class I was increased in tumour cells upon IFN-α or IFN-γ treatment, both *in vitro* and *in vivo*[107]*.* This upregulation, as well as the presence of a functional immune system were critical components of the observed IFN-γ -mediated tumour rejection[106]. To confirm these findings, it was shown that a tumour cell line engineered to express an MHC class I incompatible with the host's immune effector cells could hence not be rejected[107,102]. IFN-γ-mediated tumour rejection does not only act directly on the tumour, but also provides an appropriate inflammatory environment for the immune system to be efficiently activated. Indeed, it was shown that mice lacking STAT1 signalling, which is necessary for the immune response to IFN-γ, could not reject highly immunogenic tumours. This was due to deficiency in both cytokine production and cytolytic activity of T and NK cells[108]. It was later observed that the absence of STAT1 signalling was not intrinsically deleterious to the T cells effector functions, but was necessary in CD8α+ dendritic cells to mediate efficient antigen cross-presentation and priming of antitumour T cells[109].

However, IFN-γ signalling can sometimes make the tumour more resistant to the immune system. Indeed, mouse melanoma cells were shown to upregulate PDL-1 after IFN-γ treatment, inducing immunosuppression of tumour-specific CD8+ T cells[110]. In humans, a clinical trial evaluating the potential of IFN-γ for the immunotherapeutic treatment of ovarian cancer showed increased mortality in the treated group and had to be interrupted[111]. It was later shown that human ovarian cancer cells expressed the IFN-γ receptor and could upregulate PDL-1 upon IFN-γ treatment[112]. It is so far not well understood what are the key players determining the pro- or anti-tumoural activity of IFN-γ.

The existence of immunosurveillance in humans was tricky to demonstrate because of confounding factors, as immunosuppressed individuals are more susceptible to infections. However, some studies indicate that immunosurveillance is also relevant for humans. Indeed, immunosuppressed transplant recipients generally display an increased incidence of non-virally induced cancers[113]. For example, patients were found to have a 100-fold increase in the risk of melanoma after transplantation in Sweden[114]. Moreover, the incidence of both virally and non-virally induced cancers is increased in individuals that suffer from genetic immunodeficiencies[115].

All these data offer a glimpse of the different interactions between the immune system and the tumour and show the importance of several key components for tumour protection,

mainly inflammation via cytokines (IFN-γ) and the innate immune system (NK cells), efficient antigen presentation (dendritic cells) and a competent and persisting adaptive immune response (lymphocytes).

3.2. Immunoediting:

An apparent paradox in the immunosurveillance hypothesis is that not only immunocompromised but also immunocompetent host develop tumours. Why is that so, if the immune system is so good at preventing tumour growth? To more accurately represent the complex interaction between tumours and their environment, the cancer immunosurveillance idea was replaced by the more refined **immunoediting** concept.

Cancer immunoediting occurs in three phases. First, arising mutant cells are detected and removed by the host. This is the **elimination** phase. Secondly, there is the **equilibrium** phase, in which the host's immune system is unable to completely get rid of all malignant cells, but manages to control their growth. During this time, the immune system will exert a strong pressure on the tumour cells and only the least immunogenic mutants will survive. Depending on their tumorigenicity, these cells will go on to the third phase, the **escape**. In that final step, low immunogenic and highly malignant tumour cells are no longer controlled by the immune system and develop into a cancer[113].

First evidences of immunoediting arose from mouse tumour model. Indeed, tumours derived from immunocompetent hosts grew more aggressively than from $RAG2^{-/-}$ mice once transplanted into wild type mice[102]. This demonstrates that the adaptive immune system can shape the immunogenicity of escaping tumour cells. Additionally, it was shown that the innate immune system also influences the immunogenic phenotype of tumour cells. Indeed, tumours derived from mice lacking both the RAG2 protein and the common gamma chain ($RAG2^{-/-}$ x $\gamma c^{-/-}$) were more easily rejected once transplanted into WT mice, than tumours derived from $RAG2^{-/-}$ mice. This was due to IFN-γ produced by NK cells and polarisation of tumour associated macrophages into the antitumoural M1 phenotype[116]. These experiments show that tumour cells, which manage to escape the immune system, are selected for specific characteristics that allow them to expand better upon further transplant.

3.3. Tumour escape

Some elements that allow cancer cells to avoid immune control and increase their malignancy have been recently identified.

To escape effectors from the adaptive branch of the immune system, tumours can mutate key antigens as well as downregulate their expression of the major histocompatibility complex (MHC). Since recognition from T lymphocytes occurs through binding to the peptide-MHC, cells that express low or no MHC will be poorly recognized and lysed by cytotoxic T cells. In human tumours, complete MHC class I loss is frequent, and occurs in many types of cancer[117].

One would assume that the loss of MHC class I would induce more lysing of the tumour cells by NK cells and γδ T cells. However, it does not seem to be the case. Two mechanisms might

protect the malignant cells from NK and γδ T cell recognition. Firstly, the pro-tumoural environment is usually low on IL-12 and immunosuppressive, which is not adequate for the proper activation of NK. Secondly, NK cells also have to bind to the stress-induced ligands MICA/B via their NKG2D receptor for their activation. Consequently, many tumours lose the expression of these ligands, or even shed them in soluble form to induce a downregulation of NKG2D at the surface of NK cells before they reach the tumour cells[118,119]. These mechanisms protect the tumour cells from NK cells cytotoxicity.

Another escape technique is the secretion of **immunosuppressive cytokines** and factors to deactivate the immune response and deplete the tumour microenvironment of crucial nutrients. For example, the cytokine transforming growth factor β (TGF-β) inhibits the efficient activity of cytotoxic T lymphocytes *in vivo.* Additionally, TGF-β can convert CD4 T cells into inducible T regulatory cells (iTregs), which are in turn able to suppress the activity of other lymphocytes[120]. Moreover, this cytokine was also shown to induce the differentiation of myeloid cells into terminally differentiated myeloid mononuclear cells, which express CD39 and CD73. The CD39/CD73 enzyme pair degrades ATP into adenosine, which has further immunosuppressive effects[121]. In humans, TGF-β expression was found in several types of cancer and was associated with disease progression in breast cancer patients[122].

A further way in which tumours are able to deactivate the cytotoxic response is by catabolising the crucial amino acid tryptophan. They do so by overexpressing the enzyme indoleamine-2,3-dioxygenase (IDO) that catalyses the conversion of tryptophan to kynurenine. The depletion of tryptophan and the production of kynurenine have been shown as inducers of Th1 cell apoptosis[123]. Moreover, absence of tryptophan induces the activation of the stress-response kinase GCN2 in CD4 T cells and drives their differentiation into Tregs[124,125].

Finally, tumours are able to recruit myeloid cells that will help create an immunosuppressive microenvironment such as monocytes, which are transformed into **myeloid-derived suppressors cells** (MDSCs). Tumour cells can subvert myeloid cells via the expression of several cytokines and chemokines such as CSF1, VEGFA, SEMA3A, CCL2 and CXCL12[126]. The MDSCs in turn express inducible nitric oxide synthase (iNOS) as well as arginase 1, that both are deleterious to efficient T cell function through the production of reactive oxygen species and depletion of arginine[127]. Finally, tumours are able to express immunosuppressive ligands, such as PDL-1, which when bound to their receptor on the activated T cells, renders them tolerogenic and inactive (see Figure 4).

In addition of being able to hide from the immune system by downregulating MHC class I, tumours can directly deactivate it by creating a local **immunosuppressive environment**. They do so via the secretion of immunosuppressive factors, as well as the surface expression of inhibitory receptors and the recruitment of immunosuppressive cells. All these immunosuppressive mechanisms have to be carefully considered for the design of efficient immunotherapies.

4. Immunotherapy

The strong evidence of the importance of the immune system in mediating tumour protection promoted the development of antitumour immunotherapies. Given the fact that lymphocytes have the ability to kill tumour cells, and that CD8+ T cells infiltration correlates with good prognosis[128], the main aims of immunotherapies against cancer are both to increase the lymphocytes numbers on site, as well as to reactivate them. Indeed, lymphocytes present in the tumour microenvironment are usually of the **exhausted** phenotype and have lost most of their effector functions and tumour lysing abilities[129].

Different types of immunotherapies have been tested to boost the immune system against tumours: antibodies can either be used to target tumours epitopes and induce ADCC, or be used to block immunosuppressive pathways. Treatments with cytokines aim at systemically boosting the immune system. Cancer vaccines are made to elicit a specific response against one or several relevant tumour antigen. Additionally, adoptive cell transfer can be performed; by isolating and expanding a patient's own lymphocytes before transferring them back into the circulation. Finally, checkpoint blockade inhibitors aim to block the tumour's immunosuppressive receptors to enable the immune system to attack the tumour cells.

Originally, immunotherapies were classified in two types named passive and active immunotherapy, depending on whether the activation was mainly through the patient's endogenous immune system or via the injection of a foreign substance. However, this classification no longer holds due to the complexity of recent approaches. While therapeutic vaccines are without question "active" immunotherapy, antitumour antibody treatments, as well as cytokine treatments, which were classically designated as passive, do rely on the reactivation of endogenous immune cells for their functionality. Moreover, adoptive cell transfer techniques, in which a patient's own cells are genetically modified and reinjected into the patient, also rely on the patient's own immune cells. The passive versus active definition of immunotherapy therefore seems obsolete and does not reflect the complexity of the interactions of the different immune actors.

4.1. Antibodies

Using antibodies for tumour targeting has been of strong interest for several decades, and the biggest hurdle has been to find antigens that are specific to the tumour cells. An appropriate target for antibody immunotherapy needs to be expressed both homogeneously and at a high enough level on the surface of the tumour. Once a target that fulfils those criteria has been identified, antibodies can be produced against it, and induce three types of response: blocking a receptor necessary for the survival or growth of the tumour, inducing immune-dependant cytotoxicity via the Fc portion of the antibody, or immunomodulating T cell function (see Figure 12).

4.1.1. Monoclonal antibodies against tumour surface antigen

Several monoclonal antibodies targeting different kind of tumour antigens have shown success in clinical trials. Consequently, they have become commercially available for cancer

treatment[130]. For example, Cetuximab is a monoclonal antibody targeting the epidermal growth factor receptor (EGFR). It was found to be an efficient therapy for colorectal cancer patients, if they did not have a KRAS mutation. For the patients with wild type KRAS, treatment with Cetuximab improved the overall survival median from 4.8 months to 9.5 months[131]. This led in 2012 to the approval of Cetuximab, together with chemotherapy, for first line care of EGFR+ KRAS WT colorectal cancer patients[132]. Trastuzumab, is another FDA-approved monoclonal antibody used for the treatment of HER2+ breast cancer[133]. It showed clinical benefit in 48% of the patients whose tumours where highly positive for HER2. The efficacy of both of these treatments were linked to the inhibition of signalling, as well as antibody-dependant cell-mediated cytotoxicity.

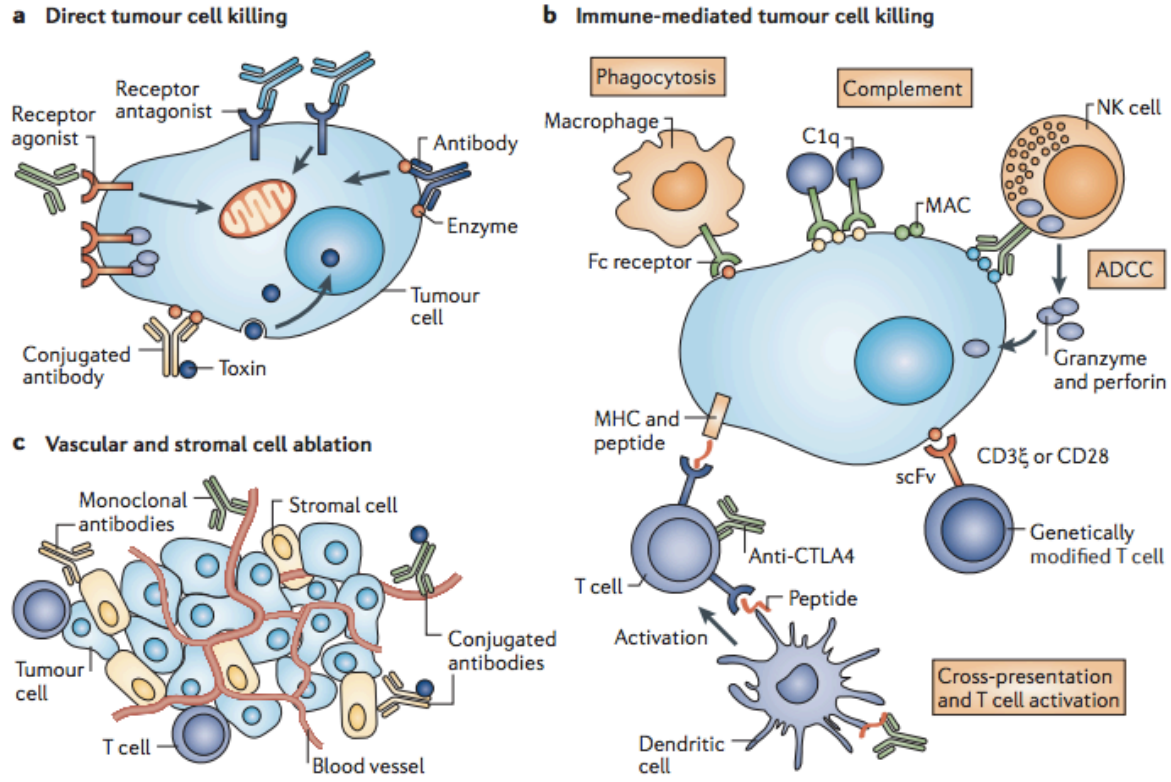

Figure 12: Mechanisms of tumour targeting by antibodies[130]

4.1.2. Bispecific antibodies

Bispecific antibodies have the advantage that they can either simultaneously target 2 antigens and thus increase the specificity of the antibody to the tumour and reduce the non-specific toxicity, or alternatively specifically attract some immune cells to the tumour site. An example from this second category is catumaxomab. This nonhumanized mouse bispecific antibody simultaneously targets EPCAM and CD3, and has a functional Fc domain to activate innate immune cells. It is used for the treatment of malignant ascites together with paracentesis (removal of fluid from the peritoneal cavity). Overall survival, as well as median puncture-free survival was significantly increased for patients treated with Catumaxomab[134]. Another bispecific antibody used in the clinic is blinatumomab. It doesn't have a functional Fc domain but it is composed of 2 single chain antibody fragments (scFv) one targeting CD19, and the other one targeting CD3. Blinatumomab was approved by the

FDA as treatment for refractory B-ALL, Philadelphia chromosome negative. Treatment with blinatumomab increased the complete response in these patients to 30% compared to 5-12% with previous chemotherapeutic regiments[135].

Other bispecific molecules have been investigated, such as ImmTACs, which comprise an antibody linked to a T cell receptor. Such a molecule, IMCgp100, was made linking an αCD3 scFv to a TCR specific for the gp100 peptide:MHC complex. It is currently in phase II trial after encouraging reports from the phase I trial, which results are not yet published[136]. Similarly to TCR-transgenic T cells, such a construct creates the challenge of having HLA-matching between the patients and the drug, as well as a high enough MHC expression on the surface of the tumour.

Additional bispecific antibodies have sought to redirect NK cells, for example by linking the Fv domains of an αCD16A and an αCD30. Such a molecule proved efficient in vitro to mediate lysis of human lymphoma cells[137]. Another investigated method to reactivate iNKT cells against tumours was to take advantage of the CD1d invariant molecule loaded with alpha-galactosylceramide (αGC), which is capable of strongly activating iNKT cells. However, iNKT cells become anergic after one stimulation with this molecule. A new approach was then investigated by fusing the αGC-loaded CD1d with an scFv against the cancer antigens Her2 or CEA. This method proved capable of initiating a potent and specific antitumour effect in a mouse model of adenocarcinoma[138].

4.1.3. Immunomodulatory antibodies

Lastly, immunomodulatory antibodies function by blocking the interaction of important immunosuppressive receptors with their ligands and thus redirecting the immune response towards inflammation. Immunosuppressive receptors are usually upregulated on the surface of activated T cells and provide a negative feedback, hence preventing an uncontrolled immune response. For this reason they have been named **immune checkpoints**. The absence of immune checkpoints can drive fatal immunopathology, for example in CTLA-$4^{-/-}$ mice[139], or in PD-$1L^{-/-}$ mice upon chronic LCMV infection[38].

CTLA-4 has two immunosuppressive functions. On one hand it binds to CD80 and CD86 and prevents their binding to CD28 for T cell costimulation (see Figure 4, page 19). On the other hand it provides immunosuppressive signalling in the T cell. CTLA-4 is constitutively expressed in Tregs, and blocking of CTLA-4 via a monoclonal antibody abrogates their suppressive function[140].

PD-1 is transiently upregulated upon T cell activation[141] and downregulated when the inflammation is resolved. However, PD-1 expression stays high in exhausted T cells upon chronic infection or in tumour infiltrating lymphocytes[142]. Several tumour cell lines were shown to either endogenously express the PD-1 ligand PD-L1, or to upregulate it upon IFN-γ treatment. Subsequent binding of PD-L1 to its receptor on T cells induced their apoptosis[143]. Indeed, tumours are able to hijack mechanisms designed to control the immune response and use them to supress the function of CTLs. Hence, researchers have sought to block the interaction of those immunosuppressive receptors with their ligands via the injection of antagonistic antibodies. This line of treatment is called **immune checkpoint blockade**.

In 1996, Leach *et al.* first demonstrated that αCTLA-4 could block the interaction of CTLA-4 its ligand and hence prevents immunosuppressive signalling as well as restores the availability of CD80/CD86 for CD28 costimulation. Treatment with this antibody induced antitumour immunity and protected the mice against a further tumour challenge[144]. A fully humanized αCTLA-4 called ipilimumab was subsequently produced and used in a phase III trial. The trial demonstrated that treatment with ipilimumab improved both overall survival and progression-free survival in metastatic melanoma patients. Moreover, 18% of the patients treatment with ipilimumab survived beyond 2 years, compared to only 5% of the patients receiving the vaccine alone[145]. Another common target of immunomodulatory antibodies is PD-1. Nivolumab and pembrolizumab both target this receptor and have been found to have an antitumour effect in advanced melanoma. Nivolumab improved both overall survival as well as progression-free survival in metastatic melanoma without a BRAF mutation compared to dacarbazine chemotherapy. Interestingly, in both treatment arms, the expression of PDL-1 on the tumour was correlated with better overall survival[146]. Pembrolizumab is currently used in 16 phase III trials for many types of cancer (breast, melanoma, lymphoma, lung ect…)[147]. In 2015, pembrolizumab was proved to be more efficient than ipilimumab in a phase III clinical trial for the treatment of advanced melanoma, as well as to induce less high-grade adverse events[148]. Additionally, pembrolizumab was shown to be more efficient than chemotherapy in patients that had ipilimumab-refractory melanoma[149]. Indeed, it had been shown that CTLA-4 and PD-1 are not redundant in their functionality, and inhibit T cell function by distinct pathways[150]. Consequently, combination therapies were experimented, and Postow *et. al.* measured an improved response rate in advanced melanoma patients treated with both nivolumab and ipilimumab compared to ipilimumab administered as a monotherapy[151].

The unprecedented efficiency of immune checkpoint blockade prompted FDA-approval of these therapies for a myriad of cancer namely non-small-cell lung cancer[152,153], kidney cancer[154], Hodgkin lymphoma[155] and bladder cancer[156], only in the last year. One question that remains unanswered is which biomarkers can discriminate responders and non-responders.

4.2. Cytokine mediated immunotherapy

It was in the late seventies that the ability of cytokines to mediate lymphocytes survival and proliferation was discovered. This new development allowed researchers to grow cells in culture for *in vitro* studies and greatly improved the understanding of lymphocytes and immune mechanisms. Following this discovery, and with the ability to produce and purify recombinant cytokines came the interest to use them in anti-cancer treatment.

Firstly, IL-2 was injected in patients with metastatic melanoma and renal cell cancers, which induced complete and durable responses in 6.6% and 9.3% of cases, respectively[157]. Systemic administration of IL-2 is a non-specific treatment, aiming at boosting the proliferation of NK and T cells. Hence, adverse toxicities were important at first, with 92% of patients experimenting grade 3/4 diarrhoea, and 81% experimenting grade 3/4

hypotension[158]. However, once appropriate dosing and administration of IL-2 were experimentally determined, adverse events following the treatment could be lowered from 2-4% fatality to less than 1%, and other non-fatal symptoms could be resolved by appropriate additional care[159].

IL-12, on the other hand, was found to have strong adverse effects that could not be controlled. IL-12 has the ability to activate NK cells and help Th1 cell differentiation. However, despite encouraging results using IL-12 therapies in animals (either via systemic injection of purified IL-12 or via local production of IL-12 in the tumour), severe toxicities were found in human trials using IL-12, which led to the immediate halt of those trials. However, more recent studies using local IL-12 expression in the tumour microenvironment, in combination with other therapies, gave encouraging results and are reviving the interest in this cytokine for cancer immunotherapy[160].

4.3. Cancer Vaccines

Vaccines are usually developed for their ability to mount an immune response against a known pathogen and prevent vaccinated individuals from further contracting a disease. Such prophylactic vaccines cannot be applied to most cancers, with the exception of virally induced cancers. For example, the human papilloma virus (HPV) is a known trigger for cervical neoplasia. Vaccines against the human papilloma virus (HPV) have been developed and could successfully prevent HPV infection as well as neoplasia development[161].

For most cancers however, research has focused on designing vaccines, which aim to set off an effective antitumour immune response once the cancer is already established. Such therapeutic vaccines can be formulated in many ways, which will affect the type of the response, whether the inflammation is more local or systemic, or if the cells are activated against only one epitope or several.

4.3.1. Peptide-based cancer vaccines

Peptide and adjuvant-based vaccines aim at igniting an adaptive immune response against a chosen target. However, most antigens expressed by the tumour are of low affinity and are subject to peripheral tolerance since the body recognizes them as “self”. Consequently, the antigen of choice for a therapeutic vaccine has to be carefully considered. Antigens of highest interest for therapeutic vaccines include mutated antigens (neoantigens), cancer testis antigens - which are expressed only in male germ cells but not adult somatic tissues with the exception of tumours - and antigens that are overexpressed in the tumour compared to normal tissue. Finally, tissue-specific antigens can be considered when the tissue happens to be dispensable for the survival of the patient, for example the prostate specific membrane antigen PSMA, or the B cell antigen CD19[162].

It was found that it is best to use long peptides in adjuvant based vaccines, as they are able to mediate both CD4+ as well as CD8+ T cell response, in opposition to short peptides which can only bind to MHC-class I and thus induce CD8+ T cell response only. When not accompanied by the presence of appropriate CD4+ T helper cells, the induced CD8+ T cell response is much less efficient and shorter-lived[163]. But even undertaking this approach, the

clinical benefits of peptide vaccines as monotherapies were limited.

4.3.2. DNA vaccines:

The delivery of tumour antigen via its genetic sequence has also been studied. Bacterial plasmids modified to include the gene of the antigen of interest can be directly injected into the muscles of patients, and some cells, such as APCs, will uptake the genetic material and start expressing the protein. It can then be presented to T cells via the classical MHC class I pathway of antigen processing and presentation. To optimize this approach, packaging vectors for DNA delivery have been engineered. For example bacterial or viral vector can be used. They possess the advantage of infecting the target cells and delivering the gene of interest more effectively. An additional advantage of this approach is the fact that pathogens carry PAMPs, which trigger an inflammatory immune response. In a recent clinical trial for the treatment of prostate cancer, Kantoff and colleagues administered subcutaneously a recombinant vaccinia virus expressing the prostate antigen PSA and three immune costimulatory molecules, namely B7.1, ICAM-1, and LFA3, together with recombinant GM-CSF. The development of autologous immunity, in the form of antibodies to the vaccinia virus, prevented boosting with the same vector. Hence, a recombinant fowlpox virus, expressing the same set of antigen and costimulants, was used in subsequent boosting vaccinations. At three years post-treatment, 30.5% of the vaccinated patients were still alive, compared to 17.5% in the control arm [164]. It should be mentioned that the control arm did not receive recombinant GM-CSF but only the empty vectors alone, which might also have influenced the results.

Of note, oncolytic viral vectors have also been used. Their advantage is that they are both directly harmful to the tumour, and at the same time inducing tumour antigen presentation in an inflammatory context, which improves the quality of the subsequent immune response. A recent clinical trial using a herpes simplex oncolytic virus expressing GM-CSF (T-VEC) to attract antigen-presenting cells to the tumour site showed encouraging results. Specifically, patients treated with T-VEC had an overall survival of 23.3 months compared to 18.9 months in the GM-CSF control arm[165].

4.3.3. Whole tumour cell vaccines

To circumvent the hurdle of the antigen's identification and selection, researchers have attempted using whole cell vaccines. Autologous tumour lysates incorporate the whole spectrum of tumour antigen and neoepitopes without the need to individually identify them. However, the success of this approach in clinical trials was limited, although slightly higher than for single peptide. A review compared the objective clinical response in 173 trials using either whole cell based approach or peptide-based approach as immunotherapies for a wide range of cancers. Whole cell vaccines had an 8.1% objective clinical response, compared to 3.6% for molecularly defined antigens. A similar result was found when the analysis was restricted to advanced metastatic melanoma (12.7% for whole cell versus 6.7% for peptides). Interestingly, according to their analysis the use of allogeneic tumour cell lines had a similar efficiency than autologous tumour lysates[166]. This would be a

good way to avoid the logistic difficulty and the high cost of using the patients own tumour lysates. Moreover, an advantage of tumour cell lines is that they can be modified to express additional immunoattractant, such as GM-CSF, which would increase further the efficiency of the vaccine. Unfortunately, most clinical trials using this technique so far did not show clinical benefits[167].

4.3.4. DCs vaccines

A more controlled way of inducing antigen presentation by professional APCs is to extract them from the patient and pulse them with the peptide or peptides of choice. Indeed, after maturation, DCs are the most potent APCs, capable of triggering robust immune response and to provide all the signals to T cells for their expansion and activation. DCs can be purified from the blood of cancer patients, maturated and pulsed with antigen or whole tumour lysate *in vitro*. Several clinical trial have been kicked off, and showed marginal improvement in terms of tumour responses[168]. A 2010 phase III clinical trial showed that Sipuleutel T, a vaccine comprising activated DCs pulsed with a prostate antigen, improved overall survival of 4 months in castration-resistant prostate cancer patients. Following the publication of these results, the FDA approved Sipuleutel T for clinical use[169].

4.4. Adoptive cell transfer

Adoptive cell transfer consists in the reinfusion of autologous immune cells, after expansion, treatment or genetic modification, into the cancer patient to mediate antitumour immunity. Some preclinical study show that the combination of radiation therapy and transfer of preactivated NK cells could mediate tumour rejection in mice[170]. Additionally, several studies have been published showing CAR-modified NK cells for the immunotherapy of cancer[171,172]. However, the vast majority of the research has focused on expanding or genetically modifying T lymphocytes, which is what we detail in this chapter.

4.4.1. Tumour infiltrating lymphocytes

As it was previously mentioned, tumours are usually infiltrated with tumour-specific CD8+ T lymphocytes (TILs). However, due to the chronic exposure as well as the immunosuppressive environment, these T cells are exhausted and hence functionally impaired. Researchers sought to isolate those TILs and expand them *in vitro*, aiming to artificially increase their total number, and to reactivate them away from the deleterious cancer environment. This allows the T cells to retrieve their functionality by providing them with the appropriate inflammatory signals. After *in vitro* expansion, the lymphocytes can be re-infused into the circulation via an intravenous injection, usually after the patient has been pre-treated with chemotherapy or irradiation to induce lymphodepletion. This technique proved very successful for metastatic melanoma, where 22% of patients receiving TILs after *ex vivo* expansion, in combination with lymphodepletion, reached complete response[173].

Unfortunately, this method can only be applied for the kind of tumours that are reasonably well infiltrated by TILs. Additionally, some tumours are simply not accessible to surgical

resection. Moreover, as previously discussed, tumours have been edited to express antigen of low immunogenicity, which means that the retrieved TILs might not have an optimal affinity to the tumour antigens. Hence, researchers aimed to genetically modify T cells to redirect them against the tumour with high affinity.

4.4.2. TCR-transgenic T cells

It was sought to use lymphocytes from peripheral blood, as they are more easily accessible. These cells can be genetically modified to enforce the expression of a tumour T cell receptor (TCR) specific for a tumour antigen. Such receptors are isolated either from patients who happen to have a high affinity CD8+ T cell clone against a shared antigen, or sometimes from mouse model. Retro- or lentiviruses are often used as a way to insert genetic material into the cells and induce the expression of the new receptor[174]. There are drawbacks in this method. Firstly different human leukocyte antigen (HLA) types exist amongst the population, and each TCR is compatible only with a certain HLA type. Secondly, TCRs are composed of 2 chains, and hence mispairing between one of the newly introduced TCR chains and the complementary endogenous TCR chain can occur and prevent the correct expression of the tumour-specific TCR. Thirdly, like naturally occurring T cells, TCR-engineered T cells depend on the expression of the peptide-MHC complex at the surface of the tumour for their activity.

4.4.3. Chimeric antigen receptors

To solve these issues, chimeric antigen receptors were created. These receptors do not occur naturally and are composed of two parts. The extracellular domain comprises the scFv of an antibody specific for an extracellular tumour antigen and is fused the intracellular CD3ζ signalling domain, as well as one or more costimulatory domains depending on the CAR generation (see Figure 13). It was observed that the addition of the costimulatory domain could efficiently compensate the fact that tumour cells generally do not express the appropriate costimulatory ligands[175]. The expression of a CAR on the surface of a lymphocyte allows for direct recognition of a non-processed antigen on the surface of the tumour, thus avoiding the issue of MHC class I expression. Moreover, proteins as well as carbohydrates and glycolipids can be recognized by CARs, which expands the list of possible targets[176].

The excitement around CARs grew strong when the results of a pilot clinical trial conducted by Carl June were published in 2011. Out of three advanced leukemia patients, 2 had a complete response and the last one a partial response[177]. Since then, more than 20 clinical trials have been launched and published, and showed great promise for the treatment of leukemia and other haematologic malignancies[178]. The adoptively transferred cells were targeting the antigen CD19, which is expressed by leukemic cells but also by normal B cells. A successful anti-CD19 CAR treatment is thus always accompanied by a long term B cell aplasia. Targeting solid tumours via CAR therapy proved more challenging and didn't yet yield such outstanding results as for leukemias[178]. A hurdle in the development of CAR T cells against solid tumours was the selection of the appropriate antigen to avoid on-target

off-tumour toxicity. A publication from 2010 reported that a metastatic colon cancer patient succumbed after treatment with CAR T cells redirected against HER2 using the scFv of the monoclonal antibody trastuzumab. CAR T cells were able to recognize HER2 expressed at low levels in the lung and induced a lethal pulmonary oedema[179]. A way to better manage the sensitivity of CAR T cells to tumour antigens, and avoid the recognition of low antigen expression might be to modulate and lower the affinity of the scFv to its ligand. In their recent publication, Liu *et. al.* showed that reducing the anti-HER2 scFv affinity prevented recognition of tumours expressing physiologic levels of the target without affecting the ability of CAR T cells to lyse tumours overexpressing HER2[180].

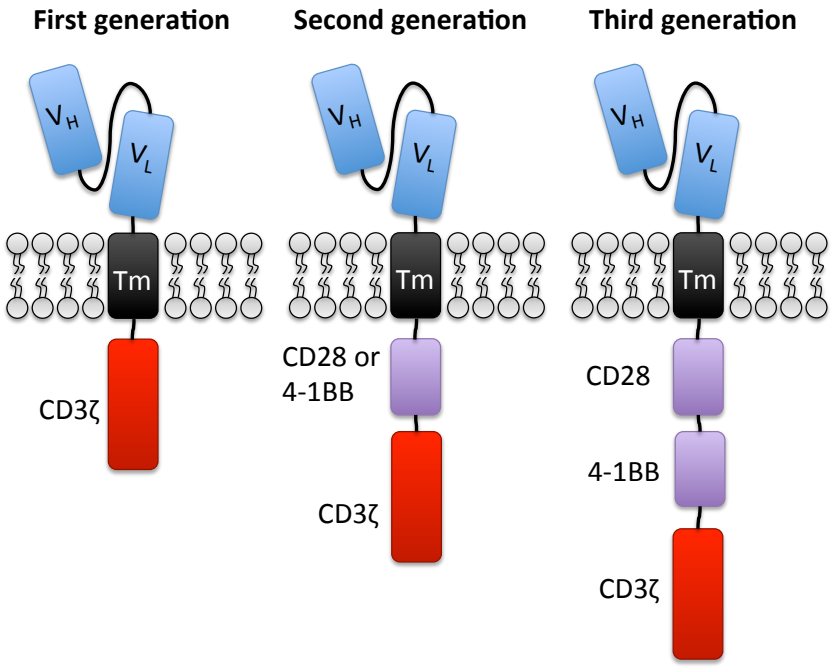

Figure 13: First, second and third CAR generations incorporate respectively none, one or two co-stimulatory domains in their intracellular moiety

4.5. Combination therapy:

All the previously mentioned approaches function via different pathways and effector cells to redirect the immune system against the tumour. To increase further the efficiency of treatments, current approaches are seeking to combine different treatments and hence augment the overall efficiency. For example, the efficiency of a whole cell cancer vaccine in preclinical model of melanoma was improved to 50% rejection using co-inhibition of both aCTLA-4 and aPD-1, compared to 10% and 25% when immune checkpoints were administered individually. In a similar manner, combining a vaccine comprising GM-CSF transduced whole tumour cells with co-blockade of CTLA-4 and PD-1 proved the most efficient for two different mouse tumour models[181]. Similarly, combining immunotherapies and tumour-targeted drugs is an approach that shows great promises, as both methods can synergistically improve the efficiency of each other[182].

Overall, many different cancer immunotherapies exist and have shown promise in clinical trials. Although this field is relatively new, there is a strong interest in further developing existing therapies, and hopefully increasing the life expectancy, as well as quality of life, of cancer patients.

The work presented in this thesis aims to improve the understanding of the role of two microRNAs, miR-17-92 and miR-155 in the functionality of CD8+ T cells. Moreover, we sought to harness miR-155 overexpression to improve adoptive cell transfer of CD8+ T cells for cancer treatment.

General aims:

Immunotherapies have been named "*breakthrough of the year*" by the journal science in 2013, in particular because of the successes of checkpoint blockade inhibitors and adoptive transfer of T lymphocytes. The second method consists in reinfusing a patient's own immune cell, after expansion and sometimes genetic modification. Although this technique is very potent in the case of melanoma and hematologic cancers, it has been less successful for solid tumours.

It is known that CD8+ T cells are important for tumour regression, as their infiltration in solid tumours can generally predict a better outcome for the patient. However, it is not fully understood what are the factors, in addition to quantity, which can drive a more potent CD8+ T cell anti-cancer response. Understanding which genes should be expressed to turn T cells into potent anti-tumour agent, is critical. Indeed, once the genetic profile of the perfect antitumour effector T cell is known, it would be possible with the current technology to force the expression of said genes and consequently to harness the power of T cells and use it in the immunotherapy of cancer.

MicroRNAs are attractive candidates for genetic modification and modulation, as they can rapidly downregulate the expression of several proteins. This allows one microRNA to simultaneously target different pathways. Since it might be necessary to change the expression of several genes in order to turn T cells into strong effectors, modulating the expression of microRNAs has recently attracted a lot of attention.

In our research, we focused on the function of one microRNA cluster and one single microRNA, miR-17-92 and miR-155. Both of them are upregulated upon CD8+ T cells activation, which could indicate an important role for CD8+ T cells effector functions.

Our aim is to study and understand the role of both of these microRNAs in the CD8+ T effector response. Moreover, to demonstrate the potency of modulating the expression of these microRNAs in CD8+ T cells to improve antitumour efficiency. Finally, we will investigate the possibility of combining the expression of a chimeric antigen receptor and the overexpressionf of a microRNA.

Thesis work:

- **Chapter I: The miR-17-92 cluster regulates naïve T cell homeostasis as well as effector and memory differentiation**

- **Chapter II: Overexpression of miR-155 in OT-1 cells enhances their responsiveness to low affinity antigen**

- **Chapter III: Overexpression of miR-155 in anti-PSMA CAR+ CD8+ T cells**

Chapter I: The miR-17-92 cluster regulates naïve T cell homeostasis as well as effector and memory differentiation

1. Aim:

This project was initiated when the information on the role of the miR-17-92 cluster was limited in CD8+ T cells. At the time, miR-17-92 was found to be upregulated in effector CD8+ T cells, and downregulated in memory CD8+ T cells[62], but its role was still undetermined.
The aim of this project was to dissect the roles of the miR-17-92 cluster, in terms of both regulation of the antigen-driven CD8+ T cell response, and CD8+ T cells homeostasis.

2. Results:

MiR-17-92 is important for CD8+ T cell homeostatic survival and expansion

Mice expressing the CD4-CRE transgene were crossed to mice bearing a floxed miR-17-92 allele. The resulting homozygous fl/fl animals expressing the CRE transgene (17-92$^{-/-}$) were used to study the effect of the absence of miR-17-92 in CD8+ T cells. This strained was crossed to the OT-1 strain, to study the antigen-specific effect of the absence of miR-17-92. Animals from both 17-92$^{-/-}$ and OT-1 x 17-92$^{-/-}$ strains were apparently healthy and had a normal life expectancy. However, when the numbers and percentages of peripheral CD8+ T cells were assessed, a significant decrease was measured compared to the WT controls (Figure 14A). To confirm that this decrease was intrinsic to the CD8+ T cells, and that there was no bias of other CD4-expressing cell types, bone marrow chimera mice were generated. CD45.1 wildtype mice were lethally irradiated and their immune system was reconstructed with a 1:1 mix of WT CD45.1/2 and 17-92$^{-/-}$ CD45.2 bone marrow (Figure 14B). The ratio of KO/WT cells was then assessed in blood and lymphoid organs 2 months post transfer. Firstly, to verify that the graft efficiency was similar for cells from the CD45.1/2 or CD45.2 hosts, the ratio of B220+ cells from both congenic markers was measured. Since B cells do not express CD4, miR-17-92 should not be deleted in this subtype and they should behave as wildtype CD45.1/2 B cells. Indeed, the ratio of B220 cells from both hosts was 1, showing that the engraftement efficiency of lymphocyte precursors was similar (Figure 14C). We then measured the ratio of cells expressing CD45.2:CD45.1/2 in the CD4 and CD8 compartment, in which miR-17-92 should be deleted. The amount of CD45.2+ cells in these 2 compartments was strongly decreased in the blood, as well as in the inguinal lymph nodes and the spleen (Figure 14C). This result shows that the reduced homeostatic levels of CD8+ T cells were an intrinsic effect of the absence of miR-17-92. Finally, to assess the survival potential of CD8+ miR-17-92$^{-/-}$ T cells, we collected splenocytes of the bone marrow

chimeras mice, and cultured them in vitro. When cultured with IL-7, these cells did as well as their wildtype counterparts – the ratio stayed stable at 1:5. However, in the absence of IL-7 miR-17-92$^{-/-}$ T cells were not able to survive like the WT T cells and the ratio sank to 1:100 (Figure 14D). This suggests that miR-17-92$^{-/-}$ T cells are impaired in their resistance to cytokine depletion.

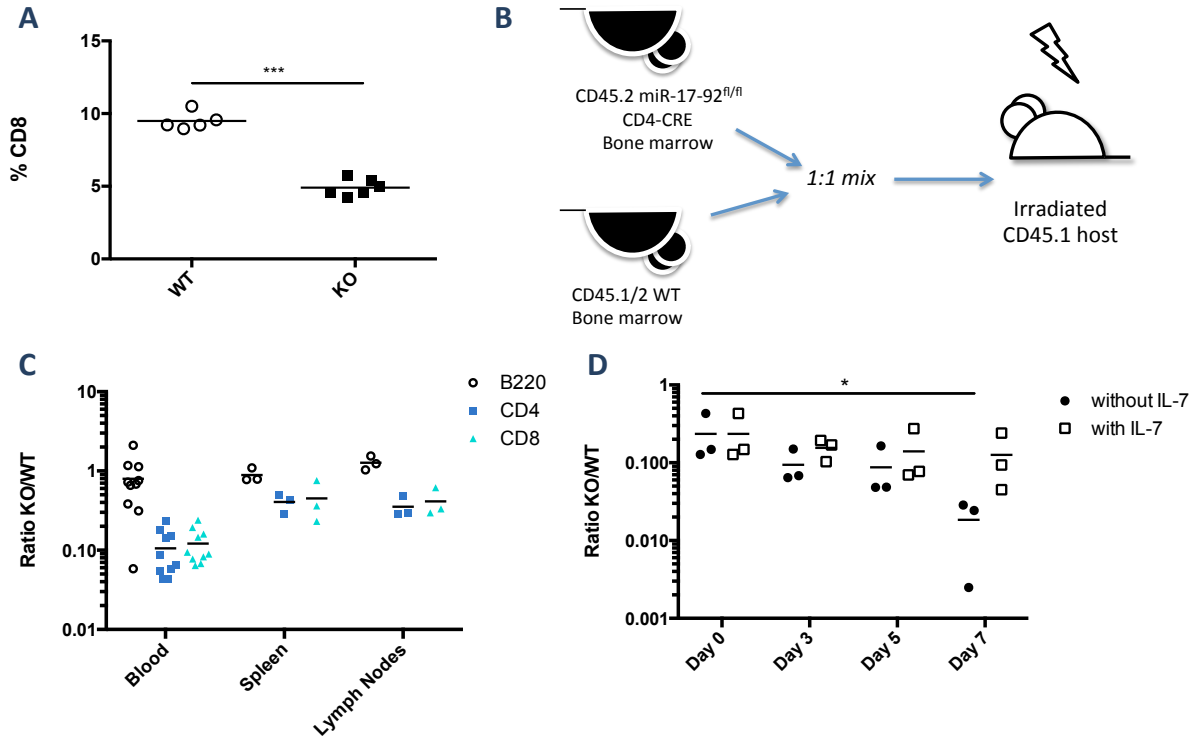

Figure 14: (A) The number and percentages of CD8+ positive T cells was measured by flow cytometry in the blood of miR-17-92$^{-/-}$ (KO) mice compared to age and sex-matched wildtype (WT) mice. (B) Bone marrow chimeras were made, by reconstituting irradiated CD45.1 mice with a 1:1 mix of WT CD45.1/2 and KO CD45.2 bone marrow. (C) 2 months after reconstitution, percentages of B220+, CD4+ and CD8+ cells in the blood, spleen and lymph nodes were assessed via flow cytometry. The ratio of CD45.1/2:CD45.2 percentages were plotted for each organ and cell subtype. (D) Splenocytes from 2-3 months old bone marrow chimera mice were plated with IL-2, supplemented or not with IL-7. The ratio of miR-17-92$^{-/-}$ KO:WT was measured by flow cytometry 3, 5 and 7 days after the start of the culture. Data presented are from one representative experiment out of 2 or 3 independent experiments.

To understand if the difference in the amount of CD8+ T cells in the periphery was due to a defect at the thymus level (development and/or egress), or in the homeostatic persistence, splenocytes were isolated and injected into RAG2$^{-/-}$ recipients (Figure 15A). Since these mice do not have any mature T cells, transferred T cells will undergo homeostatic proliferation to fill the T cell compartment. A mixture of WT and 17-92$^{-/-}$ splenocytes were injected intravenously at a 1:1 ratio. After one week, the ratio had decreased to 1:5 KO:WT CD8+ T cells in the blood of the RAG$^{-/-}$ mice (Figure 15B). This demonstrates that the ability of the 17-92$^{-/-}$ to undergo homeostatic proliferation is decreased. However, although the ratio decreased a bit more in the following weeks, miR-17-92 KO CD8+ T cells were able to survive for long periods of time, as they were still present 52 days after transfer. These surviving cells had a more naive phenotype (CD62L^{high} CD44^{low}) compared to WT CD8+ T cells that were more effector like (CD62L^{low} CD44^{high}) (Figure 15C).

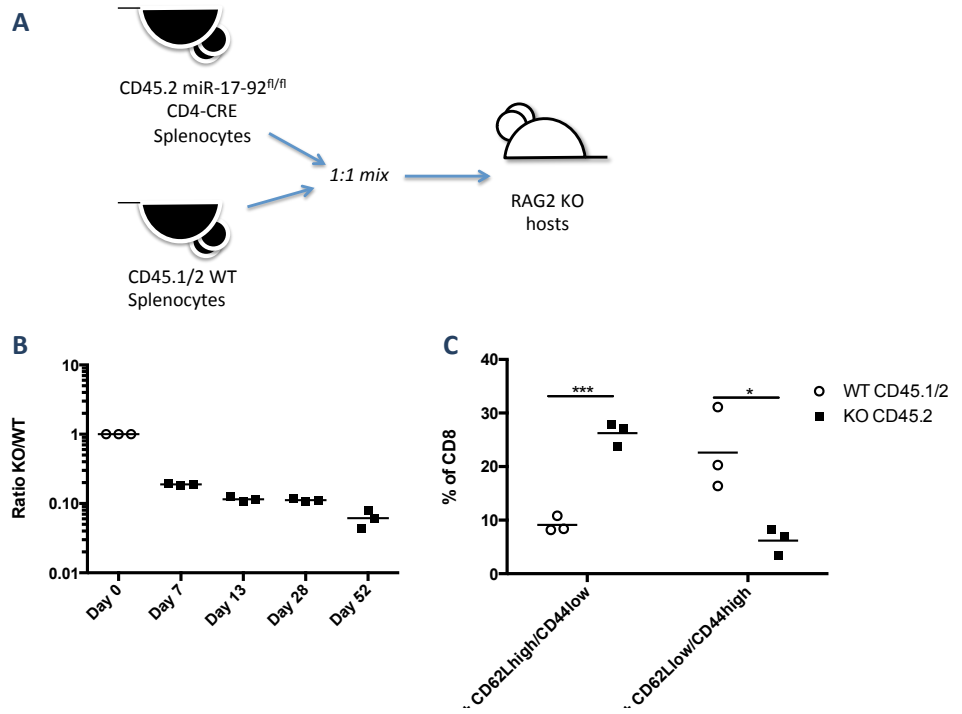

Figure 15: (A) Splenocytes from the miR-17-92$^{-/-}$ mice or WT controls were mixed at a 1:1 ratio and transferred intravenously into RAG2 KO hosts. (B) The ratio of KO:WT cells in the CD8 compartement was assessed via flow cytometry 7, 13, 28 and 52 days after the transfer in the blood of the RAG2 KO mice. (C) 4 weeks after the transfer, the phenotype of the CD8+ T cells WT or miR-17-92$^{-/-}$ was measured by flow cytometry via the surface exrpression of CD62L and CD44 on the surface of the cells. Data presented are from one representative experiment out of 2 independent experiments.

Absence of miR-17-92 impairs CD8+ T cells proliferation upon stimulation

To confirm the reduced ability of miR-17-92$^{-/-}$ CD8+ T cells to get activated and proliferate, we CFSE-labelled T cells and cultured them with 5µg/ml plate-bound CD3 and 2µg/ml soluble CD28. After 48 hours, the WT CD8+ T cells had undergone 2 to 3 rounds of proliferation, while the miR-17-92$^{-/-}$ CD8+ T cells had barely started replicating (Figure 16A). Moreover, the accumulation of miR-17-92$^{-/-}$ CD8+ T cells upon viral infection was impaired as well. MiR-17-92$^{-/-}$ and WT mice were infected with 200 plaque forming unit (PFU) LCMV and the percentage of blood CD8+ T cells was measured before the infection, as well as 6 and 8 days after (Figure 16B). Both the percentage and absolute numbers of miR-17-92$^{-/-}$ CD8+ T cells were diminished at the peak of the infection (day 8, Figure 16C). Although miR-17-92$^{-/-}$ CD8+ T cells were not able to expand to the same levels as WT, they did manage to control the viral load, as shown by similar numbers of PFU/spleen between KO and WT mice at day 8 of infection (Figure 16D).

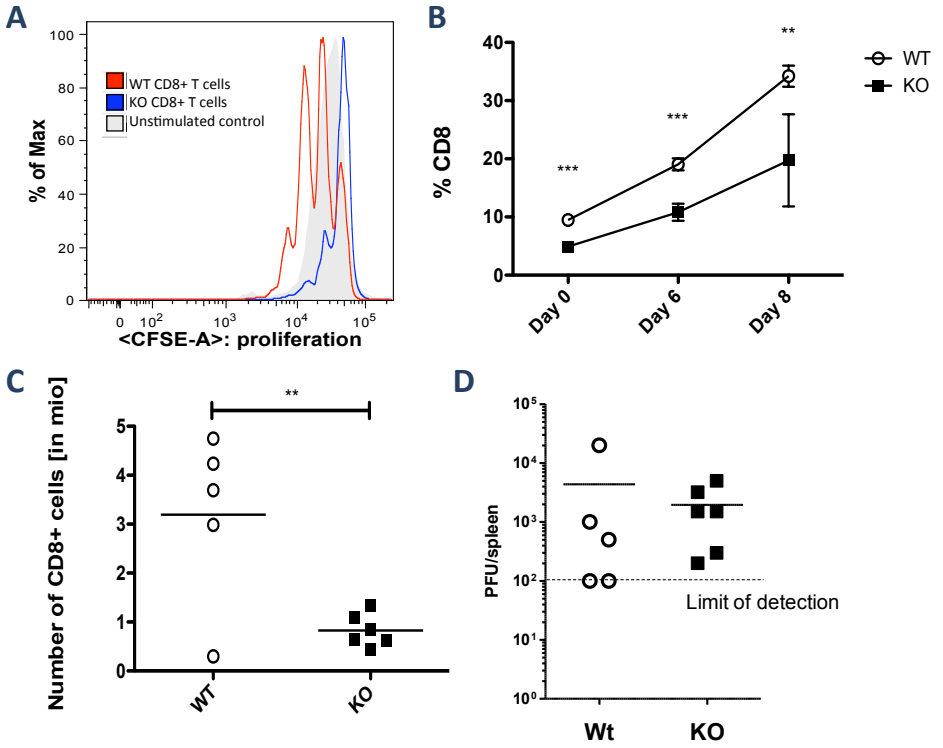

Figure 16: (A) CFSE-labelled CD8+ T cells were stimulated with αCD3/αCD28 *in vitro* and CFSE dilution was measured by FACS 2 days after stimulation. (B) Percentages of CD8+ T cells were assessed in the blood of WT and miR-17-92$^{-/-}$ mice before and 6 and 8 days after LCMV infection. (C) Numbers of CD8+ T cells at day 8 of LCMV infection were measured in the blood of WT and miR-17-92$^{-/-}$ mice. (D) Plaque forming assay allowed to determine the viral load per spleen of infected mice 7 days after infection. Data presented are from one representative experiment out of 2 or 3 independent experiments.

Absence of miR-17-92 impairs effector functions of CD8+ T cells

Three months after LCMV infection, the mice were sacrificed, and splenocytes were isolated to assess the functionality of memory CD8+ T cells. Splenocytes were restimulated with either plastic-coated αCD3 and soluble αCD28 or a mix of LCMV-specific peptides. After 5 hours of co-incubation, cytokines were stained for intracellular cytokines and analysed by flow cytometry. The percentages of IFN-γ-producing cells amongst the total CD8+ T cells population was decreased in miR-17-92$^{-/-}$ mice compared to WT, in both antibody- or

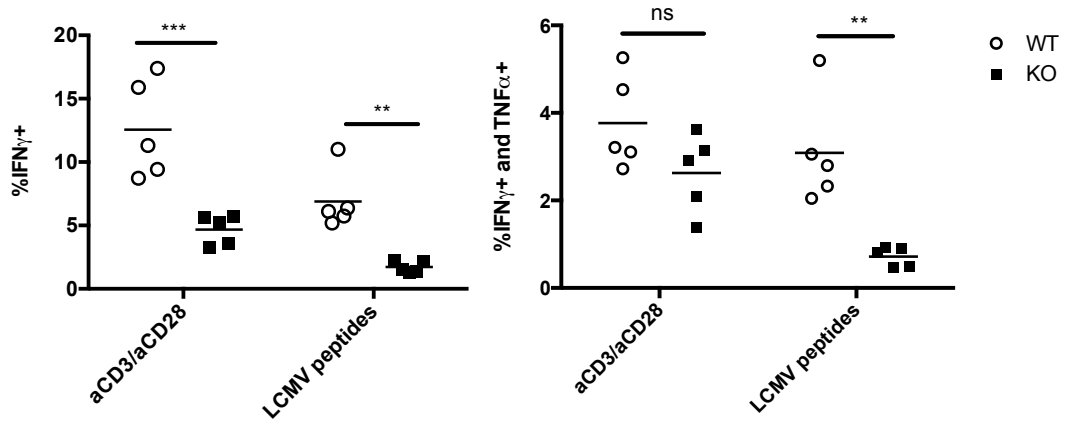

Figure 17: After 5 hours of stimulation with either αCD3 and αCD28 antibody or LCMV-specific peptides, IFN-γ and TNFα were stained intracellularly in either WT or miR-17-92$^{-/-}$ (KO) CD8+ T cells. The percentages of IFN-γ+ (left panel) and IFN-γ and TNFα double positive cells (right panel) were measured by flow cytometry. Data presented are from one representative experiment out of 2 independent experiments.

peptide-mediated stimulation (Figure 17 left panel). Moreover, the percentages of polyfunctional T cells (producing both IFN-γ and TNFα), was also decreased (Figure 17 right panel). This demonstrated impaired effector functions in CD8+ T cells in the absence of miR-17-92.

Absence of miR-17-92 intrinsically prevents CD8+ T cells accumulation upon listeria infection and promotes a central memory phenotype

Since the promoter driving CRE expression and miR-17-92 deletion is CD4, all cells that express CD4 will become knock-out for the miR-17-92 cluster. This includes T cells but also some DCs, B cells and granulocytes. To confirm that the decreased cell expansion was CD8+ T cell intrinsic, and to compare WT and KO cells in a competitive environment, we co-transferred OT-1 miR-17-92$^{-/-}$ and WT CD8+ T cells into congenically different WT hosts. OT-1 CD8+ T cells are transgenic for the Vα2 and Vβ5 TCR chains which makes them specific for the SIINFEKL, a peptide derived from the ovalbumin protein (OVA). After the intravenous transfer of 50'000 WT and KO OT-1 CD8+ T cells at a 1:1 ratio, mice were infected with a 1'000CFU dose of a strain of *L. monocytogenes* expressing the OVA protein (*Lm*-OVA). The transferred cells could be discriminated thanks to the CD45.1 and CD45.2 congenic marker. The ratio between KO and WT cells was measured 7, 13 and 28 days after listeria infection. Although the initial ratio was 1:1, it subsequently dropped to 1:10 miR-17-92$^{-/-}$:WT CD8+ T cells, showing that the ability of miR-17-92$^{-/-}$ CD8+ T cells to accumulate in response to an infection was indeed reduced compared to WT OT-1 cells (Figure 18 A). However, the ratio

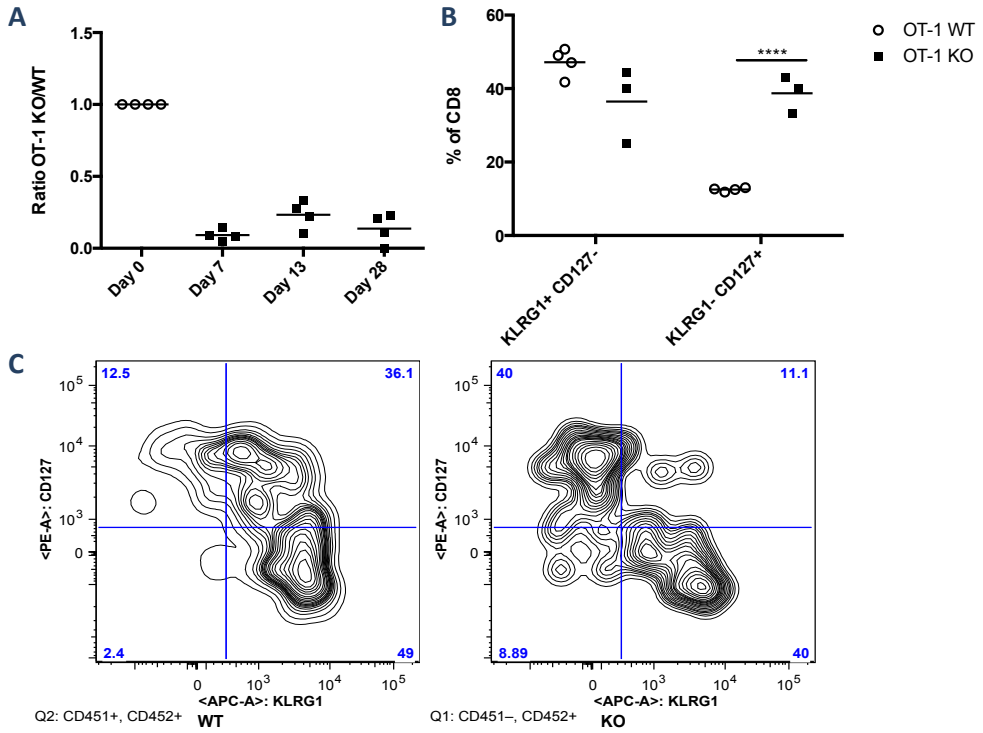

Figure 18: (A) WT or miR-17-92$^{-/-}$ (KO) cells were cotransferred into WT animals at a 1:1 ratio. Mice were subsequently infected with OVA-expressing *L.monocytogenes* (listeria-OVA). The ratio of transferred WT was measured 7, 13 and 28 days after infection. (B) The phenotype of the transferred OT-1 cells was evaluated in terms of CD127 and KLRG1 expression 4 weeks after listeria infection. (C) Representative FACS plot of CD127 and KLRG1 of OT-1 cells (left panel) WT or (right panel) miR-17-92 KO 4 weeks after listeria infection. Data presented are from one representative experiment out of 2 independent experiments.

was stably maintained until 28 days after, which indicated that KO cells are capable of forming long-lived memory cells. As a confirmation, the phenotype of miR-17-92$^{-/-}$ CD8+ T cells was skewed towards a more central memory phenotype as compared to WT cells four weeks after infection (Figure 18 B and C).

3. Discussion:

In this first chapter, we set out to understand the importance of the miR-17-92 cluster for CD8+ T cells homeostasis and response to infection.

A publication from Salaun *et. al.* firstly identified the microRNAs from the miR-17-92 cluster as some of the highest expressed in CD8+ T cells[62]. Moreover, the dynamic regulation of this microRNA cluster was found to be upregulated at the effector function, and downregulated during the memory phase[95,96]. Interestingly, different microRNAs in the cluster did not have exactly the same levels of expression at the mature microRNA level, showing intra-cluster differences in the processing efficiency, and/or half-life[96].

To understand the importance of this cluster for the functionality of CD8+ T cells, we used a CD4-CRE x miR17-92 LoxP mouse model, to delete miR-17-92 specifically. We found that the absence of miR-17-92 hindered the homeostatic persistence and proliferation of CD8+ T cells. Moreover, upon activation, their ability to proliferate as well as to produce cytokines was impaired. Finally, miR-17-92$^{-/-}$ CD8+ T cells had a more central memory phenotype than their wild type counterparts and could persist for long periods of time.

We firstly observed that there was a decrease in the percentage and numbers of CD8+ T cells in the periphery of miR-17-92$^{-/-}$ mice, compared to WT B6 mice. We also showed that this difference was intrinsic by creating bone marrow chimera mice. Indeed, although the miR-17-92 was CD4-specific, several subsets of cells express CD4 and would become miR-17-92 knock-out. This is true for CD4+ and CD8+ T cells, which become KO at the double positive stage but also other cell types such as dendritic cells and monocytes. The bone marrow chimera allowed showing that in a wild type environment, the amount of miR-17-92$^{-/-}$ CD4+ and CD8+ cells was decreased at homeostasis.

Another group using the same model of CD4-CRE, miR-17-92 LoxP to study the deletion of miR-17-92 in CD4 T cells did not report such a decrease, whether in CD4 or in CD8. However, they did not explicitly show equal numbers at homeostasis[183]. Moreover, in another study, mice reconstituted with miR-17-92$^{-/-}$ bone marrow were shown to have decreased numbers of B cells in the periphery, but no difference in the numbers of lymphocytes[85]. These results contrast with our findings. To confirm that the effect we saw was indeed due to the deletion of the miR-17-92 cluster and not to our model, it would be best to repeat the experiments using different controls. Firstly, amounts of single positive CD8+ T cells in the periphery, in terms of numbers and percentages, can vary from one strain to another. Hence, it would be preferable to use CRE^{-} littermate as WT controls instead of WT mice from a different breeding. However, this was not possible as the strain we used was CRE homozygous. Secondly, a study published in 2012 demonstrated that using the CD4 promoter for CRE

expression induces satisfying levels of gene deletion and marginal toxicity when hemizigously expressed. However, possessing 2 copies of the CD4-CRE transgene reduced the amount of thymic cellularity by 20%[184]. Unfortunately, there is a possibility that the expression of the CRE transgene would have affected the outcome of our experiments, which is why a confirmation using a different model, such as CD4-CRE hemizigous and CRE+ LoxP- controls would be desirable.

We observed that miR-17-92$^{-/-}$ were impaired in their ability to proliferate in the absence of stimulation, in RAG2 KO mice. This might be due to an impairment of the cells to differentiate into effector/effector-memory cells. Indeed, it was shown that CD4 T cells undergoing homeostatic proliferation upregulate the CD44 marker and adopt an effector-memory-like phenotype[185]. In our model, we observed that miR-17-92$^{-/-}$ CD8+ T cells did not upregulate CD44 and kept a high expression of CD62L, which is a naïve phenotype. Moreover, it was shown that overexpression of miR-17-92 skews CD8+ T cells towards terminal effectors[95]. Hence, upregulation of the miR-17-92 cluster during the effector phase might be necessary for the differentiation of CD8+ T cells.

We saw that miR-17-92$^{-/-}$ CD8+ T cells were impaired in their ability to proliferate *in vitro* and *in vivo* upon infection with LCMV or *Lm*-OVA. This reflects the findings of two other groups which used the granzymeB-CRE miR-17-92 LoxP model and observed a decrease in the accumulation of miR-17-92$^{-/-}$ CD8+ T cells upon LCMV infection[95,96]. In contrast, they showed that overexpression of miR-17-92 in CD8+ T cells increased the proliferation and accumulation at the peak of the infection and induced terminal differentiation, hence impairing the formation of memory cells[95]. In CD4+ T cells, deletion of the miR-17-92 cluster increased the sensitivity of the cells to activation-induced cell death[183]. In our *in vivo* two models of infection, it would be interesting to better discriminate between impaired proliferation or reduced survival by performing a BrdU incorporation experiment and by assessing apoptosis regulators such as BCL-2 and Bim.

Although the accumulation of miR-17-92$^{-/-}$ CD8+ T cells was decreased, some cells were able to persist for long periods of time as we could detect CD8+ T cells 4 weeks after listeria infection and 3 months after LCMV infection. These cells were mostly of the memory phenotype CD127^{high} KLRG1^{low}. However, they did not respond efficiently to restimulation. Indeed, there was a 2-fold decrease in the percentage of miR-17-92$^{-/-}$ CD8+ T cells expressing IFN-γ. This corresponds to previous finding, as Wu *et. al.* also observed a decrease in cytokine production upon miR-17-92 deletion in CD8+ T cells[95].

One possible way in which the absence of miR-17-92 might increase the differentiation into memory cells, is by its targeting of the protein PTEN. Indeed, PTEN mRNA contains sites complementary to the seed sequence of 5 of the cluster's microRNAs[95]. PTEN is a suppressor of the mTOR pathway, and it was shown that interfering with mTOR signalling via Rapamycin treatment induced a stronger CD8+ T cell memory differentiation[186]. In the absence of miR-17-92, the mTOR pathway might be decreased. To confirm this, we could quantify the amount of phosphorylated S6, a ribosomal protein phosphorylated upon mTOR signalling.

Overall, we show that miR-17-92 is a crucial microRNA cluster for the differentiation of CD8+ T cells into effector cells, and their clonal expansion. The absence of this microRNA induced CD8+ T cells to adopt a more memory phenotype, but decreased their functionality. Although overexpression of this microRNA was proven to drive CD8+ T cells towards a more terminal effector phenotype, and prevented their long term survival[95], the combination of miR-17-92 overexpression and CAR transduction in a human xenograft mouse model gave promising antitumour results[97]. Hence miR-17-92 seems to be a promising target for immunotherapy.

Overexpression of miR-17-92 has to this date not been implemented in an autologous tumour graft model. It would be interesting to test the antitumour efficiency of tumour-specific mouse CD8+ T cells overexpressing miR-17-92. Moreover, since miR-17-92 modulates the memory phenotype of CD8+ T cells, it might be interesting to try to downregulate its expression with an antagomiRs, at different timepoints after activation. It has been shown that different microRNAs in the cluster are differentially regulated and have sometimes opposing functions[90]. Hence, it might be interesting to try to target or overexpress individual microRNAs, to fine-tune the cluster's function.

While researching this subject, we were simultaneously working on a project on miR-155, which gave promising results. The miR-17-92 was subsequently dropped which explains why some important experiments have to this day not been performed.

4. Methods:

4.1. Mouse strains

MiR-17-92$^{fl/fl}$ mice from the C57BL/6J strain were purchased from Jackson Laboratory, and crossed to a CD4-CRE C57BL/6J strain, to make a mouse model miR-17-92$^{fl/fl}$ and homozygous for the CD4-CRE allele. It will be referred to as 17-92$^{-/-}$ in this report. Other models were used such as OT-1 x 17-92$^{fl/fl}$ x CD4-Cre, where the mice express an OVA-specific transgenic TCR on the surface of the CD8+ T cells. This second model will be referred to as OT-1 x 17-92$^{-/-}$. RAG2 KO mice and the congenic CD45.1 C57BL/6J mice were supplied by our local animal facility. The latter were crossed with CD45.2 C57BL/6J mice to generate CD45.1/2 mice.

4.2. Genotyping:

DNA was extracted from ear punches using the Kapa Mouse genotyping kit and following the instruction of the manufacturer (Kapabiosystems KK7352). Primers sequences and PCR programs are described in the Appendix page 88.

4.3. Infection models:

LCMV: Mice were infected intraveneously with 200PFU of LCMV of the WE strain, provided by Dietmar Zehn's laboratory at the CHUV. The immune response was assessed at the peak of infection 7-8 days after infection, usually by bleeding.

Viral titers were determined at day 7 by an LCMV focus-forming assay from splenocyte suspension (*Experiment performed by D. Utzschneider)*

Listeria Monocytogenes: Mice were infected intravenously with 1'000-2'000 CFU of *L. monocytogenes*. The immune response was assessed at day 6 or day 7 in the blood or spleen of the mice.

4.4. Bone marrow chimeras

CD45.1 C57BL/6J mice were lethally irradiated with a dose of 900rad in a Caesium-137 irradiator. Bone marrow cells from miR-17-92$^{fl/fl}$ CD45.2 or WT CD45.1/2 donor mice were isolated from femur and tibia bones with PBS flushing. Cells were subsequently washed, counted, and mixed at 1:1 ratio. A few hours after irradiation, hosts' immune system was reconstituted by intravenous injection of a total of 4 million mixed bone marrow cells. Analyses of the homeostatic reconstitution of the host's immune system were performed 2 to 3 months later.

4.5. RAG2 KO homeostatic proliferation assays

Spleens of CD45.1/2 C57BL/6J mice and CD45.2 miR-17-92$^{fl/fl}$ CD4-CRE mice were sampled and processed in a sterile manner. The percentage of CD8+ T cells in each sample was measured and 1mio of CD8+ T cells from each strain was injected i.v. in RAG2 KO mice.

4.6. Flow cytometry

Surface staining: Blood samples were directly stained in FACS buffer at 4°C for 30 minutes, before lysis of red blood cells and fixation with BD buffer (BD 349202) during 10 minutes at room temperature. Blood samples were subsequently washed twice and resuspended in FACS buffer (PBS 2%FCS 2mM EDTA).

Spleens were processed through nylon filters (100µM) in FACS buffer. Cells were pelleted in 50ml falcon tubes by centrifugation at 400xrcf for 5 minutes. Cell pellet was resuspended in 5ml RBC lysis solution (Qiagen 158904) for 10 minutes at room temperature, then washed twice. 1 to 4 mio cells were then stained in FACS buffer, washed, and then acquired on a LSR-II FACS. Lymph nodes and thymus were processed and stained in a similar way.

Intracellular cytokine staining: To assess the cytokine production of lymphocytes, 1-4 million splenocytes were plated in a 96-well plate with either 10um of a LCMV peptide mix (see supplemental methods page 89), or 10ng/ml final concentration PMA and 500ng/ml ionomycin, or 5µg/ml plate-bound CD3 and 2µg/ml soluble CD28 and placed in the incubator at 37°C. After 30 minutes, Golgistop (monensin, BD 554724) was added to the wells at a 1/1'500 dilution. The cells were placed back in the incubator for an additional 4 hours before surface staining and intracellular staining. Fixation and permeabilization for the intracellular detection of cytokines was performed with the BD kit (554714) according to the manufacturer's instruction.

4.7. Statistical analysis

When two groups were compared, the t test with a two-sided P value and a 95% confidence interval was performed. When more than two groups over time were compared, a two-way ANOVA test was performed. Figures show one representative experiment out of 2 or 3 independent experiments. The individual dots always represent biological replicates (number of mice or independent wells). When technical replicates were performed (usually in duplicates), the average of the replicates was plotted. All statistical tests were performed using the Prism version 6 software (NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.)

Chapter II: Overexpression of miR-155 in OT-1 cells enhances their responsiveness to low affinity antigen

1. Aim:

In 2013, our group published an article in the journal immunity about the role of miR-155 in CD8+ T cells. In this publication, we demonstrated that the deletion of miR-155 was intrinsically detrimental to the correct proliferation and survival of CD8+ T cells *in vivo* upon infection. Moreover, we could show that OT-1 miR-155$^{-/-}$ cells could not mediate potent antitumour response in the B16 model, upon vaccination with CpG-OVA. Using the pmel model, our collaborators demonstrated that the overexpression of miR-155 induced an ameliorated antitumour response upon vaccinia virus – hgp100 infection and systemic injection of the cytokines IL-2[81]. The aim of this project was firstly to characterize the overexpression of miR-155 in the OT-1 model and its ability to ameliorate the OT-1 cells antitumour response upon vaccination with CpG-OVA. The second aim was to take advantage of the existing pre-defined high and low affinity OVA ligands, SIINFEKL (N4) and SIITFEKL (T4) respectively, to study the implication of miR-155 overexpression for the modulation of the T cell response according the TCR ligand's affinity.

2. Results

OT-1 cells were efficiently transduced and could stably overexpress miR-155 *in vitro*

OT-1 T cells were purified from the spleens of OT-1 CD45.1 mice and transduced with either the GFP_SCR (SCR) or the GFP_miR-155 (miR-155) construct. These retroviral expression plasmids, can co-express the GFP protein and a microRNA under the same promoter called PGK. Two days after transduction, the percentage of transduced cells was assessed via flow cytometry by measurement of GFP+ cells. It was on average 90% (Figure 19A). After transduction (day 4-day 10), miR-155 levels were measured by qPCR. The amount of miR-155 expressed in resting OT-1 cells transduced with miR-155 was increased 30-50 fold compared to the levels in SCR transduced cells (Figure 19B). To assess the influence of the endogenous upregulation of miR-155 upon activation, total levels of miR-155 were measured in OT-1 SCR or miR-155 cells after activation with either a 1:1 ratio of αCD3/αCD28-coated magnetic beads or the B16-OVA cell line at a 1:5 target:effector ratio. The levels of miR-155 were indeed increased upon activation in both the OT-1 SCR and the OT-1 miR-155, which is not surprising since the endogenous miR-155 is upregulated upon

activation. However, 1 day after activation, the level of miR-155 in the miR-155 transduced cells was only 2-fold higher compared to the SCR-transduced cells. This indicates that the transgene expression does not increase with activation, and that when the endogenous levels of miR-155 are high, the overexpression of miR-155 only minimally affects the total amount of microRNA. This is true for both non-specific activation (Figure 19C) and activation with a cell line expressing the high affinity target OVA (Figure 19D).

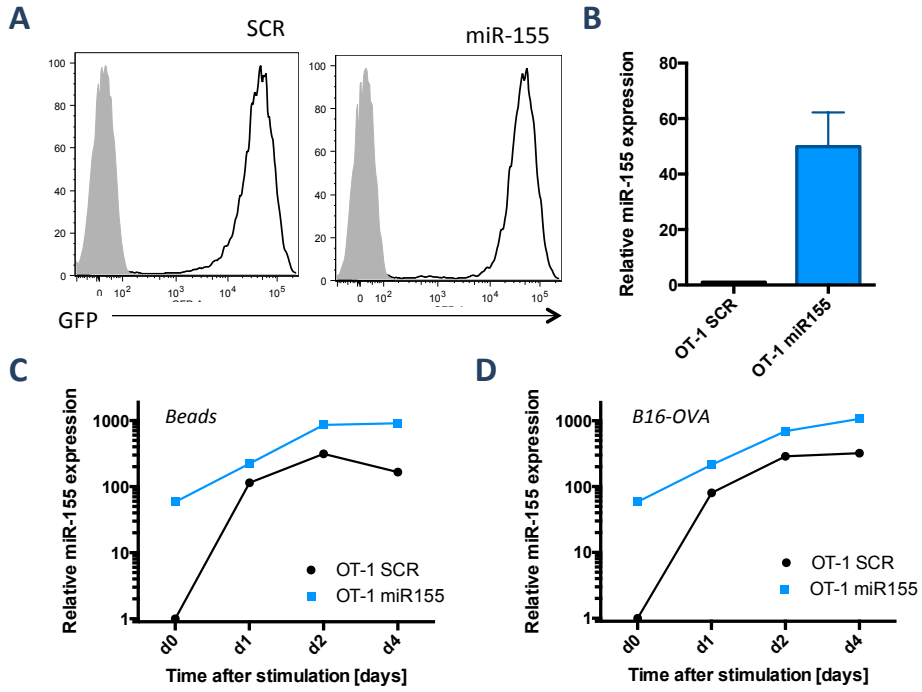

Figure 19: (A) 1 representative FACS plot showing GFP expression in OT-1 cells 2 days after transduction (B) qPCR measurement of miR-155 levels in SCR or miR-155 resting cells. (N=3) (C) qPCR of miR-155 levels before and 1, 2 and 4 days following coculture with αCD3/αCD28 coated beads or (D) B16-OVA. Experiment representative of 2 independent experiments.

MiR-155 overexpression increases the proliferation and accumulation of OT-1 cells *in vivo* in the blood and spleen of infected and vaccinated mice

In vivo, miR-155 overexpression allowed OT-1 cells to respond more efficiently to an infection with *Listeria monocytogenes* expressing the ovalbumin protein (*Lm-OVA*). Indeed, OT-1 cells percentages, measured as the percentage of CD45.1+ GFP+ double positive cells in the CD8+ gate, were increased at the peak of the infection in the spleen (Figure 20A). The overexpression of miR-155 had an impact only during the late phase of the expansion, as there was no difference either in percentage or numbers of OT-1 miR-155 4 days after infection. However, 6 days after *Lm-OVA* infection, both numbers and percentage of OT-1 miR-155 were increased compared to OT-1 SCR. This was due to an increased proliferative ability. Indeed, at day 6 post infection, 13% of OT-1 miR-155 cells had proliferated 2.5 hours after BrdU injection, whereas only 4% of OT-1 SCR had proliferated in the same time (Figure 20B). OT-1 miR-155 cells could respond more efficiently to vaccination with CpG and OVA. Indeed, both the percentages (Figure 20C) and numbers (Figure 20D) of OT-1 miR-155 cells were increased in the blood of vaccinated animals compared to OT-1 SCR. This

demonstrates the miR-155-mediated increased ability to accumulate is independent of a systemic infection (Figure 20 C and D).

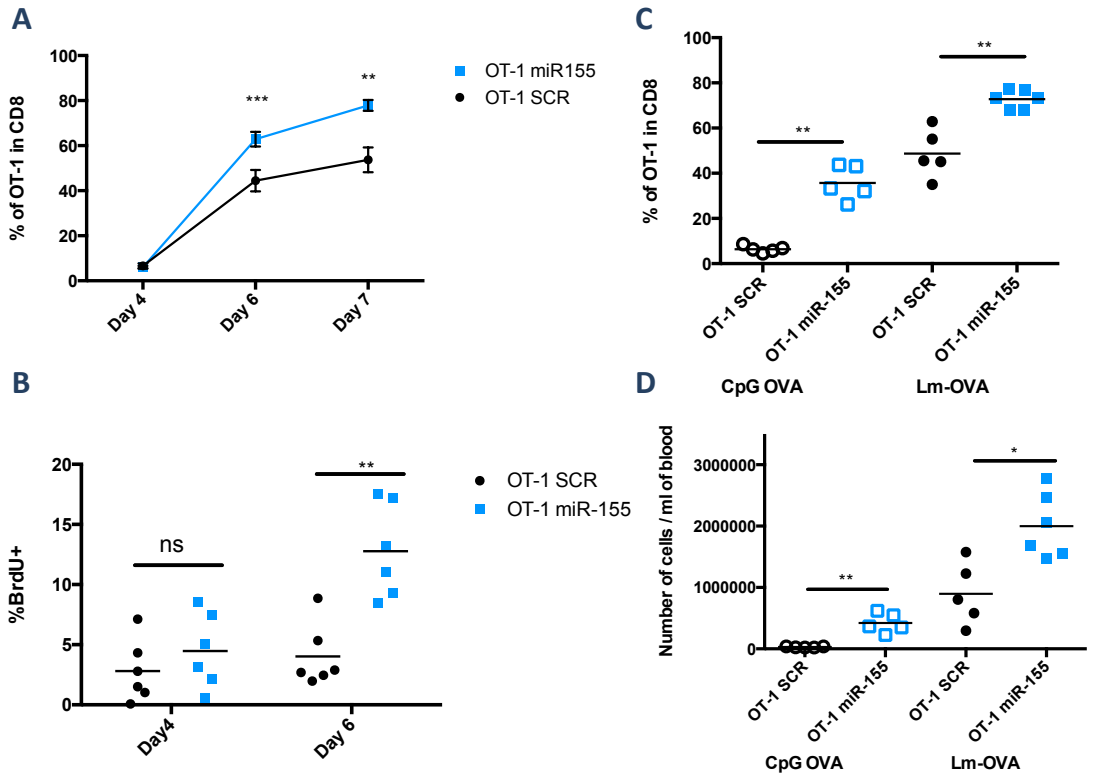

Figure 20: (A) Percentages of GFP+ OT-1 cells amongst total CD8 were measured in the spleen of Lm-OVA infected mice 4, 6 and 7 days after infection. (N=6) (B) The proliferation of OT-1 SCR and OT-1 miR-155 was assessed by intracellular staining of BrdU positive cells 2.5 hours after peritoneal BrdU injection, 4 and 6 days after Lm-OVA infection. (C) Percentages of OT-1 cells amongst CD8+ T cells were measured in the blood of mice 7 days after vaccination with CpG-OVA or infection with Lm-OVA. (D) Absolute numbers of OT-1 SCR and OT-1 miR-155 were calculated in the blood of mice 7 days after listeria infection using trucount FACS tube. Experiment representative of two independent experiments.

MiR-155 overexpression in OT-1 cells marginally improves their ability to control tumour growth

WT CD45.2 mice were engrafted subcutaneously in the flank with 0.1 million (mio) B16-OVA cells in suspension in PBS. Three days later, T cells were purified from the spleen of a CD45.1 OT-1 mouse and activated in vitro with αCD3/αCD28 beads. The next day, the T cells were transduced either with the pMGP-SCR or pMGP-miR155 plasmid. Six days after the tumour graft, mice received an intravenous injection of 0.1 mio OT-1 SCR or OT-1 miR-155. The next day, mice either received an intravenous injection of 2'000 cfu of Lm-OVA, or a subcutaneous injection, at the base of the tail, of CpG and OVA. The timecourse of the experiment is summarised in Figure 21A. Although, as shown previously, the proliferation and accumulation of cells was increased at the peak of the response (Figure 20), the tumour protection was only mildly improved when mice were transferred with OT-1 miR-155 and infected with Lm-OVA (Figure 21B). Furthermore, there was no improvement upon cell transfer and vaccination (Figure 21C). Indeed, although the accumulation of OT-1 miR-155 in

the periphery was increased compared to OT-1 SCR, the percentage of OT-1 miR-155 in the TILs was similar as the one from OT-1 SCR (data not shown).

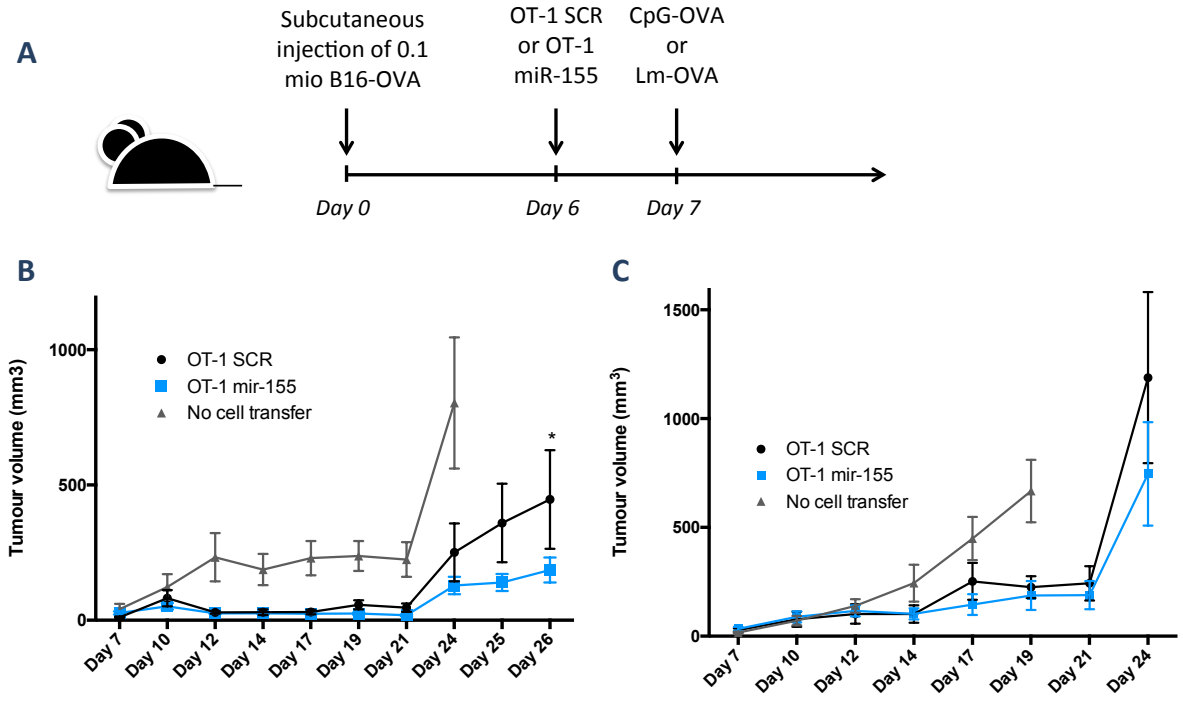

Figure 21: (A) B6 mice were engrafted subcutaneously at day 0 with 1x10^5 B16-OVA (n=5-6). At day 6, 1x10^5 OT-1 T cells were transferred intravenously. At day 7, mice were either infected with Lm-OVA or vaccinated with CpG OVA. Tumour volumes were measured with a caliper every other day from day 7-post engraftement until the end of the experiment for (B) mice infected with Lm-OVA or (C) mice vaccinated with CpG OVA. The graphs are one experiment representative of two independent experiments.

Upon cotransfer, OT-1 miR-155 cells preferentially accumulate in the tumour when compared to OT-1 SCR

As variation within animals or differences in tumour sizes could affect the ability of the CD8+ T cells to infiltrate tumours, independently of their phenotype, we sought to compare the OT-1 SCR and OT-1 miR-155 cells directly in the same host. In this situation, the OVA-specific CD8+ T cells will have to compete with one another for space and survival signals, making the conditions more stringent. In this purpose, we cotransferred OT-1 CD45.1/2 SCR and OT-1 CD45.1 miR-155 cells intravenously in CD45.2 tumour-bearing mice. The mice were subsequently either treated with PBS, or vaccinated with CpG + OVA, or infected with *Lm-OVA*(Figure 22). 7 days after the vaccination or infection, the mice were sacrificed and the percentages of OT-1 SCR and OT-1 miR-155 were measured in the blood and tumour via FACS staining. In this setting, the ability of OT-1 miR-155 CD8+ T cells to accumulate in the blood (Figure 22 left panel) or in the tumour (Figure 22 right panel) 7 days after either vaccination or infection significantly surpassed that of the OT-1 SCR cells. This contrasts with the previous observation that the tumour invasion was similar in a non-competitive setting, showing that the OT-1 miR-155 cells performed better under the competitive pressure.

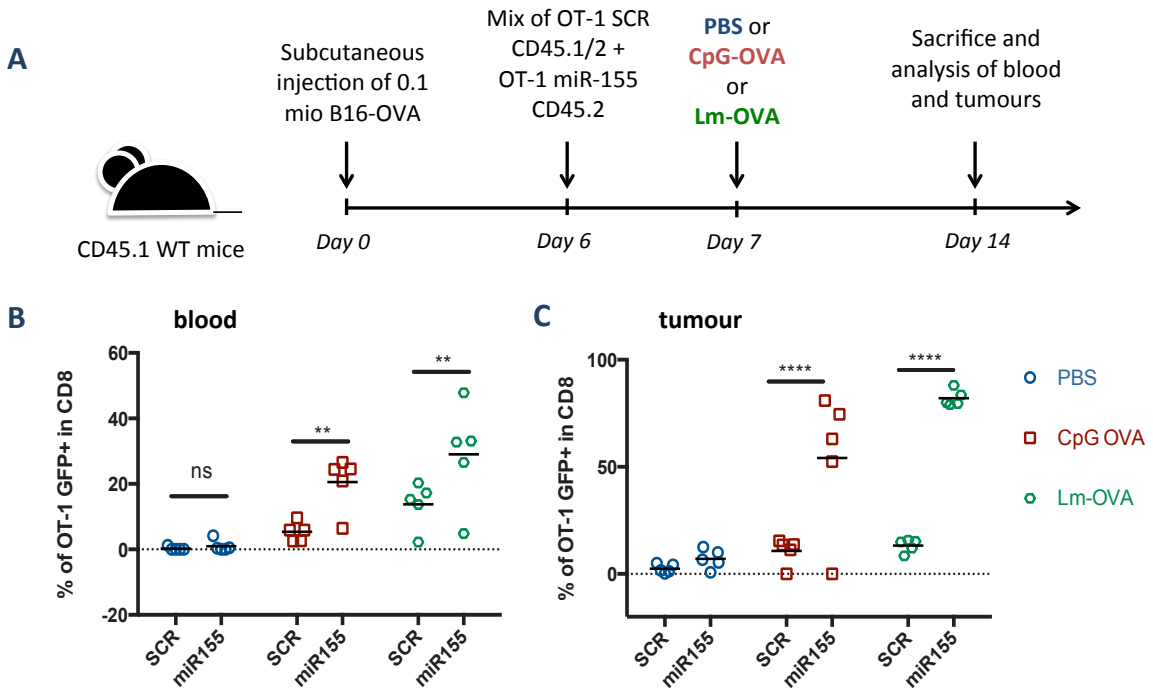

Figure 22: (A) CD45.2 OT-1 cells were transduced with miR-155 and CD45.1/2 OT-1 cells were transduced with SCR and cotransferred in CD45.1 tumour bearing mice. (B) 7 days after vaccination or infection, mice were sacrificed and GFP+ OT-1 cells were quantified in the blood. (C) Tumour infiltrating lymphocytes were purified and stained and GFP+ OT-1 cells were quantified. Graphs representative of one experiment.

We then assessed the effector functions of the OT-1 cells by restimulating them with the high affinity SIINFEKL (OVA N4) peptide for 4 hours and subsequently staining intracellularly for IFN-γ and TNF-α. At the peak of the infection, both OT-1 miR-155 and SCR extracted from the tumour could respond to restimulation and express IFN-γ efficiently (Figure 23A). Moreover, OT-1 miR-155 cells were more polyfunctional than the controls, as a higher percentage could also express TNF-α (Figure 23B). Finally, we stained for the surface markers PD-1, CD62L and CD44 and could not see striking differences in the tumour, whereas in the blood miR-155 had an increased expression of CD44 and decreased expression of CD62L (data not shown). The expression of CD8α (Figure 23C) and CD8β (Figure 23D) were both systematically increased in the tumour-infiltrating OT-1 miR-155 cells, both in the vaccination and in the infection setting. This was also the case for circulating cells (data not shown).

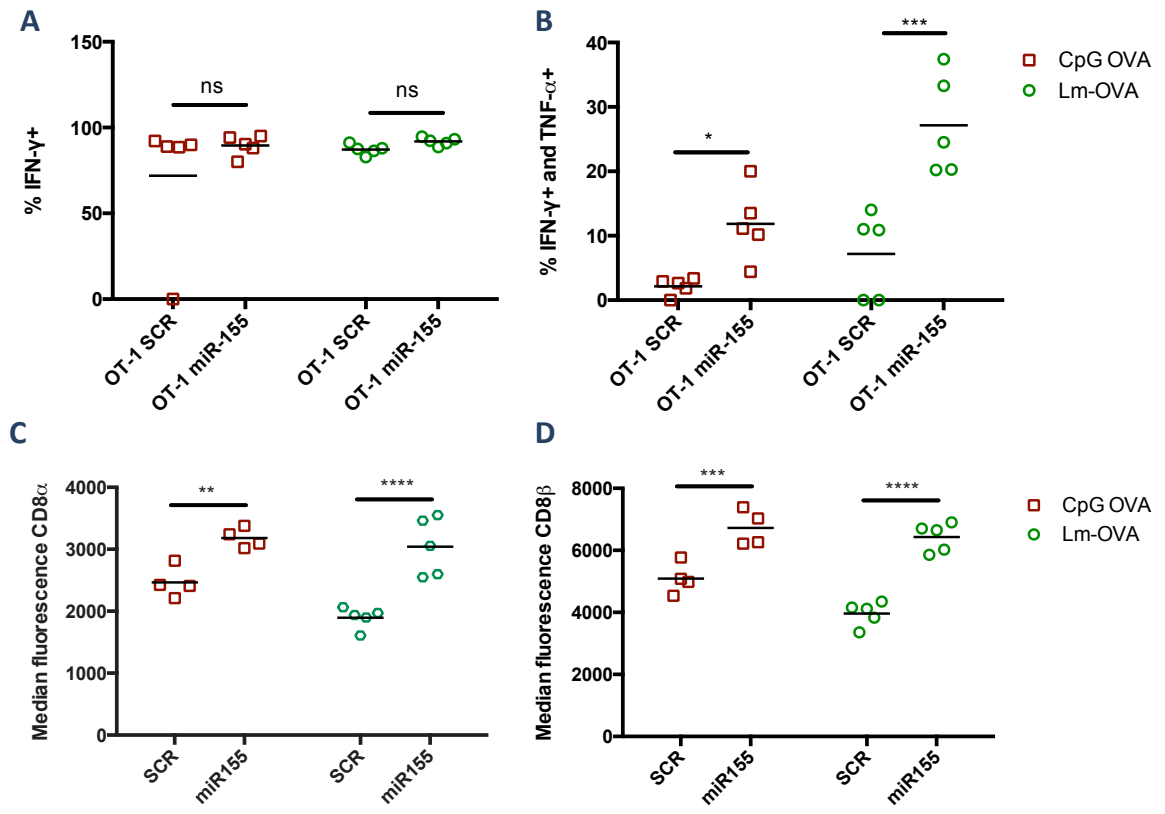

Figure 23: (A)-(B) OT-1 SCR and OT-1 miR-155 were extracted from the tumours 7 days after vaccination or infection and restimulated with the N4 peptide before intracellular cytokine staining. (C) Expression of CD8α on the surface of OT-1 cells was quantified by the FACS staining and measure of the median fluorescence via flow cytometry. (D) Expression of CD8β on the surface of OT-1 cells was quantified by the FACS staining and measure of the median fluorescence via flow cytometry. Graphs representative of one experiment.

Cells overexpressing miR-155 have an increased glycolytic capacity and non-glycolytic acidification rate

We then sought to characterize the metabolic capacities of the OT-1 miR-155 cells and see if they differed from the OT-1 SCR. To do so we performed seahorse assays to evaluate the OT-1 cells capacity to perform glycolytic respiration from a glucose supplemented medium, and to upregulate their glycolytic respiration upon stimulation with αCD3/αCD28 stimulation with beads (Figure 24A). We also tested CD8+ T cells from a miR-155 KO breeding, either homozygous for the miR-155 deletion (miR-$155^{-/-}$) or possessing both wildtype alleles (miR-$155^{+/+}$). To measure the ability of the CD8+ T cells to perform non-glycolytic acidification, and to respond to stimulation in the absence of glucose, we performed a glycolysis stress assay. The cells were plated in medium depleted of glucose, but containing glutamine. They were subsequently activated with αCD3/αCD28-coated beads. Afterwards, glucose, oligomycin and 2DG were added to the wells and the extracellular acifification rate (ECAR) was measured at least 3 times (Figure 24B). Upon activation, all cell subtypes could increase their glycolytic metabolism. This increase was more marked in the presence of glucose in the medium (Figure 24A). The addition of oligomycin blocks oxidative phosphorylation and reveals the glycolytic capacity. It was increased in OT-1 miR-155 cells, especially in the glycolysis stress assay (Figure 24B). The

basal glycolysis, before activation, was augmented in OT-1 miR-155 cells in the presence of glucose (Figure 24C) and also in its absence (Figure 24D), showing that the OT-1 miR-155 cells were more efficient at non-glycolytic acidification. To assess the activity of the mTOR pathway in those cells, we measured the amount of phosphorylated ribosomal protein S6, which is downstream of the mTORC1. OT-1 SCR and OT-1 miR-155 cells were fixed and intracellularly stained 1 hour and 15 minutes after coculture with αCD3/αCD28. The amount of phosphorylated S6 was increased in OT-1 miR-155 upon activation (Figure 24E).

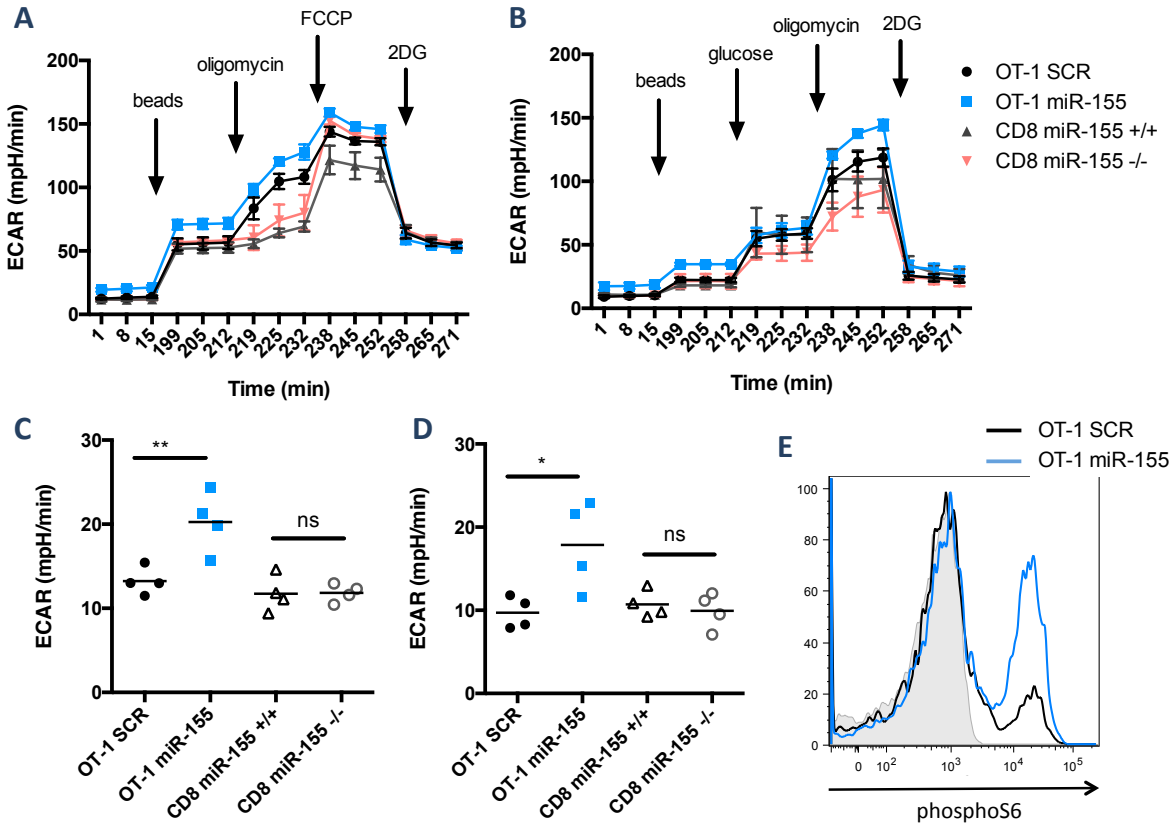

Figure 24: (A) Extracellular acidification rate (ECAR) was measured every 7 minutes in a 96-well plate using a seahorse machine. αCD3/αCD28 beads, oligomycin, FCCP and 2DG were sequentially added to the wells and subsequent ECAR was measured at least 3 times. The starting medium contained 10mM glucose and 2mM glutamine (B) ECAR was measured using a seahorse machine before and after the addition of αCD3/αCD28 beads, glucose, oligomycin and FCCP. (C) The average ECAR of the three measured basal values was plotted for each well for the glucose-supplemented medium (D) The average ECAR of the three measured basal value was plotted for each well for the medium without glucose. (E) The phosphorylation of the ribosomal protein S6 was assessed by phosphostaining and measured by FACS 1h15 after OT-1 SCR and OT-1 miR155 activation with αCD3/αCD28 beads. Graphs of one experiment representative of three independent experiments.

Overexpression of miR-155 in OT-1 cells improves their ability to mediate protection against tumours expressing a low affinity antigen

We were surprised to observe that the OT-1 miR-155 cells did not significantly improve the tumour protection against the B16-OVA antigen upon priming through a systemic infection with Lm-OVA, or through vaccination with CpG OVA. Indeed, previously published results with the demonstrated a dramatic improvement of the CD8+ T cells-mediated antitumour response upon miR-155 overexpression[81]. However, that study was not using OT-1 cells but pmel cells. The pmel model takes advantage of self-tolerant mgp100-specific CD8+ T cells,

which do not protect against the mouse gp100 (mgp100)-expressing B16 tumours, unless stimulated by an infection with a vaccinia virus expressing the human gp100[187]. Once the tolerance is broken thanks to the infection and the higher affinity hgp100 antigen, pmel CD8+ T cells can recognize and destroy tumour cells expressing the mgp100. Our high affinity OT-1 model was thus different compared to the original study. Moreover, the upregulation of the endogenous miR-155 has been shown to be correlated with the strength of the affinity of the TCR to the peptide:MHC complex[81]. In our hands, we could confirm via the transfection of OT-3 cells, which have a low affinity to OVA, that the endogenous miR-155 was less upregulated upon activation with the peptide. Hence, the difference after activation between the OT-3 cells overexpressing miR-155 or the ones transduced with the SCR was greater compared to OT-1 SCR and miR-155 activated with B16-OVA (Supplementary Figure 34). To understand if the affinity to the target antigen was a determining factor in the improved CD8+ T cell function upon miR-155 overexpression, we took advantage of the SIINFEKL (N4) and SIITFEKL (T4) peptides. The SIINFEKL is the endogenous ovalbumin peptide, and is of high affinity to the OT-1 TCR. The SIITFEKL is an altered peptide ligand that has a much decreased affinity to the OT-1 TCR, at the limit between negative selection and self-tolerance[18]. We subcutaneously engrafted C57BL/6J mice with $1x10^5$ B16 tumour cells expressing the endogenous ovalbumin (B16-N4) on the right flank, and $1x10^5$ B16 tumour cells expressing the altered low affinity ovalbumin (B16-T4) on the left flank. As usual, OT-1 cells were either transduced with SCR or miR-155 and were injected intravenously 6 days post graft. Then, vaccination was performed with CpG and N4 the following day (Figure 25A). As previously observed, the overexpression of miR-155 did not improve the protection against B16-N4 tumours (Figure 25B). However, 21 days after engraftement, the B16-T4 treated with OT-1 miR-155 CD8+ T cells were significantly smaller than the ones treated with the OT-1 SCR cells (Figure 25C). As a conclusion, in this model, the overexpression of miR-155 could improvethe antitumour efficiency of OT-1 cells only in the context of tumours expressing a low affinity antigen.

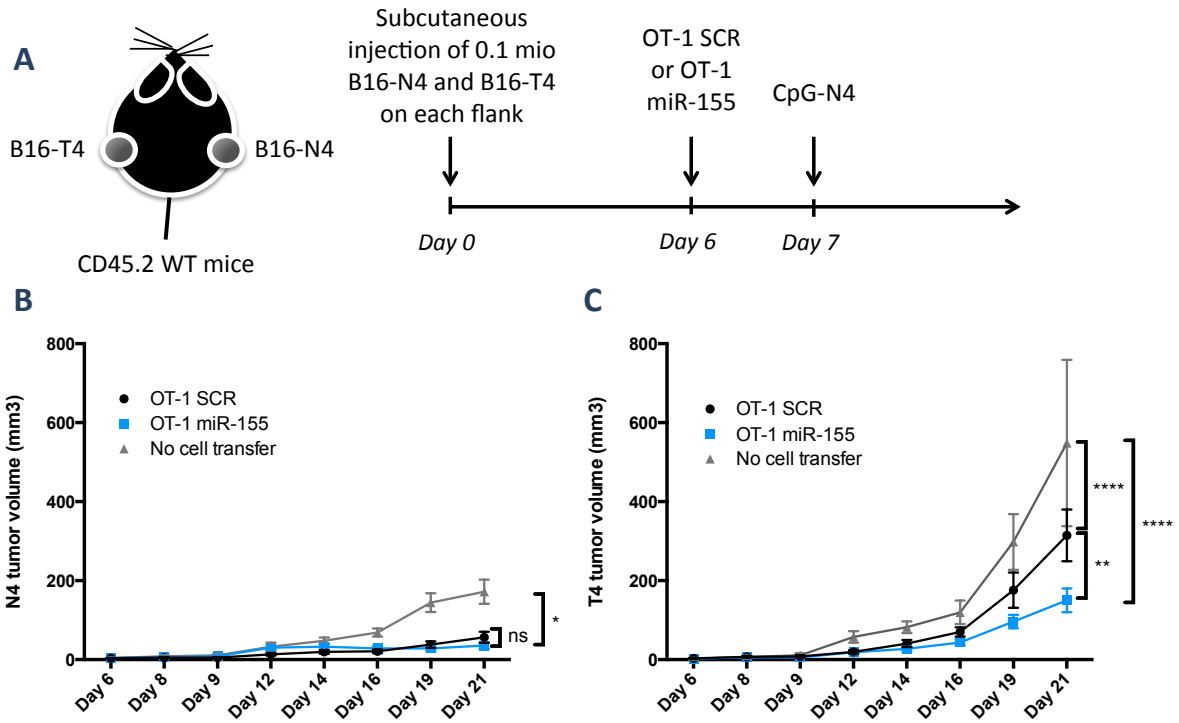

Figure 25: (A) B6 mice were engrafted subcutaneously on the left flank with B16-T4 and on the right flank with B16-N4. 6 days after the graft, the mice were injected intravenously with 1x10^5 OT-1 SCR or OT-1 miR-155 cells, or PBS. The following day, they were vaccinated with CpG and the high affinity OVA peptide, SIINFEKL (N4). (B) B16-N4 tumour growth in the different treatment groups was measured every 2 days with a manual caliper. (C) B16-T4 tumour growth was also measured every 2 days. 2-way ANOVA and tukey's multiple comparison test was used to compute the statistical significance at day 21 post graft for the different treatment groups (N=6). Graphs of one experiment representative of 2 independent experiments.

We also measured the percentage of OT-1 miR-155 in T4 tumour at the end of the experiment (22 days after engraftement), and we could observe that it was higher compared to OT-1 SCR. In the spleen and N4 tumours of the same mice, however, there was no difference between the amount of OT-1 SCR and OT-1 miR-155 cells (Figure 26A). We repeated the experiment but sacrificed the animals 7 days after vaccination, at the peak of the immune response to assess the OT-1 cells distribution and functionality in the different organs. As expected, the OT-1 miR-155 cells were strongly enriched in the spleen of the vaccinated animals, compared to the OT-1 SCR, showing an increase of the circulating OT-1 miR155 cells (Figure 26B). Moreover, this increase was also reflected in the tumour draining lymph nodes (dLNs), on both flanks of the mice, although the T4 dLNs (Figure 26E) seemed to be attracting less cells that the N4 dLNs (Figure 26C).

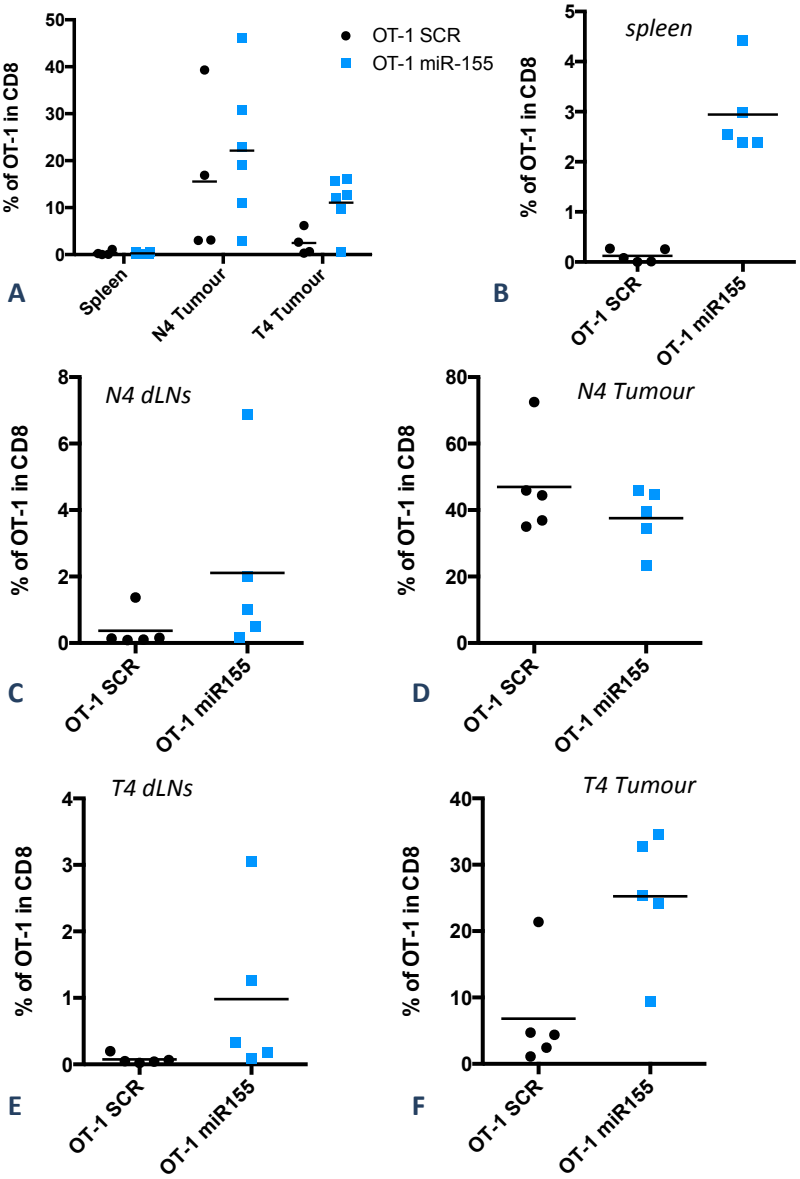

Figure 26: (A) percentages of OT-1 cells amongst CD8+ gate 22 days after engraftment. The differences in population are non significant. Percentages of OT-1 in the CD8 gate 7 days after CpG and N4 vaccination in the (B) spleen, (C) N4 tumour axillary and inguinal draining lymph nodes, (D) N4 tumour, (E) T4 tumour axillary and inguinal draining lymph nodes, (F) T4 tumour. Graphs of one experiment representative of 2 independent experiments.

Most interestingly, although the N4 dLNs were enriched in OT-1 miR-155 cells compared to the OT-1 SCR controls, the N4 tumours contained similar numbers of OT-1 miR-155 compared to OT-1 SCR (Figure 26D). In the B16-T4 tumours however, the amount of OT-1 miR-155 was increased compared to the controls, showing a local effect, specific of the low-affinity antigen, in the ability of miR-155 overexpressing T cells to either survive or expand better (Figure 26D).

miR-155 overexpression increases the tumour infiltration and functionality of OT-1 cells upon low affinity vaccination

Finally, we looked at the ability of the overexpression of miR-155 to increase the response to a low affinity priming, by vaccinating the mice bearing N4 and T4 tumours with either CpG and the T4 peptide or CpG and the N4 peptide. As a result, the tumour invasion of OT-1 miR-155 T cells was increased in both the N4 and T4 tumours. Showing that upon low affinity antigen priming, OT-1 overexpressing miR-155 are advantaged compared to OT-1 SCR for the invasion of both high and low affinity tumours 7 days after vaccination (Figure 27A). Moreover, the functionality of the OT-1 miR-155 cells was augmented in the N4 tumours, at the peak of the response following both N4 and T4 vaccination. The percentage of cells producing IFN-γ was higher in the OT-1 cells transduced with miR-155 (Figure 27B), and there was a tendency for more polyfunctionality as well as the percentage of IFN-γ and TNF-α double positive cells was augmented (Figure 27C). This demonstrates the potential of miR-155 to rescue low affinity T cells, not only in their effector functions but also during the priming of the response. These findings confirm miR-155 as an interesting candidate for immunotherapy, as circulating T cells are usually of low affinity to tumour associated antigens because of central tolerance. Overexpressing miR-155 could potentially rescue those low affinity T cells and make them more efficient in controlling tumour growth.

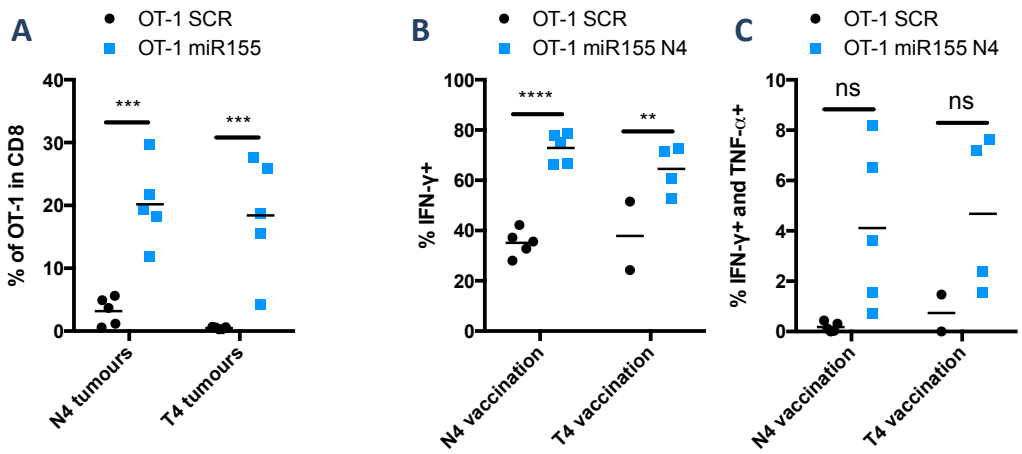

Figure 27: (A) Percentages of OT-1 cells in the CD8 gate 7 days after CpG + T4 vaccination measured in N4 and T4 tumours. Percentages of OT-1 cells in the N4 tumours producing (B) IFN-γ and (C) both IFN-γ and TNF-α, assessed by intracellular cytokine staining. Graphs representative of 1 experiment.

3. Discussion:

We have shown that the overexpression of miR-155 in OT-1 T cells improves their ability to respond to antigen stimulation upon vaccination and infection, both in terms of proliferation and cytokine production. Moreover, the ability of OT-1 miR-155 cells to infiltrate tumours was ameliorated in the context of either competition via the co-injection of OT-1 SCR cells, or upon low affinity vaccination with the SIITFEKL (T4) peptide and CpG. Finally, OT-1 miR-155 T cells were capable to infiltrate B16 tumours expressing the T4 ligand more efficiently, and could consequently mediate a better tumour control in this model. In contrast, when OT-1 cells were optimally activated by a high affinity peptide, overexpressing miR-155 was unhelpful in terms of antitumour effect against the B16 tumours expressing the high affinity SIINFEKL (N4) ligand.

We and others have shown that the upregulation of miR-155 is proportional to the affinity of the TCR to the peptide:MHC ligand, as OT-1 T cells express miR-155 at higher levels when stimulated with the N4 ligand *in vitro* compared to the T4 ligand[81]. Additionally, stimulation with A2/NY-ESO-$1_{157–165}$ multimers induced a higher miR-155 upregulation in human CD8+ T cells transduced with TCRs of stronger affinity to the NY-ESO peptide[188]. Moreover, miR-155 is upregulated dynamically *in vivo* during the course of an infection. For example in effector cells sorted from LCMV infected mice, miR-155 expression peaked at day 6 post infection, and was subsequently downregulated[81]. In our OT-1 model, we observed a reduction in the difference in miR-155 levels between miR-155 and SCR OT-1 cells when the endogenous miR-155 was upregulated after *in vitro* stimulation. The 50-fold difference in miR-155 levels in resting cells became a 2-fold difference after one day of stimulation with αCD3/αCD28-coated beads or B16-OVA. Hence, adding more miR-155 might be less useful to the cells during the peak of endogenous miR-155 expression. We did not yet measure the expression of miR-155 in SCR and miR-155 transduced OT-1 T cells *in vivo* upon listeria infection. However, we did observe that transduction with miR-155 induced an advantage in the proliferation and accumulation of OT-1 cells at the peak of the *in vivo* response to vaccination and infection, but not in the early time point, 4 days after infection. This might be because the endogenous miR-155 is downregulated in the SCR cells 6 and 7 days after infection, but not in the miR-155 transduced cells, which continuously overexpress miR-155. To confirm this hypothesis, it would be interesting to extract RNA from SCR or miR-155 OT-1 cells sorted from the spleen of infected mice, and measure the dynamic regulation of miR-155 upon listeria infection *in vivo.*

MiR-155 expression has been previously shown to have an effect on the metabolism of tumour cells. For example, high expression of either miR-155 or mTORC1 is associated the aggressive ERα negative tumour phenotype in breast cancer patients[189]. Moreover, it was shown that miR-155 could modulate mTOR signaling in breast cancer cells. Indeed, overexpression of miR-155 in MCF-7, an ER^+ tumour cell line with low basal miR-155 levels, downregulated the Rictor protein levels and hence both increased mTORC1 signaling and blocked the mTORC2[189]. The involvement of miR-155 in the modulation of mTOR signalling in CD8+ T cells is not yet published. Here, we demonstrated that miR-155 overexpression

could enhance mTOR signalling upon stimulation. Indeed, phosophorylation of the ribosomal protein S6, which is a downstream marker of mTOR activity, was increased upon OT-1 miR-155 cells activation. It would be interesting to confirm if in our model the increased mTOR pathway was also due to a decrease in the rictor protein expression. We know that mTOR signalling is associated with a more effector phenotype, as blocking of mTORC1 via rapamycin increased the amount of memory T cells in mice upon vaccinia virus infection[186]. In line with this observation, the OT-1 miR-155 T cells had lower expression of the marker CD62L compared to the OT-1 SCR in the periphery at the peak of the infection (data not shown).

The metabolic switch from oxidative phosphorylation towards glycolysis upon activation of the CD8+ T cells has been shown to be crucial for their functionality[34]. A recent publication indicated that miR-155 might be important in the regulation of cell metabolism. Researchers showed that in the MCF-7 breast cancer cell line, the expression of miR-155 was associated with a higher aerobic glycolytic activity and an increase in expression of the glucose transporter GLUT1. Interestingly, in the same study the high miR-155 cells were also more resistant to the glycolysis inhibitor 2-DG, demonstrating that they possessed a better ability of to perform metabolic switch between glycolysis and oxidative phosphorylation. Consequently, inhibition of miR-155 sensitized the cells to 2-DG treatment[190]. In our OT-1 cell model we observed an increase in the basal glycolysis of OT-1 miR-155 cells both in the presence and absence of glucose, indicating an increased ability for non-glycolytic acidification, potentially via the conversion of the glutamine present in the media into lactate. Moreover, the OT-1 miR-155 also demonstrated a higher glycolytic capacity upon activation. It would be interesting to investigate more precisely which metabolic pathways are involved in these changes.

Both numbers and percentages of OT-1 miR-155 T cells were increased *in vivo* at the peak of vaccination with OVA CpG and infection with Lm-OVA. We showed in a previous publication that miR-155 could downregulate the expression of the protein Suppressor of cytokine signalling 1 (SOCS-1). This downregulation was advantageous both for the proliferation of the CD8+ T cells and for their ability to mediate tumour control. Indeed, pmel CD8+ T cells transduced with a shRNA targeting SOCS-1 had an increased accumulation at day 4 post hgp100 vaccinia virus infection and could subsequently decrease the growth of melanoma. It is likely that the difference observed in our model is also linked to the downregulation of SOCS-1. Hence, it would be interesting to confirm by measuring SOCS-1 mRNA levels by qPCR, or protein levels by western blot.

Although OT-1 miR-155 cells were increased in numbers and percentages in spleen and draining lymph nodes of both tumours, overexpression of miR-155 lead to a higher accumulation of cells only in the low-affinity tumours. One explanation might be that in a high affinity setting, the OT-1 SCR cells are already being highly stimulated and are producing high level of miR-155 as a consequence. Hence, they are already making the best of the limited amount of survival signals in the immunosuppressive tumour environment. In this context, miR-155 constitutive expression likely did not confer an improved capacity to

OT-1 T cells to infiltrate tumours. Moreover, OT-1 miR-155 cells only mediated a better protection against tumour expressing the low affinity ligand T4, and not the high affinity ligand. This is somewhat surprising since we and others showed that stimulated OT-1 miR-155 are capable of producing more IFN-γ and TNF-α as compared to OT-1 SCR cells[82,83]. One would expect higher cytokine production to be helpful for the OT-1 cells tumour-lysing ability, even without an increase in accumulation. However, we confirmed *in* vitro that the cytotoxic abilities of OT-1 SCR and OT-1 miR-155 cells did not differ, which means that this increase of cytokine production was either compensated by another mechanism (higher rate of activation induced cell death) or was not useful in this context. As we discussed in the introduction, IFN-γ signalling is known to mediate PDL-1 upregulation in some tumour cell lines. We did not observe a change in the PD-1 expression levels on the surface of the tumour infiltrating OT-1 miR-155 cells compared to OT-1 SCR cells. However, we did not measure if PDL-1 expression increased on tumour cells *in vivo* upon OT-1 miR-155 cell transfer. If it is the case, it could explain why the higher IFN-γ production by miR-155 CD8+ T cells did not result in a better tumour protection. Additionally, it would be interesting to measure the proliferative ability of OT-1 cells within the tumour tissue, to see if the tumour environment would specifically prevent the OT-1 miR-155 cells from proliferating, as compared to lymphoid organs.

In contrast, there were more OT-1 miR-155 cells than OT-1 SCR cells infiltrating OVA-expressing tumours in the cotransfer setting. This might be due to proliferation kinetics, with miR-155 proliferating faster and reaching the tumour earlier. Once in competition with SCR OT-1 cells for cytokines and survival signal, miR-155 cells are likely to do better due to their better ability to respond to low levels of IL-2 via the downregulation of SOCS-1 and the phosphorylation of STAT5[81]. We already observed that, in optimal culture conditions with high levels of cytokines, OT-1 miR-155 cells did not differ significantly in their survival or proliferative ability compared to OT-1 SCR. To confirm that miR-155 OT-1 cells are more successful in a competition setting, we plan to co-culture OT-1 SCR and OT-1 miR-155 in the presence of limiting amounts of cytokines.

Importantly, while miR-155 overexpression in OT-1 T cells did not improve their antitumour activity against tumours expressing the bona fide OVA epitope N4 (SIINFEKL), it did so against tumours expressing the low-affinity antigen T4 (SIITFEKL). This might explain the discrepancy we observe between our results, which show close to no tumour protection in the B16-N4 model, and the results of Ji *et. al.* which show a dramatic increase in tumour protection in the pmel model upon miR-155 transduction[83]. Indeed, pmel T cells are tolerant to the mouse gp100 antigen (mgp100), and do not respond to either endogenous mgp100 expression, or vaccination with a mgp100 expressing virus[187]. However, infection with vaccinia virus expressing the human gp100 induces a potent response of the pmel CD8+ T cells, which are then able to destroy tumours expressing the mgp100 antigen. The low affinity SIITFEKL (T4) antigen that we used in our OT-1 model is of comparable affinity to the mgp100 in the pmel model. Indeed, it was shown that expression of SIITFEKL in foetal thymic organ culture did not induce the negative selection of single positive OT-1

thymocytes, when expressed at low doses[18]. Hence, overexpression of miR-155 might ameliorate the antitumour response only in a low affinity setting, both for the pmel and the OT-1 model. However, in the pmel model of Ji *et. al.*, transfer of genetically modified CD8+ T cells was accompanied with infection with a vaccinia virus and, in some experiments, systemic injections of IL-2[81]. We plan to investigate if we obtain similar results as Ji *et. al.* when we overexpress miR-155 in pmel cells and vaccinate the mice with CpG and hgp100, instead of infecting them with the vaccinia virus. This would allow us to confirm that the discrepancies we observe in our respective results are due to the affinity of the model TCR used, and not to the difference of the *in vivo* T cell priming.

Interestingly, we could observe in our system that overexpression of miR-155 did not only allow a better response to a target cell expressing a low-affinity antigen, but also improved the response when the OT-1 miR-155 cells were primed with CpG and the T4 peptide.

There might be two explanations for the miR-155 mediated improvement of the low affinity response. Firstly, as we previously mentioned, low affinity interaction between the TCR and the peptide:MHC complex induces lower levels of the endogenous miR-155. This might be due to the IRF4 binding to the BIC promoter[191], as it has been shown that IRF4 is a key element in the affinity-mediated regulation of CD8+ T cells responses[192]. In that context overexpressing miR-155 might be more useful and rescue the cells that have been sub-optimally activated via a low-affinity ligand.

Additionally, we observed downstream effects of miR-155 overexpression, which might have an effect on the TCR:MHC functional avidity. Indeed, we measured an increased surface expression of both CD8α and CD8β at resting phase. This might have an effect, not on the TCR affinity for the peptide:MHC, as the cells have an identical transgenic TCR, but on the functional avidity of the TCR to the peptide:MHC complex. Indeed blocking the interaction between CD8α and the MHC abrogated the ability of CD8+ T cells to kill target cells expressing a low affinity ligand, but had no effect on their ability to kill target cells expressing a high affinity antigen[193]. Hence, although CD8-binding to the MHC molecule seems to be dispensable for high affinity ligands, it is necessary to stabilize the interaction between the TCR and a low affinity ligand. A higher surface expression of these two molecules might improve the OT-1 cells avidity to low affinity ligand. To test this hypothesis, we are planning to measure the upregulation of miR-155 in CD8+ T cells upon binding to different concentration of either N4 or T4 peptides, and to assess if CD8-blockade affects the outcome. Moreover, we would like to take advantage of CD8β KO T cells, and transduce them with our miR-155 construct, in order to determine their ability to improve low-affinity antitumour effect *in vivo*.

Overall, our results demonstrate that overexpression of miR-155 in tumour-specific CD8+ T cells is a promising approach for cancer immunotherapy. Indeed, a better ability of CD8+ T cells to expand in a competition setting might be useful in human patients to circumvent the need for lymphodepleting treatments before adoptive cell transfer. This would improve the safety of the procedure. Moreover, endogenous tumour antigens are often of low affinity. Hence, overexpressing miR-155 would help naturally-occurring tumour-specific CD8+ T cells

respond better, even in the absence of genetic modification to improve the TCR's affinity. This would prove useful in therapies using the culture and reinjection of tumour infiltrating lymphocytes, by rescuing those of low affinity. In our hands and in the hand of others, mir-155 overexpression in CD8+ T cells did not induce the occurrence of a lymphoproliferative disease in the short term[83]. This is an encouraging sign for the safety of the procedure, as miR-155 overexpression has been associated with several malignancies. However, the long-term safety of miR-155 overexpression for a clinical use would have to be further validated before its implementation.

4. Methods:

4.1. Mouse strains:

OT-1 mice transgenic and H-2Kb/OVA-specific TCR and pmel mice transgenic for the H-2Kb/pmel 17/gp100 epitope were bred in house until 6-15 weeks of age and used to obtain OT-1 and pmel CD8+ T cells. OT-3 mice of low affinity OVA-specific TCR, were obtained from Dietmar Zehn's laboratory. Pmel mice producing CD8+ T cells specific for the mouse gp100 protein were bred in house. C57BL/6J mice were purchased from Harlan. C57BL/6J expressing the CD45.1 or CD45.1/2 congenic markers were bred in house.

4.2. Cell lines

B16-F10 mouse melanoma cell line was used either as such or transduced to express the SIINFEKL (N4) or SIITFEKL (T4) OVA protein. B16-OVA implicitly refers to the high affinity SIINFEKL ovalbumin protein.

4.3. Production of retroviral particules

10^7 phoenix-ECO cells were plated in a T150 flask the day before the transfection. Transfection with a mix of 15µg of either pMGP-SCR or p-MGP-M155 (Addgene 26527) and 15µg of the pCL-ECO packaging plasmid was performed with the JetPEI kit (Chemie Brunschwig 101-10) following manufacturer's instruction. Phoenix-ECO media was discarded and replaced with 19ml of fresh media 24 hours after transfection. The media was collected 48- and 72 hours after transfection and concentrated via ultracentrifugation for 2 hours at 11'000xrcf. Retroviral particules were carefully resuspended in mouse RPMI and snap-frozen in dry ice to be subsequently stored at -80°C until use.

4.4. Transduction of mouse T cells and maintenance in culture

Spleens of OT-1 mice were dissociated, and T cells isolated using the mouse T cell isolation kit (stemcell 19851). T cells were subsequently activated by co-culturing them with αCD3/αCD28-coated magnetic beads (Lifetechnologies 11452D) at a 2:1 beads:T cell ratio. Usually, $0.5x10^6$ T cells were plated in a 48-well plate in mouse RPMI (see supplementary methods page 89) supplemented with 50IU IL-2. On the same day, non-treated 48-wells plate were coated with retronectin at a 20µg/ml concentration and left overnight at 4°C. 20h after T cell activation, the retronectin-coated plates were washed with PBS and blocked

for 30min at RT with PBS 2%BSA. The plates were washed again before the addition of the concentrated virus. If the volume of virus added on the plate (determined by prior virus titration) was inferior to 250µl, the final volume was adjusted to 250µl with medium. The retronectin-coated plates containing the virus were then centrifugated for 90minutes at 32°C at 2'000xg. At the end of the centrifugation, the activated T cells from each 48-well were resuspended and were directly added on top of the corresponding well in the virus-coated plate. The cells + virus were centrifugated for 10min at 25°C at 200x rcf and then directly placed in the incubator at 37°C. The next day, 500µl of mouse RPMI supplemented with 50IU/ml IL-2 was added on top of the transduced cells. The percentage of transduced cells was assessed 2 days after transduction via flow cytometry detection of GFP expression. At this time, the cells were either used for *in vivo* tumour challenge, or kept in culture. The cells *in vitro* were switched to mouse RPMI + 10IU IL-2/ml + 10ng/ml IL-7 + 10ng/ml IL-15 at day 2 post transduction, and to mouse RPMI + 10ng/ml IL-7 + 10ng/ml IL-15 at day 4 post transduction. Transduced mouse CD8+ T cells could be kept in culture in IL-7 and IL-15 and used for *in vitro* assays for 2-3 weeks after transduction.

4.5. Tumour challenge

C57BL/6J mice 6-10 weeks old were subcutaneously engrafted with 10^5 B16-OVA (N4) or B16-T4 tumour cells in 100 or 200 µl PBS. 6 days later, 10^5 mio OT-1 cells (usually around 90% SCR or miR-155 transduced) were transferred intravenously in the tail vein. 7 days after the tumour graft, mice were either infected or vaccinated. Infections were performed via the intravenous injection of 2'000 PFU *L. monocytogenes* genetically modified to express the OVA protein either in the high affinity natural N4 epitope or the low affinity T4 variant. Vaccination was performed as a subcutaneous injection at the base of the tail. The vaccination mix incorporated 10µg N4 or T4 peptide with 50µg CpG in PBS. Tumour growth was measured using a manual caliper from day 6-post engraftement and every 2-3 days after that until the end of the experiment. Tumour sizes were calculated in mm^3 as the product of length, width and height, divided by 2.

4.6. Organ processing for *in vitro* analysis

Spleens were mashed through a 100µm filter, and treated with red blood cell lysis buffer for 5minutes before staining. Lymph nodes were mashed through a 40µm filter and stained directly. Tumours were cut in small pieces with scissors, and put into a MACS c-tube with 2.5ml of plain DMEM containing enzymes from the MACS 130-096-730 kit. Tumours were then mechanically processed using the gentleMACS Dissociator (# 130-093-235) and the m_impTumor_02 programm. Afterwards, tumours were incubated at 37°C for 20 minutes, rolling constantly. Finally, tumours were again mechanically processed using the program m_impTumor_03. Resuspended tumours were filtered through 40µm, before pelleting by centrifugation. Debris were removed by percoll purification. The tumour cell pellet was firstly resuspended in a 15ml falcon in 40%percoll in PBS at 37°C. Then 4ml of 70% percoll in PBS was carefully layered on top of the 40% percoll. The tubes were subsequently centrifuged at 975x rcf for 20 minutes, without brake and with level 6 acceleration. Tumour

infiltrating lymphocytes were isolated from the middle phase of the density gradient, and washed 2x before staining or restimulation for the detection of cytokine production. Both the axillary and inguinal lymph nodes on the same flank as the tumour were sampled and defined as tumour draining lymph nodes (dLNs) in the results.

4.7. Functional assays

Detection of proliferation in vivo via bromodeoxyuridine (BrdU) incorporation: Mice were injected with 1.8mg BrdU diluted in PBS intraperitoneally 2.5 hours before organ collection and analysis. Organs were processed as described previously. After staining for surface markers, cells were fixed and firstly the cell membrane was permeabilized, then the nuclear membrane was permeabilized using the Cytoperm Plus (BD 561651). Add DNAse at a concentration of 0.3µg/µl and incubate for 1 hour at 37°C. Then add the anti-BrdU PE antibody in diluted in Perm/Wash buffer and incubate xx minutes at room temperature. Wash 2x before resuspension and acquisition via flow cytometry.

4.8. FACS

Trucount beads quantification of absolute number of cells/ml of blood: Absolute numbers of cells in the blood were quantified thanks to Trucount beads (BD 340334). 50µl of blood was added to the Trucount tubes containing a defined number of beads. 50µl of FACS buffer containing a 2x concentrated amount of antibodies was added directly to the blood and beads mix and incubated at 4°C for 20 minutes. 400µl of 1x FACS lysing buffer (BD 349202) was then added to the stained blood. No wash was performed before acquisition, in accord with the manufacturer's instruction, to maximize the measurement accuracy.

After acquisition the total number of cells/ml of blood was calculated as such:

$$= \frac{Number\ of\ recorded\ cells}{Number\ of\ recorded\ beads} * total\ number\ of\ beads\ in\ tube * 200$$

Intracellular cytokine staining: To assess intracellular cytokine production, 10ng/ml final concentration PMA and 500ng/ml ionomycin, or 10µM of SIINFEKL peptide were added to plated splenocytes or tumour infiltrating lymphocytes in a 96-well plate and placed in the incubator at 37°C. After 30 minutes, Golgistop (monensin, BD 554724) was added to the wells at a 1/1'500 dilution. The cells were placed back in the incubator for an additional 4 hours before surface staining and intracellular staining. Fixation and permeabilization for the intracellular detection of cytokines was performed with the BD kit (554714) according to the manufacturer's instruction.

CFSE labelling: Count cells in suspension and transfer desired number to a 15ml falcon tube. Pellet cells by centrifugation, discard supernatant and resuspend thoroughly in 1ml/mio cells pre-warmed 37°C PBS solution containing CFSE diluted to a working concentration of 1µM. Incubate the cells for 20 minutes at 37°C, under constant rolling motion, protected from light. Add 1volume of ice-cold FCS, and incubate cells on ice for at least 5 minutes. Fill the rest of the tube with complete medium, 14 ml final. Centrifuge cells, discard supernatant and resuspend cells in fresh complete medium. The cells can now be counted, used for flow cytometry and cocultures.

4.9. qPCR

Cells were collected, resuspended in RNAlater (Ambion AM7020) and stored at -20°C for further RNA isolation. Subsequently, RNA was purified in 40µl H2O using the miRVana (Ambion AM1561) kit and following manufacturer instructions. MiR-specific reverse transcription was performed on 5µl of the extracted RNA and using Taqman primer pairs (Taqman SnoRNA202 1232, Taqman mmu-miR155 2571) and the Taqman reverse transcription kit (Lifetechnologies 4366597) following manufacturer instructions. Finally, qPCR was run using 96-well optical plates (Lifetechnologies 4346906) in a 7500 Fast Real-Time PCR machine (Thermofisher 4351106). The qPCR mix volume was of 10µl/well and comprised 2µl of the RT reaction mixed with the primers and Taqman Fast Universal Master Mix (Lifetechnologies 4352042). All qPCR reactions were performed in 2 technical replicates, and the values were excluded if the difference in C_T was higher than 0.2 or if one of the C_T value was higher than 33. The mean of the technical replicates of the control gene (Sno202) was substracted to the mean of the technical replicates of the gene of interest (miR-155) to calculate the ΔC_T. Fold changes in expression where then calculated using a control sample for normalization as such:

$$Fold\ change\ of\ miR155\ expression\ in\ (x) = 2^{\Delta Ct(control\ sample) - \Delta Ct(sample\ x)}$$

4.10. Statistical analysis

Experiments showed are representative experiments of usually 2 replicates. Statistical tests performed were 2-way ANOVAs followed by a Tukey's multiple comparison test to compare the different groups between them.

When two groups were compared, the t test with a two-sided P value and a 95% confidence interval was performed. When more than two groups over time were compared, a two-way ANOVA test was performed, followed by a Tukey's multiple comparison test. Figures show one representative experiment out of 2 or 3 independent experiments. The individual dots always represent biological replicates (number of mice or independent wells). When technical replicates were performed (usually in duplicates), the average of the replicates was plotted. All statistical tests were performed using the Prism version 6 software (NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.)

Chapter III: Overexpression of miR-155 in anti-PSMA CAR+ CD8+ T cells

1. Aim:

Recent studies have shown the potential of chimeric antigen receptor (CAR) therapy for the treatment of various cancers, especially haematological malignancies. However, designing effective CAR treatments against solid tumours has proven difficult to achieve. Indeed, solid tumours induce a strongly immunosuppressive microenvironment that infiltrating T cells need to overcome in order to perform their cytolytic activity.

Our aim is to improve the antitumour efficiency of an existing CAR against a solid tumour by overexpressing miR-155. We used a CAR targeting the prostate specific marker antigen (PSMA) as our model CAR to test our hypothesis. PSMA is a protein that is overexpressed in the endothelium of advanced prostate cancer, as well as in the neovasculature of several other cancers, but not in normal endothelium[194]. It was previously shown that the anti-PSMA chimeric antigen receptor can specifically lyse PSMA+ cells, and induce tumour regression *in vivo* in xenograft mouse models[195–197]. The results of a Phase I clinical trial showed that T cells transduced with an anti-PSMA CAR could engraft efficiently in prostate cancer patients, and mediate some partial responses[198]. We sought to ameliorate the antitumour efficiency of CAR T cells redirected against PSMA by overexpressing miR-155, as a proof of concept of the potential of combining miR-155 overexpression with CAR transduction.

2. Results:

Efficient and stable transduction of CD8+ T cells from peripheral blood of healthy donor with the PSMA-CAR-miR-155 or -SCR construct

We obtained the lentiviral construct expressing the anti-PSMA CAR of the second generation from the laboratory of Prof. Coukos in U-Penn. We subsequently modified the plasmid to incorporate the miR-155 and SCR sequence after the CAR sequence, so that both the CAR and miR-155 would be co-expressed in the transduced cells (see page 90 for microRNAs sequence and Figure 28A for a schematic view).

Peripheral blood from healthy donors was collected from the Lausanne transfusion blood center and CD8+ T cells were purified (see Methods 4.3, page 83). CD8+ T cells were transduced with either the SCR- or the miR-155 CAR construct and CAR expression on the surface of the cells was measured 5-7 days after transduction by flow cytometry. Usually CAR+ CD8+ T cells comprised 30-50% of the total population. These cells were subsequently expanded by coculture with the MS1_PSMA cell line. After re-expansion, the CAR+ population usually amounted to 80-90% of the whole population (Figure 28B). Transduced cells could be restimulated with irradiated MS1_PSMA or irradiated PBMCs and kept in culture for up to 3 weeks. CAR expression at the surface of the cells was stable during this time lapse.

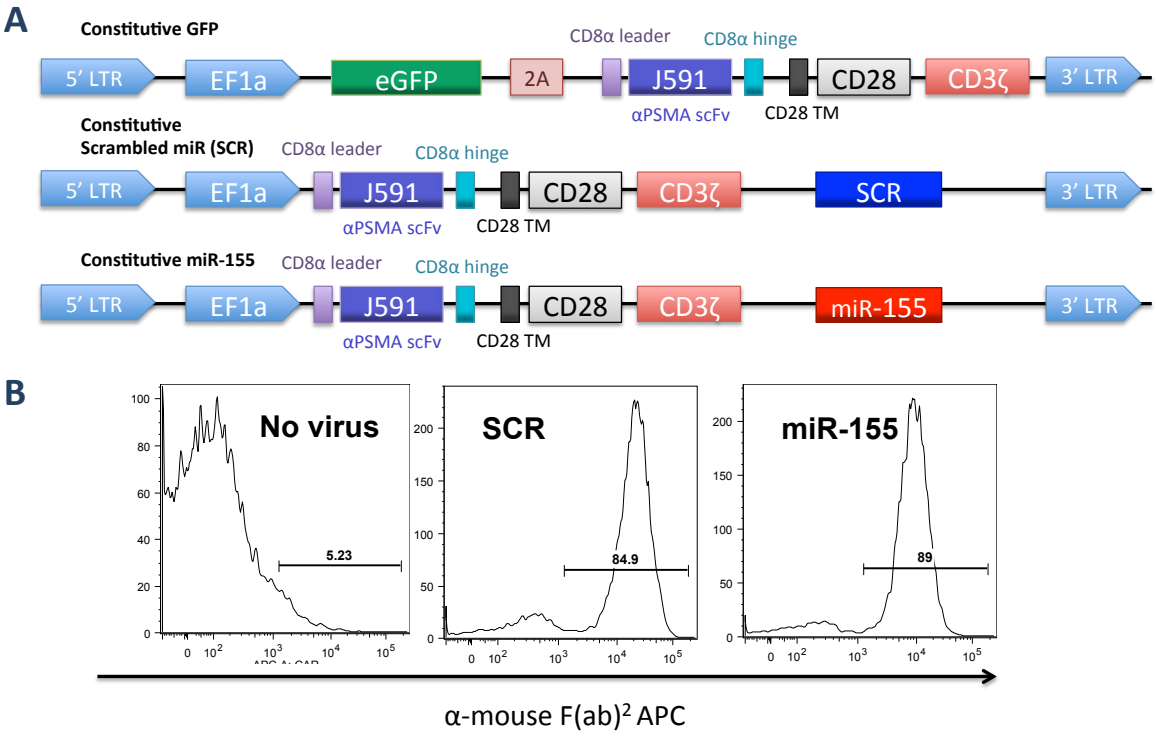

Figure 28: (A) Scheme of the three lentiviral plasmids incorporating both the αPSMA CAR and either the green fluorescent protein (GFP) or the microRNA miR-155 or the scrambled control (SCR) under the control of the EF1α promoter. (B) Human CD8+ T cells were transduced with the lentiviral constructs and surface expression of the αPSMA CAR was detected by flow cytometry using an APC-conjugated anti-mouse F(ab)2 antibody.

MiR-155 was augmented at baseline in cells transduced with the miR-155_CAR construct

After transduction and restimulation, CD8+ T cells were left in culture with 150IU IL/2 for 13 days and restimulated with either MS1_PSMA at a 5:1 T cells:MS1_PSMA ratio, or αCD3/αCD28 beads at a 1:1 ratio. Before restimulation, the difference in miR-155 expression between CAR_SCR and CAR_miR-155 cells was of 3-fold. Upon activation, the endogenous miR-155 was highly upregulated and masked the effect of constitutive overexpression. 1 day after activation, the difference was only 1.5-fold, and even less later in the response (Figure 29).

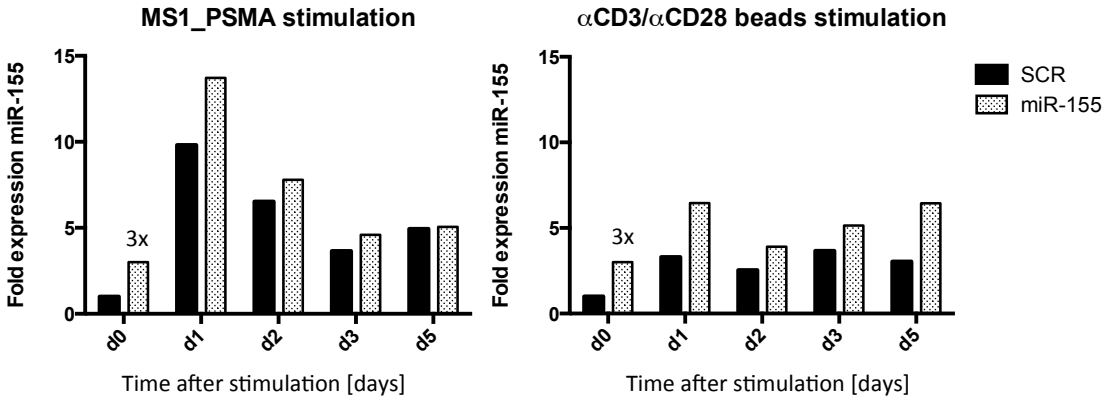

Figure 29: MiR -155 levels were measured at baseline, as well as 1, 2, 3 and 5 days after stimulation with either MS1_PSMA (left panel) or αCD3/αCD28 beads (right panel), and normalised to OT-1 SCR levels before stimulation

The proliferative ability of CAR_miR-155 T cells was augmented *in vitro*

CAR+ CD8+ T cells were marked with cell trace Violet dye and then stimulated either specifically by coculture with MS1_PSMA or non-specifically by αCD3/αCD28-coated magnetic beads. Three days after restimulation, the CAR_miR-155 T cells had replicated more than the CAR_SCR controls, both when stimulated with beads or with the PSMA+ cell line (Figure 30). This shows that the miR-155 transduced CAR cells were able to proliferate faster than their SCR counterparts.

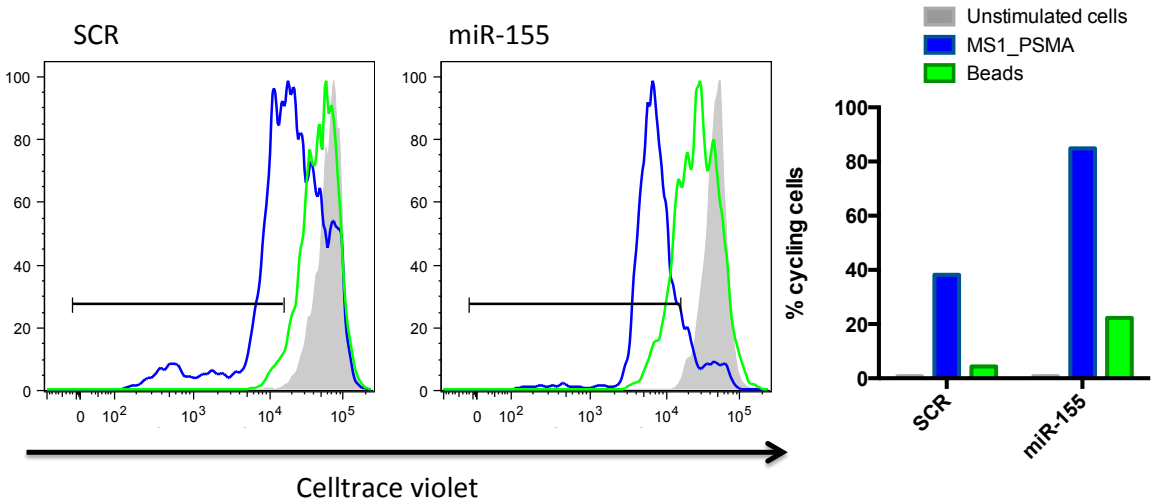

Figure 30: Celltrace Violet dilution upon T cell proliferation measured 3 days after restimulation either with αCD3/αCD28 beads or MS1-PSMA

Lysis of target cells in vitro was unchanged upon miR-155 overexpression

The ability of CAR+ T cells to lyse cells expressing the PSMA protein was measured by chromium release assay. Cells from either the Cal 27 or MS1 cell line, genetically modified to express PSMA or not, were loaded with ^{51}Cr for 1 hour and then cocultured with different amounts of effector cells (CAR+) for 4 hours. The CAR+ cells were able to lyse efficiently the cells expressing the PSMA protein, and had a low level of non-specific lysis. However, all constructs were similar in their ability to lyse target cells, which shows that overexpression of miR-155 did not affect the lysis ability of the cells in vitro (Figure 31).

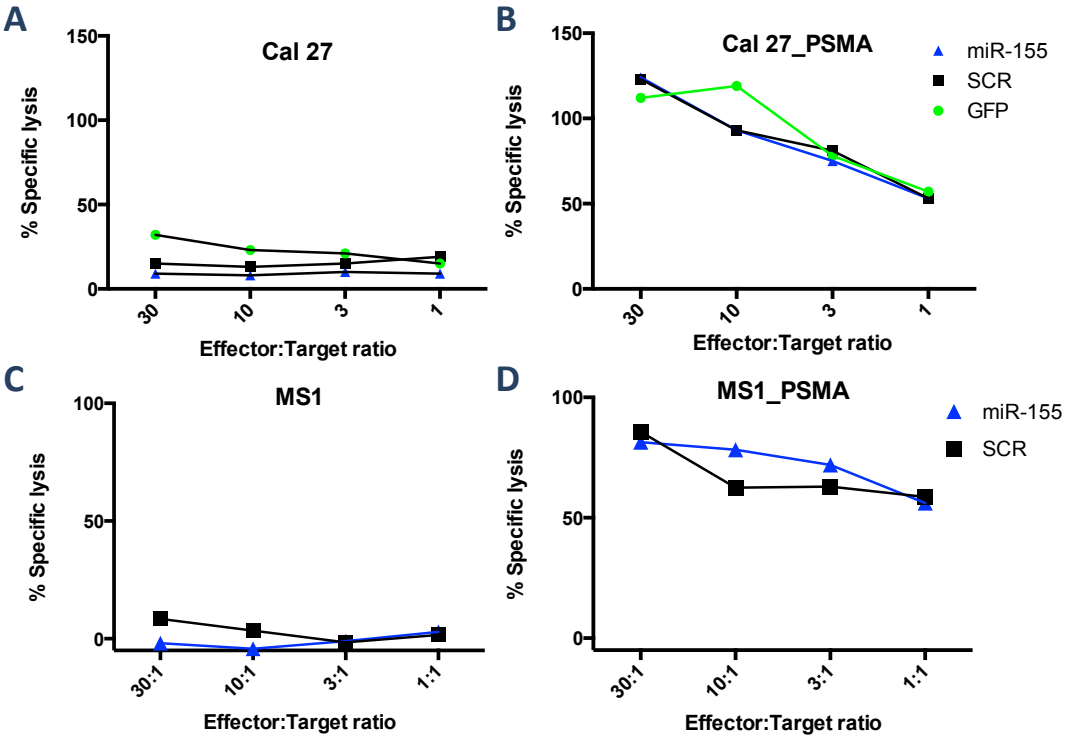

Figure 31: (A) Cal 27, (B) Cal 27_PSMA, (C) MS1 and (D) MS1-PSMA, were marked with Cr51 and then incubated at different effector:T cell ratio for 4 hours. Specific lysis was calculated as explained in the methods section.

MiR-155 CAR T cells did not improve tumour protection *in vivo* compared to SCR CAR T cells

To assess the ability of our CAR+ T cells to mediate tumour regression *in vivo*, we engrafted NSG mice with a mixture of MS1_PSMA and matrigel. After 2 weeks, tumours were palpable and their growth was measured every week using the IVIS imager. Luciferin was injected intraperitoneally and the luminescence was measured in anesthetized live animals(see Supplementary Figure 35). 4.5 and 5.5 after tumour graft, two sequential intravenous transfers of respectively $2.5x10^6$ and $5x10^6$ CAR+ T cells were done in tumour bearing animals (Figure 32). Mice were either injected with SCR_CAR, miR-155 CAR, or received no cell transfer (PBS). Injection of anti-PSMA-CAR+ T cells mediated a strong tumour regression. Indeed, a 20-fold decrease in luminescence was observed one week after CAR T cell transfer. However, the addition of miR-155 did not increase the ability of CAR+ T cells to

mediate tumour regression in this model (Figure 32). Moreover, the long-term tumour was not improved, as tumours started growing back in both groups after a couple of weeks.

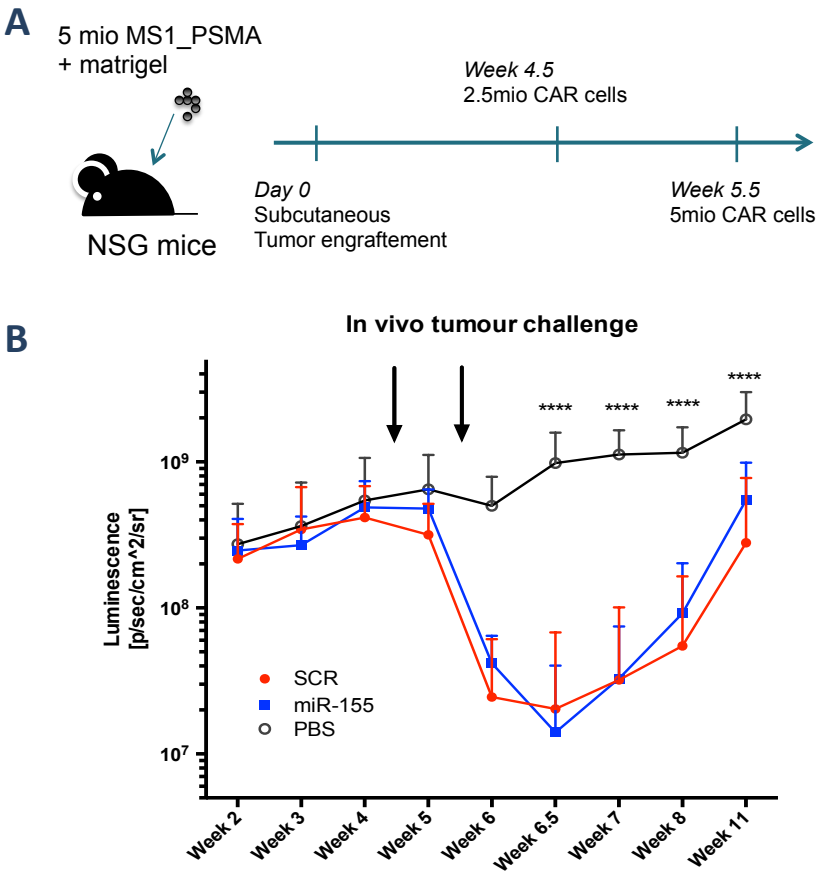

Figure 32: (A) 5x10^6 MS1_PSMA cells were subcutaneously engrafted in the flank of NSG mice. 4.5 and 5.5 weeks after tumour graft, 2.5x10^6 and 5x10^6 CAR+ T cells were transferred intravenously in tumour bearing mice. (B) Tumour growth was measured via luminescence every week. Statistical significance calculated with Tukey's multiple comparisons test (n=7-10)

3. Discussion:

In this project we wanted to assess the ability of miR-155 overexpression to increase the antitumour efficiency of anti-PSMA CAR CD8+ T cells. We observed that CD8+ T cells co-expressing the CAR and miR-155 had a higher proliferative ability *in vitro*, but did not differ in their tumour lysing ability. In mouse xenograft model, we showed that anti-PSMA CAR CD8+ T cells could efficiently mediate tumour regression. However, concomitant miR-155 overexpression did not improve the antitumour efficiency of the CAR in this model.

The lentiviral construct we developed, incorporated both the anti-PSMA CAR sequence, as well as the whole miR-155, so that both the CAR and the microRNA would be co-expressed. To make our lentiviral construct, we inserted the original miR-155 pre-miRNA sequence, and added 200 bp on each side to ensure correct processing. It was shown in a previous publication that at least 40bp on each side of the stem-loop sequence were necessary for a

correct processing of the pre-miRNA into a functional mature microRNA[46]. Moreover, the promoter EF1α, has been shown to be able to mediate good expression of microRNAs[199]. The miRNA sequence was added directly after the CAR sequence. Theoretically, the microRNA miR-155 should be correctly processed from the CAR mRNA, as it was shown that it is possible for pri-miRNA to function both as mRNAs as well as microRNA precursors[200]. However, when we measured the increase in miR-155 expression in cells transduced with the miR-155 construct, there was only a 3-fold increase compared to the cells transduced with the control plasmid. This is surprising, as in the retroviral construct p-MGP miR-155 used in OT-1 cells to overexpress miR-155, the pri-miRNA sequence was added directly after the GFP sequence, and both the protein and mature microRNA were very efficiently produced. Indeed we could measure a strong GFP signal, as well as a 30-fold increase in miR-155 production (Figure 19). However, one cannot exclude that CARs, which are complex proteins, might impair microRNA processing. In another study, in which miR-17-92 and the anti-EGFRvIII CAR were co-expressed in human T cells, the CAR and microRNA were encoded in different lentiviral plasmids[97]. This is something we could try in our next experiments.

Another possibility is that the endogenous miR-155 was highly upregulated in CAR+ CD8+ T cells in culture, and hence would mask the expression of the transgenic miR-155. It is likely that the endogenous miR-155 is high, since CAR CD8+ T cells were kept *in vitro* with high doses of IL-2, and frequently restimulated. Indeed, it was shown in a malignant T cell line that the presence of Il-2 and IL-15 could upregulate miR-155 expression via an increase in STAT5 signaling[72]. Of note, when the endogenous levels of miR-155 in our CAR SCR cells were compared to that of naive cells from the peripheral blood, a two-fold increase was observed (data not shown). Eventually, we do not know how many extra copies of miR-155 this baseline 3-fold increase represents, which is why we decided to investigate anyway if this increase was enough to induce a biological difference in our model.

Indeed, we could see an improvement in the proliferative ability of the CAR miR-155 cells *in vitro* compared to the CAR SCR cells, both with CAR signalling through PSMA binding or TCR cross linking via αCD3/αCD28 binding. However, no increase in cytokine production or target cell killing was observed *in vitro*. Finally, we did not see an increase either in proliferation or in tumour control *in vivo*. Hence, miR-155 overexpression did not improve the αPSMA CAR antitumour effect in this model. However, there are still some questions that remain to be answered. Firstly, miR-155 effect acts mainly by increasing cytokine signalling, via the downregulation SOCS-1, as we previously discussed, and the increase of phosphoSTAT5 levels in response to IL-2. Extrinsic cytokines are crucial for the miR-155-mediated antitumour response. Indeed, the antitumour efficiency of mouse CD8+ T cells overexpressing miR-155 was strongly reduced when those cells were transferred in IL-$15^{-/-}$ x IL-$7^{-/-}$ KO hosts[83]. For this reason, it would be more appropriate to test the proof of principle of miR-155 and CAR co-expression for antitumour therapy in a complete mouse model, and not in a human xenograft model. In that case we could use immunoreplete animals, because we would not need to worry about the transferred human CD8+ T cells being rejected. It is

possible that the overexpression of miR-155 does not bestow an advantage to CD8+ T cells functionality *in vivo* in NSG mice.

Secondly, we now know that the affinity of the TCR to its peptide:MHC complex determines the extent of miR-155 upregulation. We can only speculate as whether the affinity of the CAR scFv to its ligand is also correlated to miR-155 expression. And whether miR-155 overexpression would be more helpful in a CAR bearing a scFv of lower affinity, in the same manner that overexpression of miR-155 was more beneficial for a low affinity TCR.

Researchers have become interested in low affinity CARs recently, as they might be more sensitive in their discrimination of high and low tissue expression of the target antigen. This is indeed an issue, as many antigens that are overexpressed in tumours, can be expressed at low levels in other parts of the body. This type of situation can have dramatic consequences. For example, a patient succumbed to an anti-HER2 CAR treatment, after the CAR+ T cells started attacking their lungs, where low levels of HER2 were expressed. The subsequent pulmonary oedema was fatal[179]. In the case of low affinity CARs, it was demonstrated that they have the ability to discriminate between high and low surface expression of the antigen[180]. In this context, it would be interesting to test if overexpression of miR-155 is helpful for low affinity CARs.

4. Methods:

4.1. Construction of a chimeric antigen receptor plasmid containing miR-155

The group of G.Coukos in U-penn, Philadelphia, provided the anti-human PSMA chimeric antigen receptor[197]. It is composed of a mouse antibody scFv (J591) specific for an extracellular epitope of human PSMA[201]. This scFv is fused to a CD8 hinge as the transmembrane domain and spacer[195]. In addition, it comprises the intracellular CD3ζ signalling domain as well as the CD28 costimulatory domain[202]. To allow for proper CAR extracellular localisation and anchorage in the membrane, a CD8a leader sequence is present at the 5' position of the CAR.

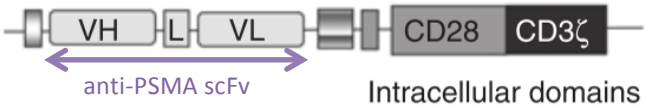

Figure 33: Chimeric antigen receptor specific for PSMA

We subsequently inserted in the original vector and inserted either the full pri-microRNA sequence, with an additional 200 base pairs on each side of the mature microRNA to ensure correct processing, or a scrambled microRNA sequence as a control. Finally, we made a vector expressing the Green Fluorescent Protein (GFP) and the CAR under the same EF1α promoter, separated by a 2A sequence (see Figure 28A page 77).

4.2. Production of lentiviral particles for transduction

HEK 293T/17 cells were cultured in complete RPMI. 10^7 cells were plated in the afternoon before the day of transfection. The following morning, the DNA construct containing the

CAR (15 µg), together with packaging plasmids Gag/pol (7µg), Vsvg (7µg) and Rev (3.5µg), was transfected into the cells with the JetPEI® kit (Chemie Brunschwig 101-10). Virus was collected at 24- and 48 hours and concentrated by ultracentrifugation at 11'000x rcf for 2 hours before use or freezing.

4.3. Isolation and transduction of CD8+ T cells from peripheral blood of healthy patients

Peripheral blood mononuclear cells (PBMCs) were extracted from healthy donor blood provided by the local blood transfusion centre. 15ml of buffycoat was mixed with 15ml of PBS and carefully layered on top of 15ml of a density gradient media (Lymphoprep, Axonlab 1114547) in a 50ml Falcon tube. The cells were subsequently centrifuged 20min at 2200 rpm (600xg) without break. The PBMCs sedimented as an intermediate band, were recovered and washed in PBS 1x.

Human CD8+ T cells were negatively selected from PMBCs by magnetic sorting (STEMCELL 19053). Then, they were activated by αCD3/αCD28 beads (Dynabeads 11161D) for 24h in human RPMI (see Supplemental methods) containing 150 international units (IU) IL-2/ml before being infected by lentiviral particles. Infection was conducted by adding fresh or frozen virus on the cells together with 8µg/ml polybrene, followed by centrifugation of the cell plate for 90 minutes at 800x*g*. The transduction efficiency was assessed by surface staining of the CAR for 30min on ice, with an anti-mouse F(ab)2 antibody from Jackson Immunosearch (115-606-072).

4.4. Human CD8+ T cell maintenance in vitro

Cells were kept in 150IU IL-2 in human RPMI at a density of 10^6 cells/ml of medium for 7-12 days after activation. Once the cells stopped proliferating, they were restimulated. The cells were either non-specifically stimulated with irradiated PBMCs from 2 donors (feeders) at a 1:1 ratio T cell:feeder and PHA (2µg/ml) or CAR+ T cells were specifically expanded with MS1_PSMA and feeders at a 5:5:1 ratio T cell:feeder:MS1_PSMA.

4.5. Cell lines

The MS1 mouse pancreatic islet endothelial cell line was provided by the Prof. Coukos laboratory in U-Penn. The MS1_PSMA cell line was co-transduced with two lentiviral construct, one expressing the *firefly luciferase* and the fluorescent protein DsRed, and the other expressing the human PSMA. The Cal 27 human epithelial cell line was provided by Dr. Rivals. Cal 27_PSMA cells were obtained by transducing the Cal 27 cells with a lentiviral vector expressing the human PSMA.

4.6. Mouse tumour models

The severely immunosuppressed strain of mice NOD SCID common gamma KO (NSG) was used as a xenograft model. 6-10 weeks old mice were engrafted with 5 million of either MS-1 or MS-1 PSMA cells were engrafted. Injections were performed subcutaneously in the flank in a 1:1 mix of matrigel (Chemie Brunschwig 356234) and PBS. Every week, tumour size

was measure by luminescence by intraperitoneal injection of 200µl luciferin (15µg/ml) and using a the Xenogen IVIS Lumina II machine. The images were analysed using the Living Image® Software.
4-5 weeks after tumour graft, 5 million CAR+ T cells were transferred intravenously.

4.7. qPCR

Cells were collected, resuspended in RNAlater (Ambion AM7020) and stored at -20°C for further RNA isolation. Subsequently, RNA was purified in 40µl H2O using the miRVana (Ambion AM1561) kit and following manufacturer instructions. MiR-specific reverse transcription was performed on 5µl of the extracted RNA and using Taqman primer pairs (Taqman RNU44 1094, Taqman hsa-miR155 2623) and the Taqman reverse transcription kit (Lifetechnologies 4366597) following manufacturer instructions. Finally, qPCR was run using 96-well optical plates (Lifetechnologies 4346906) in a 7500 Fast Real-Time PCR machine (Thermofisher 4351106). The qPCR mix volume was of 10µl/well and comprised 2µl of the RT reaction mixed with the primers and Taqman Fast Universal Master Mix (Lifetechnologies 4352042). All qPCR reactions were performed in 2 technical replicates, and the values were excluded if the difference in C_T was higher than 0.2 or if one of the C_T value was higher than 33. The mean of the technical replicates of the control gene (RNU44) was substracted to the mean of the technical replicates of the gene of interest (miR-155) to calculate the ΔC_T. Fold changes in expression where then calculated using a control sample for normalization as such:

$$Fold\ change\ of\ miR155\ expression\ in\ (x) = 2^{\Delta Ct(control\ sample) - \Delta Ct(sample\ x)}$$

4.8. Functional assays

Celltrace staining: The proliferative capacity of transduced cells was assessed by staining them with the Celltrace Violet dye (Lifetechonologies C34557) following manufacturer's instructions and culturing the cells at a 1:10 ratio with MS1 or MS1_PSMA or at a 1:1 ratio with anti-CD3/anti-CD28 beads or on their own. After 3 days of coculture, the cells were resuspended and stained with an APC conjugated goat anti-mouse f(ab)^2 antibody and a dead cell marker. The samples were analysed on a LSR-II flow cytometer.
Chromium release assay: Target cells were loaded with chromium ^{51}Cr, and then cocultured with CAR-transduced T cells at different ratios. After 4 hours, the supernatants were collected and transferred to a LumaPlate-96 for radioactivity detection from a TopCount NXT plate reader. The lysis percentage was calculated as follows: $100\times\frac{experimental-spontaneous\ release}{total-spontaneous\ release}$. Spontaneous release was calculated for each cell line as the recorded signal in a well without target cells. Total release was calculated as the recorded signal for a well where all the cells were lysed by addition of HCl.

General conclusion

During my thesis project, I studied CD8+ T cells and the importance of microRNAs on their functionality and their antitumour ability.

First, we used a CD4 conditional knockout of the miR-17-92 cluster, to observe the functionality of CD8+ T cells in its the absence. In line with other publications using similar models, we found that the absence of miR-17-92 impaired the CD8+ T cells in their proliferative ability upon activation, and decreased their production of cytokines. Moreover, the CD8+ T cells were impaired in their effector cells differentiation, and showed a phenotype of central memory cells $CD127^{high}$ $KLRG1^{low}$. Overexpression of miR-17-92 was shown by other groups to induce more terminal differentiation of the CD8+ T cells as well as increased proliferation. One publication showed improved efficiency of human CAR+ T cells that were cotransduced with a construct overexpressing miR-17-92 in a mouse xenograft model. However, whether overexpression of miR-17-92 improves the antitumour efficiency of CD8+ T cells in a complete mouse model is still not known and would be interesting to investigate.

Second, we overexpressed miR-155 in OT-1 CD8+ T cells and assessed their response to infection and their ability to control tumour growth. CD8+ T cells overexpressing miR-155 had an increased proliferative capacity and cytokine production upon activation. However only the tumours that expressed the low affinity SIITFEKL (T4) OVA altered peptide ligand were controlled better by miR-155 OT-1 CD8+ T cells. Our findings indicate that miR-155 overexpression rescues CD8+ T cells that have been sub optimally activated or restimulated by a low affinity ligand. Cytotoxic T cells specific to tumour antigens are often of low affinity because of central tolerance. Hence, overexpressing miR-155 shows promise in restoring the functionality of those tumour-specific low affinity T cells.

Third, we assessed miR-155 overexpression in human CD8+ T cells expressing the anti-PSMA CAR. We managed to induce a small increase of miR-155 levels in those cells. This increase was sufficient to improve the ability of CAR+ CD8+ T cells to proliferate *in vitro* upon activation. We then measured the ability of miR-155 CAR+ CD8+ T cells to mediate tumour protection in a mouse xenograft tumour model. Although CAR+ CD8+ T cells could mediate strong tumour regression, overexpression of miR-155 did not improve the efficiency of the anti-PSMA CAR in this model.

Perspectives

In this research, we demonstrated the importance of microRNAs for efficient CD8+ T cell effector response as well as antitumour efficiency. We were able to show that it is possible to modulate the expression of the microRNA miR-155 and hence increase the performances of effector CD8+ T cells. Finally, we showed that overexpression of miR-155 impacts the antitumour efficiency of genetically modified CD8+ T cells in the context of adoptive cell transfer.

Adoptive cell transfer has gained momentum as a treatment against cancer thanks to the recent results of clinical trials using tumour infiltrating lymphocytes or lymphocytes transduced to express a chimeric antigen receptor. However, both of these methods have proved successful only for a limited range of cancers. TILs transfer induced a complete response in 22% of advanced melanoma patients, whereas CAR therapies were very successful for haematological cancers. In the case of TILs, most of the CD8+ T cells are naturally cancer-specific because these cells are found within the tumour microenvironment. In the case of CAR therapy, the T lymphocytes are genetically modified to become cancer-specific via the expression of the chimeric receptor. In both case, modulating the expression of genes to improve the effector performance could ameliorate currently available treatments.

Although we could not demonstrate that overexpression of miR-155 was therapeutically helpful in the case of CAR therapy, we argue that miR-155 is a strong candidate for improving the antitumour efficiency of CD8+ T cells in the context of adoptive cell transfer. Indeed, we showed that miR-155 overexpression simultaneously impacted several aspects of the T cells functionality; namely the response to low affinity antigen, the production of cytokine upon activation, and the metabolic program. Moreover, miR-155 overexpressing cells could be efficient against cancer cells expressing a low affinity antigen, which is often the case for naturally occurring tumour associated antigens, and for some neoantigens as well.

One important aspect that was not directly addressed in the scope of this thesis is the establishment of a long-term protection in cancer patients. Indeed, future treatments should not only be efficient in their immediate ability to reduce the cancer burden, but should also induce a complete curative response in patients. It has been proposed that immunotherapies are a good method to achieve such a goal if they can trigger the formation of long-lived memory lymphocytes. Modulating the expression of microRNAs might actually be helpful in this quest of memory lymphocytes. Indeed, we observed that the absence of miR-17-92 drove cells towards a more memory phenotype. Other current research show that it is possible to tweak the CD8+ T cells towards a more memory phenotype by blocking the mTOR pathway. Combining the expression of some microRNAs to boost effector functions, with the expression or repression of others to induce more memory might be an interesting approach to reach the goal of a long-term cure.

Another hurdle for the clinical application of adoptive cell transfer therapies is their high cost for each patient. Indeed, lymphocytes have to be isolated and cultured for each patient individually, and this sometimes for weeks. Although we did not directly address this aspect, the fact that the T cells overexpressing miR-155 proliferate more could mean that less T cells are needed to reach the same therapeutic goal. This would save costs, as those cells would necessitate less time *in vitro* before the transfer.

Finally, cancer is a very dynamic disease, which is able to adapt rapidly to changing conditions. Consequently, a strong immune attack against malignant cells often induces them to differentiate into a more immunosuppressive phenotype. For this reason, using therapies in combination seem to be the best next step for cancer treatments. Simultaneously increasing the inflammation and depriving the cancer of its immunosuppressive defences shows a lot of promise. It is, to my knowledge, the way that should be pursued in the future of cancer medicine.

Appendix

1. Supplemental methods:

1.1. PCR primers

CD4-CRE

FW – 5’ GTT CGC AAG AAC CTG ATG CAC A 3’

Rev – 5’ CTA GAG CCT GTT TTG CAC GTT C 3’

miR-17-92

FW - 5 ‘ TCG AGT ATC TGA CAA TGT GG 3’

Rev - 5’ TAG CCA GAA GTT CCA AAT TGG 3’

miR-155

FW : 5’ GTG CTG CAA ACC AGG AAG G 3’

Rev : 5’ CTG GTT GAA TCA TTG AAG ATG G 3’

Mut: 5’ CGG CAA ACG ACT GTC CTG GCC G 3’

1.2. PCR programs:

CD4			CRE
#1	95°C	5min	
#2	95°C	15sec	
#3	60°C	20sec	
#4	72°C	20sec	repeat from #2 34 times
#5	72°C	5min	

miR-17-92			
#1	94°C	5min	
#2	94°C	15sec	
#3	60°C	15sec	
#4	72°C	15sec	repeat from #2 38 times
#5	72°C	5min	

miR-155			
#1	94°C	5min	
#2	94°C	15sec	
#3	61°C	15sec	
#4	72°C	20sec	repeat from #2 34 times
#5	72°C	5min	

1.3. Media

Complete RPMI
500ml RPMI
50ml FBS (Gibco 10270106)
5ml Penicillin-streptomycin (Gibco 15140-114)
5ml HEPES (Animed 5-31F00-H)
500µl 2β-mercaptoethanol (Sigma M-7522)

Medium for primary human T cells (human RPMI):
500ml RPMI (Gibco 61870-010)
5ml Penicillin-streptomycin (Gibco 15140-114)
5ml L-Glutamine (Gibco 25030-024)
5ml Non essential amino acids (Gibco 11140-035)
5ml HEPES (Animed 5-31F00-H)
5ml Na Pyruvate (Gibco 11360-039)
500µl 2β-mercaptoethanol (Sigma M-7522)
40 ml Human Serum (isolated in house)

Medium for primary mouse T cells (mouse RPMI)
500ml RPMI (Gibco 61870-010)
50ml FBS (Dutscher S1810-500)
5ml Penicillin-streptomycin (Gibco 15140-114)
5ml L-Glutamine (Gibco 25030-024)
5ml Non essential amino acids (Gibco 11140-035)
5ml HEPES (Animed 5-31F00-H)
5ml Na Pyruvate (Gibco 11360-039)
500µl 2β-mercaptoethanol (Sigma M-7522)

1.4. LCMV peptide mix

KAVYNFATM	GP33
FQPQNGQFI	NP396
SGVENPGGYCL	GP276
SLLNNQFGTM	NP166
CSANNSHHYI	GP92
ISHNFCNL	GP118
YTVKYPNL	NP205
NISGYNFSL	NP235
GVYQFKSV	GP70

1.5. Sequences of miR-155 and SCR from CAR construct

Mir-155 (pre-miRNA in capitals + 200bp flanking sequences on each side)

aggatttaatgagctccttcctttcaacagaaaatggactattttcctttcagatttactatatgctgtcactccagctttataaccgcat
gtgcatacacaaacatttctttctctcttgcaggtggcacaaaccaggaaggggaaatctgtggtttaaattctttatgcctcatcctc
tgagtgctgaaggcctgctgtaggctgtatgCTGTTAATGCTAATCGTGATAGGGGTTTTTGCCTCCAACTGACT
CCTACATATTAGCATTAACAGtgtatgatgcctgttactagcattcacatggaacaaattgctgccgtgggaggatgacaa
agaagcatgagtcaccctgctggataaacttagacttcaggctttatcatttttcaatctgttaatcataatctggtcactgggatgttc
aaccttaaactaagttttgaaagtaaggttatttaaaagatttatcagtagtatcctaaatgcaaacattttc

SCR miR-155 (pre-miRNA in capitals + 200bp flanking sequences on each side)

aggatttaatgagctccttcctttcaacagaaaatggactattttcctttcagatttactatatgctgtcactccagctttataaccgcat
gtgcatacacaaacatttctttctctcttgcaggtggcacaaaccaggaaggggaaatctgtggtttaaattctttatgcctcatcctc
tgagtgctgaaggcctgctgtaggctgtatgCTGAAGGTGACGGACGTTGCAATATCTTTTGCCTCCAACTGAG
ATATTGCAACGTCCGTCACCTTGtgtatgatgcctgttactagcattcacatggaacaaattgctgccgtgggaggatgac
aaagaagcatgagtcaccctgctggataaacttagacttcaggctttatcatttttcaatctgttaatcataatctggtcactgggatg
ttcaaccttaaactaagttttgaaagtaaggttatttaaaagatttatcagtagtatcctaaatgcaaacattttc

2. Supplemental Data:

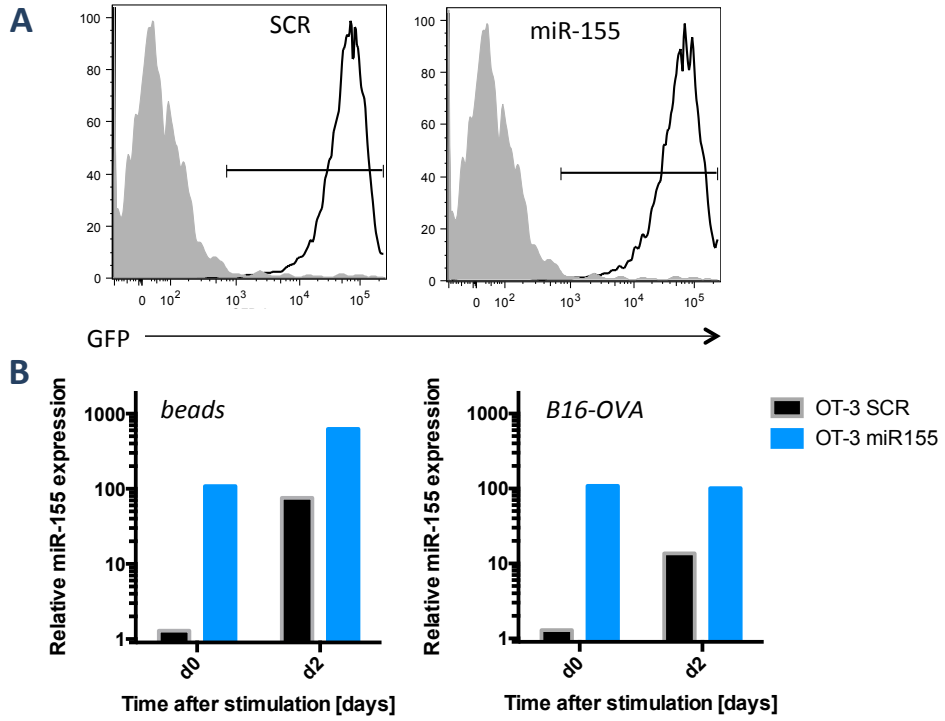

Figure 34: (A) OT-3 cells were transduced with either the pMGP-SCR (left panel) or pMGP-miR-155 (right panel) construct. (B) The expression of miR-155 was measured by qPCR in resting cells or 2 days after coculture with either beads (left panel) or B16-OVA cells (right panel).

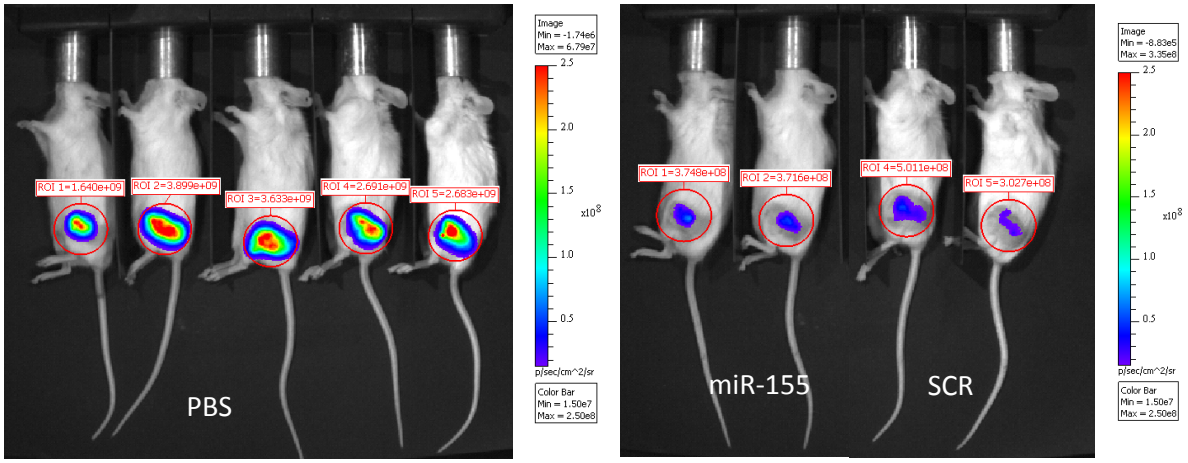

Figure 35: Living Image® analysis of pictures taken with the Xenogen IVIS Lumina II. On the left are untreated animals, on the right, treated animals with either CAR_SCR or CAR_miR-155.

3. Publication:

I was in charge of measuring the levels of miR-155 in mouse T cells via qPCR after *in vivo* infection and cell sorting, or after *in vitro* stimulation with N4 or T4 peptides. I also measured the levels of the SOCS1 mRNA via qPCR. Consequently, I was responsible for the figures 1B, C and D and the figure 6A.

Cell PRESS

Immunity
Article

MicroRNA-155 Is Required for Effector CD8$^+$ T Cell Responses to Virus Infection and Cancer

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SUMMARY

MicroRNAs (miRNAs) regulate the function of several immune cells, but their role in promoting CD8$^+$ T cell immunity remains unknown. Here we report that miRNA-155 is required for CD8$^+$ T cell responses to both virus and cancer. In the absence of miRNA-155, accumulation of effector CD8$^+$ T cells was severely reduced during acute and chronic viral infections and control of virus replication was impaired. Similarly, *Mir155*$^{-/-}$ CD8$^+$ T cells were ineffective at controlling tumor growth, whereas miRNA-155 overexpression enhanced the antitumor response. miRNA-155 deficiency resulted in accumulation of suppressor of cytokine signaling-1 (SOCS-1) causing defective cytokine signaling through STAT5. Consistently, enforced expression of SOCS-1 in CD8$^+$ T cells phenocopied the miRNA-155 deficiency, whereas SOCS-1 silencing augmented tumor destruction. These findings identify miRNA-155 and its target SOCS-1 as key regulators of effector CD8$^+$ T cells that can be modulated to potentiate immunotherapies for infectious diseases and cancer.

INTRODUCTION

CD8$^+$ T cells are essential effectors in immune responses to intracellular pathogens and cancer (Zhang and Bevan, 2011). Upon stimulation, antigen-specific CD8$^+$ T cells massively expand and differentiate into inflammatory cytokine producing, cytolytic T cells able to eliminate virally infected or transformed cells. As the antigen is cleared, the majority of specific CD8$^+$ effector T cells die (Marrack and Kappler, 2004), whereas only a small number of memory cells survive. The CD8$^+$ T cell response is influenced by a series of costimulatory (and inhibitory) ligands and by multiple soluble mediators such as interleukin-2 (IL-2) (Boyman and Sprent, 2012). The latter is essential for sustaining an efficient effector response, whereas other cytokines such as IL-7 and IL-15 play crucial roles for the survival of naive or memory T cells (Cui and Kaech, 2010). Several studies have identified key molecular factors involved in the differentiation from naive to effector CD8$^+$ T cells, but the contribution of microRNAs (miRNAs) has just begun to be investigated (Almanza et al., 2010).

miRNAs are a class of small, noncoding RNAs that impart posttranscriptional gene regulation (Bartel, 2004) through several mechanisms including translational repression and messenger RNA (mRNA) degradation (Djuranovic et al., 2011). They are important in many physiological processes, in carcinogenesis (Calin and Croce, 2006), and in the immune system (Xiao and Rajewsky, 2009). Early studies in mice deficient for Dicer, an RNase III enzyme important for mature miRNA production, revealed that miRNAs are involved in CD4$^+$ T cell differentiation and strongly influence CD8$^+$ T cell responses (Muljo et al., 2005; Zhang and Bevan, 2010). Specific miRNAs were shown to regulate both lymphocyte development and function. For instance, miRNA-181a influences thymocyte selection by modulating the expression of molecules involved in T cell receptor (TCR) signaling (Li et al., 2007). Moreover, the miRNA-17~92 cluster regulates B cell development (Ventura et al., 2008), autoimmunity, and T helper 1 (Th1) cell differentiation (Jiang et al., 2011; Xiao et al., 2008).

miRNA-155 is upregulated upon lymphocyte activation (Haasch et al., 2002) to control cell proliferation and differentiation (O'Connell et al., 2008; Turner and Vigorito, 2008). For instance, miRNA-155 regulates B cell proliferation, malignancy, and antibody production, at least in part through inhibition of activation-induced cytidine deaminase and PU.1 expression (Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2007). In CD4$^+$ T cells, miRNA-155 has been shown to suppress differentiation of naive cells into Th2 by downregulation of c-Maf, to promote Th17 cell-mediated inflammation (Kurowska-Stolarska

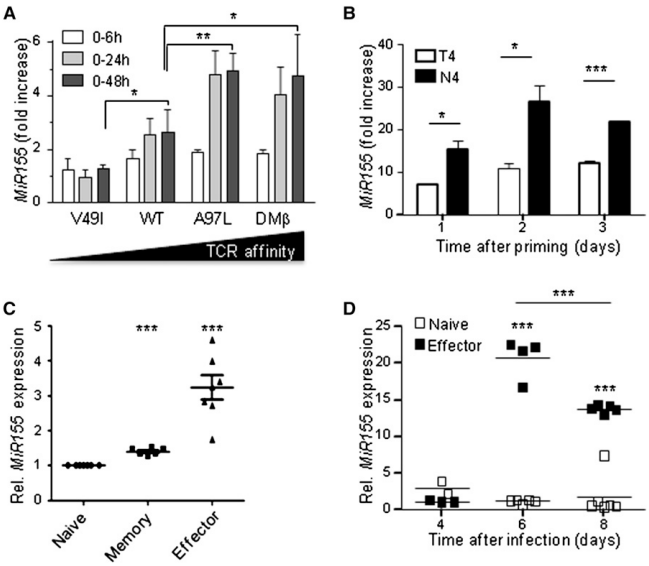


Figure 1. miRNA-155 Expression Is Regulated at Various Stages of CD8+ T Cell Differentiation in a TCR-Affinity-Dependent Manner

(A) Quantification of miRNA-155 in human CD8+ T cells transduced with TCRs of increasing affinity following TCR stimulation with multimers (n = 3 experiments) as fold increase relative to unstimulated clones.

(B) miRNA-155 expression of naive mouse OT-1 T cells stimulated with splenic dendritic cells pulsed with the natural SIINFEKL (N4) or weaker SIITFEKL (T4) altered peptide ligand as relative to day 0 unstimulated cells. Data are representative for triplicates in one out of two experiments.

(C) miRNA-155 concentrations in naive, central memory, and effector CD8+ T cells sorted at day 8 after LCMV WE infection as fold change relative to naive cells.

(D) Relative miRNA-155 expression in splenic naive and effector CD8+ T cells sorted from LCMV-infected mice. Symbols represent individual mice and the line is the mean ± SEM. Data are representative for two independent experiments. For human BIC expression, also see Figure S1.

et al., 2011; O'Connell et al., 2010) and to inhibit interferon-γR (IFN-γR) expression (Banerjee et al., 2010; Martinez-Nunez et al., 2011). In addition to direct modulation of cytokine receptor expression, miRNA-155 shapes cytokine signaling in several cell subsets via downregulation of SMAD2 (Louafi et al., 2010) and suppressor of cytokine signaling (SOCS-1) (Lu et al., 2009; O'Connell et al., 2010; Wang et al., 2010). Despite the evidence for an important role of miRNA-155 in a wide spectrum of immune compartments, it is not known whether this miRNA, which is highly expressed in antigen-experienced CD8+ T cells (Salaun et al., 2011), influences CD8+ T cells in vivo. In the present study, we have investigated the role of miRNA-155 during CD8+ T cell responses to viral infection, vaccination, and cancer.

RESULTS

CD8+ T Cells Dynamically Regulate miRNA-155 Depending on the Magnitude of TCR Stimulation or Their Differentiation State

The strength of TCR signaling has a major impact on the magnitude of CD8+ T cell expansion, but not on their differentiation (Zehn et al., 2009). We sought to investigate whether the strength of TCR stimulation affects the expression of miRNA-155 in CD8+ T cells by transducing human CD8+ T cells with variants of a NY-ESO-1-specific TCR of increasing affinity for its ligand (Derré et al., 2008; Schmid et al., 2010). Comparatively, a mutated low-affinity TCR failed to upregulate miRNA-155 within 48 hr, whereas TCR variants of higher affinities induced higher levels of miRNA-155 than the wild-type (WT) (Figure 1A). Thus, miRNA-155 expression increased in a TCR affinity-dependent manner in human CD8+ T cells. A similar upregulation was observed for the pri-miRNA-155 noncoding RNA transcript, BIC (see Figure S1 available online). To see whether miRNA-155 was also regulated in an affinity-dependent manner in mouse CD8+ T cells, we activated naive OT-1 T cells with splenic dendritic cells (DC) loaded with the WT peptide SIINFEKL (N4) or the weaker altered peptide ligand SIITFEKL (T4) (Daniels et al., 2006). To exclude miRNA-155 contamination from the DCs, we used *Mir155*$^{-/-}$ DCs, which retain normal antigen-presenting capabilities (O'Connell et al., 2010). Exposure of OT-1 cells to the WT natural peptide resulted in a strong upregulation of miRNA-155, whereas a weaker TCR stimulation by the T4 peptide was less effective (Figure 1B). To assess miRNA-155 regulation in vivo, we analyzed naive (CD62L^{+}CD44^{-}), effector (CD62L^{-}CD44^{+}) and central memory (CD62L^{+}CD44^{+}) CD8+ T cells following lymphocytic choriomeningitis virus (LCMV) infection (200 pfu of WE strain). Compared to their naive counterparts, miRNA-155 was strongly upregulated in effector cells and to a lower extent in central memory CD8+ T cells 8 days postinfection (Figure 1C). A more detailed kinetics of miRNA-155 regulation during LCMV infection revealed that numbers of effector cells peaked on day 6 but stayed low in naive cells (Figure 1D). These results demonstrate that miRNA-155 is induced in effector CD8+ T cells depending on the strength of stimulation and differentiation.

miRNA-155 Promotes the Accumulation of Anti-Viral Effector and Central Memory CD8+ T Cells

To determine the role of miRNA-155 in activated CD8+ T cells, we monitored the expansion of effector cells following acute LCMV WE strain infection in the presence or absence of miRNA-155. Percentage, number, and phenotype of naive *Mir155*$^{-/-}$ CD8+

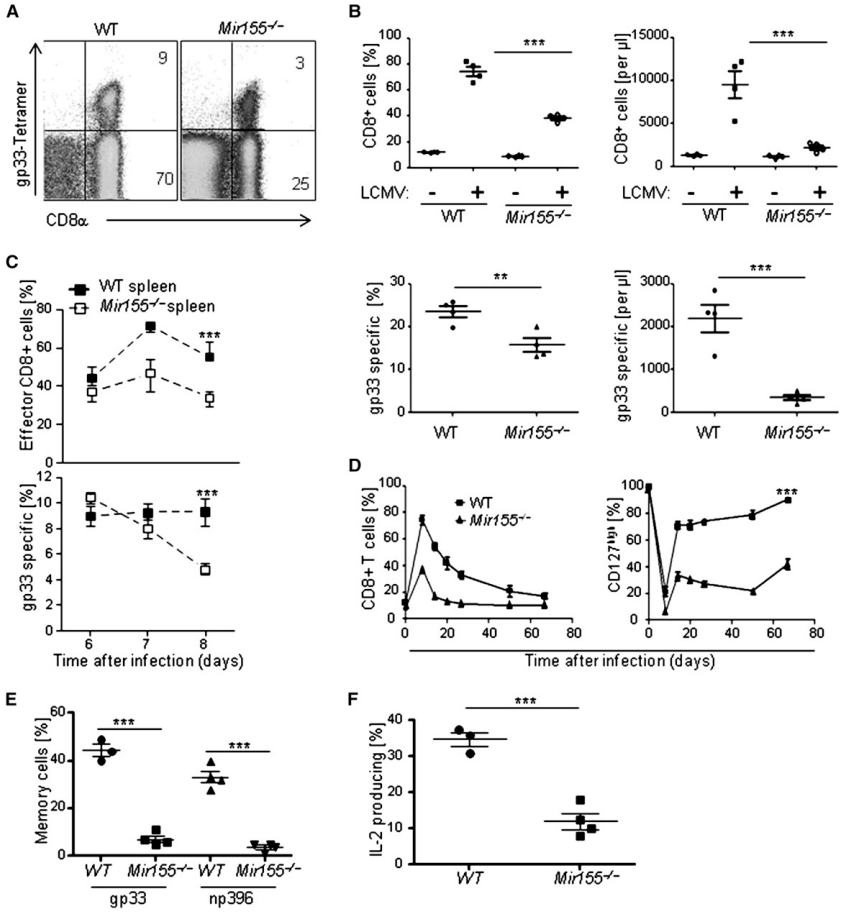


Figure 2. miRNA-155 Is Required for Optimal Effector CD8$^+$ T Cell Accumulation and Memory Cell Differentiation during Acute LCMV Infection

(A) Splenocytes from day 8 infected WT and *Mir155*$^{-/-}$ mice were stained with K^b/LCMV gp33 tetramers and anti-CD8α.

(B) Blood percentages and numbers of CD8$^+$ (upper panels) and gp33 tetramer$^+$ cells (lower panels) on day 8 of infection.

(C) Percentages of CD44^{hi}CD62L^{lo} effector CD8$^+$ T cells gated on lymphocytes in WT and *Mir155*$^{-/-}$ spleen cells (upper) and of gp33 tetramer$^+$ cells within the CD8$^+$ T cells (lower panel) at days 6 to 8.

(D) Percentages of total CD8$^+$ T cells (left) and CD127$^+$ cells within tetramer gp33$^+$ CD8$^+$ cells (right panel) in blood at given time points.

(E) Percentage of liver CD127^{hi}CD62L^{hi} tetramer gp33 and np396$^+$ memory cells and (F) IL-2 production upon gp33 peptide restimulation of splenocytes within IFN-γ-positive CD8$^+$ T cells at 3 months past infection. Symbols represent individual mice, and the line is the mean +/− SEM. Representative results of one out of four (A–C) to two (D–F) independent experiments with three to five mice are pictured. Also see Figure S2.

T cells in blood and spleen did not differ from those in WT mice before infection (Figure S2A; data not shown). In contrast, both percentage and number of total CD8$^+$ T cells, as well as virus gp33 tetramer specific CD8$^+$ effector T cells, were substantially reduced in spleen and blood of *Mir155*$^{-/-}$ mice at the peak of the response (Figures 2A and 2B). Following the expansion of CD44$^+$ effector cells in the blood and spleen from days 6 to 8, we observed impaired effector CD8$^+$ cell accumulation in spleen, liver, and blood of *Mir155*$^{-/-}$ mice (Figure 2C; data not shown). Despite a defect in the magnitude of effector T cell responses,

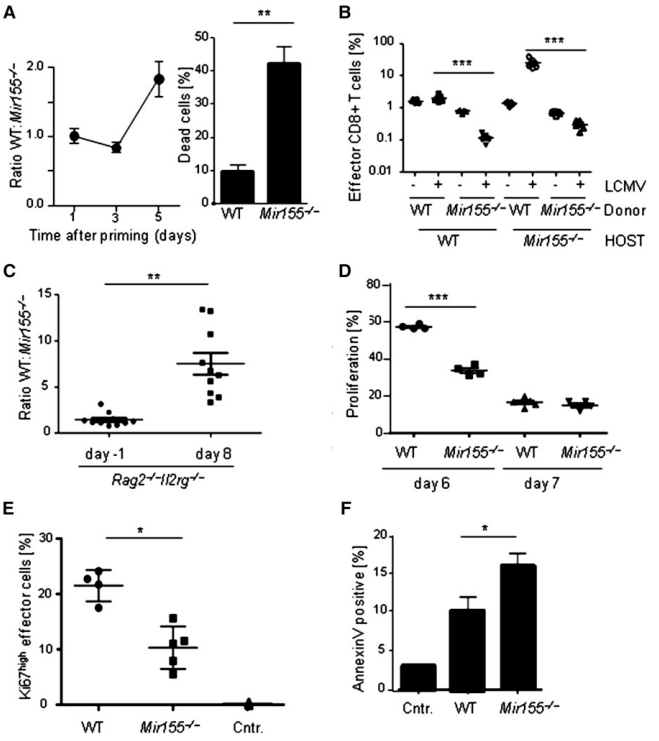


Figure 3. A Cell Intrinsic Role for miRNA-155 in Promoting Effector CD8+ T Cells

(A) Congenically marked WT and *Mir155*$^{-/-}$ OT-1 cells were competitively cocultured with peptide-pulsed dendritic cells, and the ratio of populations is pictured at indicated time points (left). On day 5, percentage of trypan blue cells harvested from either WT or *Mir155*$^{-/-}$ cultures was counted (right graph). Pooled data from three representative experiments are pictured.

(B) CD8+ T cells from WT and *Mir155*$^{-/-}$ mice were cotransferred into WT or deficient hosts before LCMV WE infection, and percentages in blood at day 8 were measured.

(C) A 1:1 mix of WT and *Mir155*$^{-/-}$ splenocytes was adoptively transferred into Rag2 and IL2Rγ double deficient mice, which were infected with LCMV WE 2 months after transfer. CD8+ effector T cell ratios at days −1 and 8 postinfection are pictured.

(D) Proliferating BrdU-positive splenic CD44^{hi} CD8+ effector T cells at days 6 and 7 postinfection.

(E and F) At the same time, (E) cells were stained for the proliferation marker Ki67 (day 7) and (F) apoptotic cells were identified by AnnexinV staining. Symbols represent individual mice, and the line is the mean ± SEM. Representative results from two (B and C) to three (D–F) experiments are pictured.

Mir155$^{-/-}$ animals were capable of controlling viral replication and clearing the virus (Figure S2B), as also confirmed by the lack of CD44 upregulation on adoptively transferred naive LCMV-specific P14 T cells (Figure S2C). In line with this result, CD8+ T cells differentiated into phenotypically and functionally cytolytic effector cells similar to WT cells during LCMV infection (Figures S2A, S2D, and S2E). Interestingly, circulating T cells in *Mir155*$^{-/-}$ mice exhibited not only a defect in the expansion at the peak of the immune response but also a more rapid contraction compared to WT animals (Figure 2D). Moreover, CD127+ CD62L+KLRG1− memory cells were strongly reduced in the gp33 and np396 tetramer+ CD8+ T cells in blood, liver, and spleen of *Mir155*$^{-/-}$ mice 3 months after infection (Figure 2E; Figure S2F; data not shown). Consistent with these findings, IL-2 production, a hallmark of central memory cells, was strongly diminished in *Mir155*$^{-/-}$ mice after stimulation with gp33 peptide (Figure 2F; Figure S2F). In this immune memory context, it is of interest that we observed a deficient CD4+ effector T cell activation on day 8 of the response in *Mir155*$^{-/-}$ mice (data not shown). Altogether, these results demonstrate that miRNA-155 is crucial for a robust T cell expansion, but not effector functions, as well as for a memory phenotype response upon an acute LCMV infection.

Intrinsic Expression of miRNA-155 in CD8+ T Cells Promotes Proliferation and Limits Apoptosis of Effector CD8+ T Cells

To investigate whether the defective expansion of CD8+ T cells was cell intrinsic, we cocultured naive WT and congenic *Mir155*$^{-/-}$ OT-1 CD8+ T cells together with peptide pulsed dendritic cells and analyzed the OT-1 cell ratio. After 5 days, WT CD8+ T cells outnumbered *Mir155*$^{-/-}$ cells and the abundance of dead cells was strongly increased among *Mir155*$^{-/-}$ T cells (Figure 3A). To assess these parameters in vivo, we cotransferred equal numbers of congenic polyclonal WT and *Mir155*$^{-/-}$ CD8+ T cells into either WT or *Mir155*$^{-/-}$ hosts, which were then infected with LCMV. Despite the initial low frequency of WT CD8+ T cells transferred in miRNA-155 ablated hosts (about 1% of CD8+ T cells in blood before infection), these cells expanded to about 30% of the CD8+ T cells at the peak of the response. In contrast, the frequency of *Mir155*$^{-/-}$ CD8+ T cells that transferred into WT hosts decreased upon infection (Figure 3B), clearly demonstrating a stronger response of WT compared to *Mir155*$^{-/-}$ CD8+ T cells. When Rag2 and γ common (γc) chain deficient hosts were engrafted with a 1:1 mix of WT and *Mir155*$^{-/-}$ splenocytes, both populations reached similar frequencies after 2 months, indicating comparable homeostatic expansion (Figure 3C). However, following LCMV infection, WT T cells again showed an advantage in expansion over their *Mir155*$^{-/-}$ counterparts. To determine the basis for the impaired accumulation of virus-specific CD8+ T cells in the absence of miRNA-155, we pulsed LCMV infected WT and *Mir155*$^{-/-}$ mice with bromodeoxyuridine (BrdU)

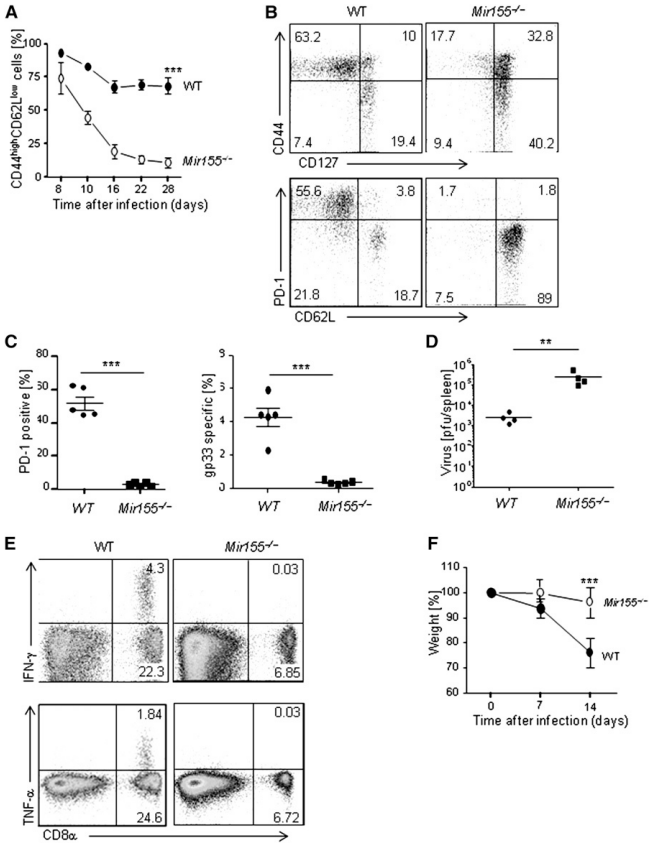


Figure 4. miRNA-155 Drives Survival of Effector Cells and Sustains the Anti-Viral Response in a Chronic LCMV Infection

WT and *Mir155*$^{-/-}$ mice were infected with LCMV clone 13 and phenotype and expansion of effector cells was monitored by flow cytometry.

(A) Effector CD44^{hi}CD62L^{lo} within blood CD8^{+} T cells at indicated time points.

(B and C) At 5 weeks, splenic CD8^{+} cells were analyzed for the indicated activation markers and gp33 tetramer^{+} cells shown as (B) flow cytometry dot blots from representative mice and (C) graph from one representative out of three experiments.

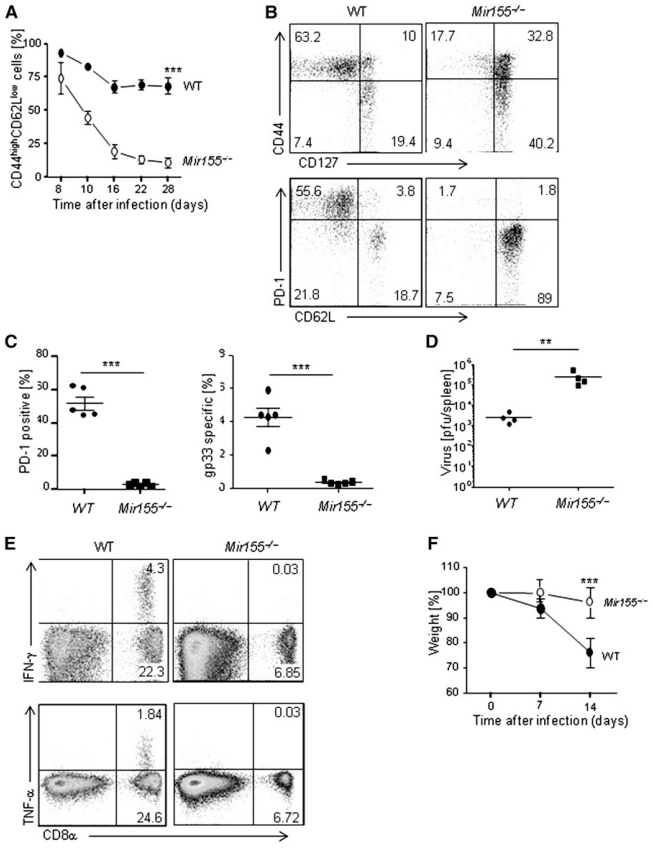
(D) Virus titer and (E) cytokine response upon stimulation with a peptide cocktail was determined in the blood at 2 and 3 months after infection, respectively.

(F) Weight of mice was monitored the first 2 weeks postinfection. Symbols represent individual mice, and the line is the mean (C and D). Error bars are given as ± SEM. Shown are representative results from one out of two experiments with three to five mice per group.

and measured proliferation and apoptosis 4 hr later. We found that the proliferation of *Mir155*$^{-/-}$ CD44^{+} effector CD8^{+} T cells was decreased compared to WT cells 6 days after infection (Figure 3D). Additionally, the frequency of proliferating Ki67^{+} cells within the CD44^{+}CD62L^{-} effector CD8^{+} T cells was reduced in *Mir155*$^{-/-}$ mice (Figure 3E). Finally, we observed an increased frequency of AnnexinV^{+} apoptotic cells in *Mir155*$^{-/-}$ compared to WT effector CD8^{+} T cells 7 days after infection (Figure 3F). Altogether, these data demonstrate a cell-intrinsic role of miRNA-155 in the proliferation and survival of effector CD8^{+} T cells in response to LCMV infection, but not for homeostatic expansion in lymphopenic hosts.

miRNA-155 Is Crucial for Effector CD8^{+} T Cell Accumulation and Virus Control in Chronic LCMV Infection

On the basis of the strong impairment of effector CD8^{+} T cell accumulation in low dose LCMV infection, we asked how *Mir155*$^{-/-}$ mice would respond to high dose and long-lasting antigen exposure, which characterizes chronic infections and cancer. Mice were inoculated with 2 × 10^{6} pfu of LCMV clone 13, causing a chronic infection for several weeks (Moskophidis et al., 1993; Salvato et al., 1991). Whereas WT mice mounted a robust effector CD8^{+} T cell response with high percentages of CD44^{+}CD62L^{-} effector cells that were maintained over time, *Mir155*$^{-/-}$ mice progressively lost effector CD8^{+} T cells (Figure 4A). Interestingly, the remaining CD44^{+} cells in spleen showed high CD127 and CD62L expression, reminiscent of a memory phenotype (Figure 4B). Percentages and numbers of gp33 tetramer-positive cells were also strongly decreased in deficient mice 5 weeks and 3 months postinfection (Figures 4B and 4C; data not shown). At this time, we could not detect cells capable of producing effector cytokines in response to a cocktail of LCMV peptides in miRNA-155 ablated mice, confirming the loss of most virus-specific *Mir155*$^{-/-}$ CD8^{+} T cells and ruling out TCR downregulation that may appear as tetramer-negative T cells (Figure 4E; data not shown). Whereas about 50% of WT cells remained positive for PD-1, associated with T cell exhaustion, PD-1 was barely detectable on *Mir155*$^{-/-}$ CD8^{+} T cells 5 weeks after infection (Figure 4C). Importantly, virus titers were elevated 5 weeks and 2 months postinfection in miRNA-155 ablated mice (Figure 4D; data not shown). Finally, WT but not miRNA-155 ablated mice showed symptoms of immunopathology such as shivering, hunching, and weight loss, suggesting a lower inflammatory response in the absence of miRNA-155 (Figure 4F; data not shown). These data demonstrate an important role of miRNA-155 in maintenance and survival of CD8^{+} effector T cells, as well as virus control in chronic virus infections.



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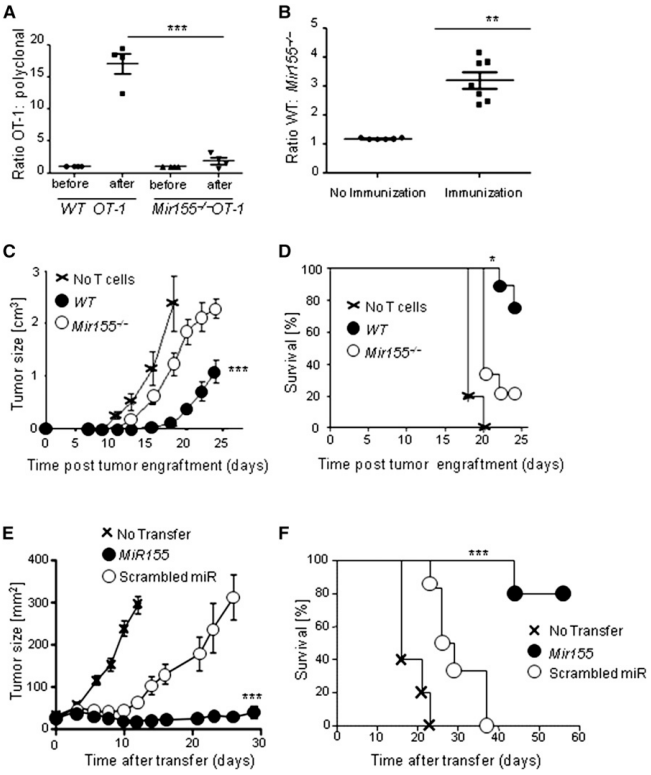

Figure 5. miRNA-155 Is Crucial for the CD8+ T Cell Response to Peptide Vaccination and Tumor Challenge

(A) Congenically marked OT-1 and polyclonal CD8+ T cells from WT or *Mir155*$^{-/-}$ backgrounds were transferred into WT hosts, which were immunized with OVA peptide and CpG in IFA. Shown is the ratio of antigen-specific versus polyclonal cells in the draining lymph nodes 4 days later. Symbols represent individual mice, and the line is the mean ± SEM.

(B) Ratio of blood WT and *Mir155*$^{-/-}$ OT-1 CD8+ T cells cotransferred into WT hosts, which were immunized as in (A) on day 7. Symbols represent individual mice, and the line is the mean ± SEM. Data from one out of two (A) to three (B) independent experiments are pictured.

(C and D) Upon adoptive transfer of WT or *Mir155*$^{-/-}$ OT-1 cells, mice were engrafted with B16 melanoma cells expressing OVA and immunized 1 week later. Tumor growth (C) and survival (D) are pictured (n = 5–9 mice per group, data from one out of two independent experiments) displayed as mean ± SEM.

(E and F) TCR transgenic pmel CD8+ T overexpressing miRNA-155 or control scrambled miRNA were transferred into B16 tumor-bearing mice and (E) tumor growth and (F) survival of mice are shown from one representative out of two experiments with five to eight mice per group displayed as mean ± SEM. Also see Figure S3.

miRNA-155 Expression in CD8+ T Cells Is Crucial for Efficient Immunization and Cancer Immunotherapy

Because we observed an important role of miRNA-155 to sustain CD8+ T cell responses to chronic infection, we looked at the impact of miRNA-155 on CD8+ T cell-dependent antitumor immunity, which requires robust CD8+ T cell responses. We examined the role of miRNA-155 in CD8+ T cells for vaccination, a clinically relevant setting characterized by limited adjuvant-induced inflammation. Polyclonal and OVA-specific OT-1 CD8+ T cells (either WT or *Mir155*$^{-/-}$) were cotransferred into WT mice before immunization with OVA peptide adjuvanted with IFA and CpG-ODNs. While the ratio of WT OT-1 to polyclonal cells strongly increased following immunization, there was only a minor increment in the ratio of *Mir155*$^{-/-}$ OT-1 to polyclonal WT cells (Figure 5A). We next cotransferred OT-1 cells from both WT and *Mir155*$^{-/-}$ backgrounds into WT mice before immunization. WT and *Mir155*$^{-/-}$ cells were found in similar proportions indicating a comparable survival after adoptive transfer. Following immunization, however, WT cells accumulated more efficiently than *Mir155*$^{-/-}$ cells (Figure 5B), whereas upregulation of CD44 and the proportion of cells producing IFN-γ were comparable (Figures S3A and S3B). Now we were interested to see whether miRNA-155 would also be critical for tumor control by CD8+ T cells and engrafted B16 melanoma upon transfer of naive WT or *Mir155*$^{-/-}$ OT-1 T cells and therapeutic vaccination 1 week later.

Compared to WT, *Mir155*$^{-/-}$ CD8+ T cells were less effective in inhibiting tumor growth and ensuring the survival of tumor-challenged mice (Figures 5C and 3D). Given the inability of T cells to expand and control tumor growth in the absence of miRNA-155, we hypothesized that enforcing miRNA-155 expression would augment the antitumor activity of CD8+ T cells. Pmel-1 TCR transgenic CD8+ T cells specific for the melanoma antigen gp100 were transduced with a retrovirus encoding miRNA-155 or scrambled miRNA and adoptively transferred into tumor-bearing mice in conjunction with gp100 vaccination and IL-2. There were no phenotypic differences between control and miRNA-155 overexpressing T cells prior to adoptive transfer (Figure S3C). However, overexpression of miRNA-155 greatly enhanced the antitumor responses compared to scrambled miRNA control (Figure 5E). Notably, 80% of mice receiving miRNA-155 transduced T cells survived for over 60 days, whereas all mice treated with control cells or left untreated had to be euthanized after less than 40 days due to tumor size (Figure 5F). Taken together, these results show that upon vaccination, *Mir155*$^{-/-}$ CD8+ T cells differentiated into effector cells but failed to accumulate in normal numbers. Consequently, the antitumor response was strongly dependent on miRNA-155 and could be therapeutically boosted by enforced miRNA-155 expression.

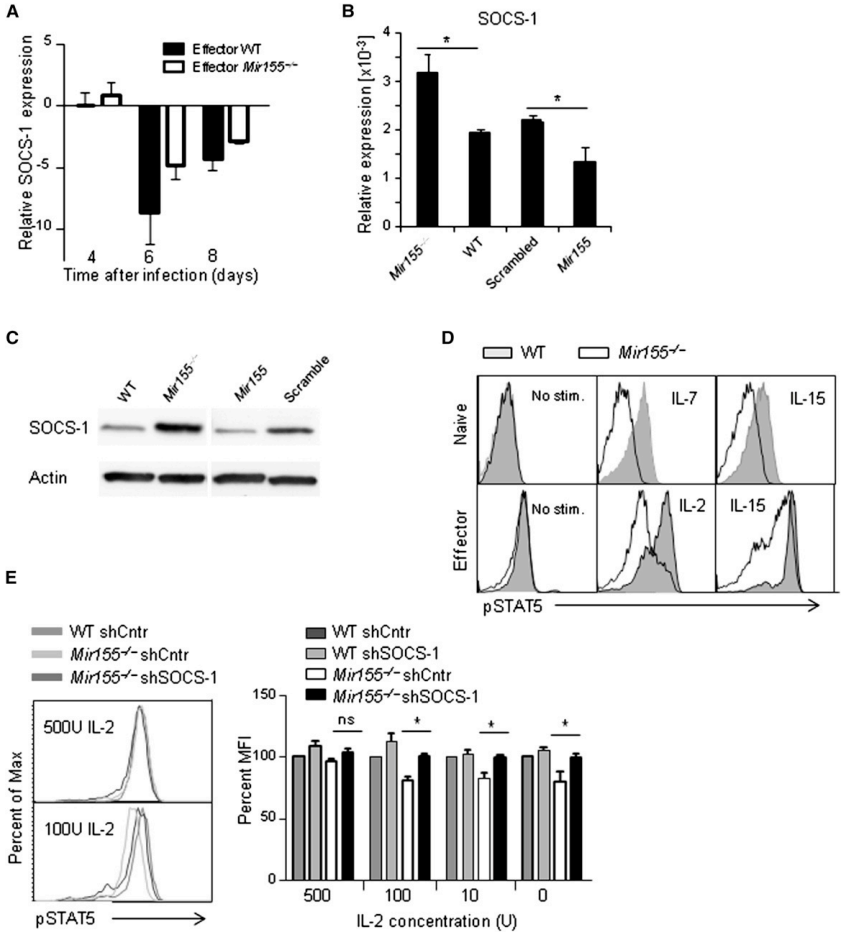

Figure 6. SOCS-1 and miRNA-155 Modulate the Antiviral CD8+ T Cell Response and Cytokine Signaling

(A) SOCS-1 mRNA concentrations were measured upon LCMV WE infection in purified effector ($CD44^{hi}CD62L^{lo}$) WT and *Mir155*$^{-/-}$ splenic $CD8^+$ T cells by qPCR relative to naive $CD8^+$ T cells from noninfected mice.

(B and C) Regulation of SOCS-1 by miRNA-155 in naive $CD8^+$ T cells from WT and miRNA-155 deleted mice, as well as after retroviral transfection with miRNA-155 overexpressing or control vectors, was tested by (B) qPCR (shown as relative to β-actin) and (C) immunoblot.

(D) Naive or effector T cells from LCMV-infected mice were stimulated with indicated cytokines, and pSTAT5 was measured by flow cytometry.

(E) WT and *Mir155*$^{-/-}$ T cells were transduced with control or shSOCS-1 lentivirus and pSTAT5 response to IL-2 is shown. The table gives the percentages of MFI normalized to the MFI measured in WT sh-control cells set to 100%. Representative data from two (A–C) to three (D and E) experiments are pictured as mean ± SEM. Also see Figure S4.

Targeting of SOCS-1 by miRNA-155 in Effector CD8+ T Cells Enables Cytokine Responsiveness and Accumulation

miRNA-155 has been shown to regulate γc chain cytokine signaling by targeting SOCS-1 expression (D'Souza and Lefrançois, 2003; Lu et al., 2009; Wang et al., 2010). We assessed SOCS-1 regulation in splenic effector $CD44^+CD62L^-CD8^+$ T cells during the response to acute LCMV infection of WT and *Mir155*$^{-/-}$ mice. We found that both WT and *Mir155*$^{-/-}$ $CD8^+$ T cells downregulated SOCS-1 on days 6 and 8 compared to $CD62L^+CD44^-$ naive $CD8^+$ T cells from noninfected mice (Figure 6A). To more directly test whether SOCS-1 was regulated

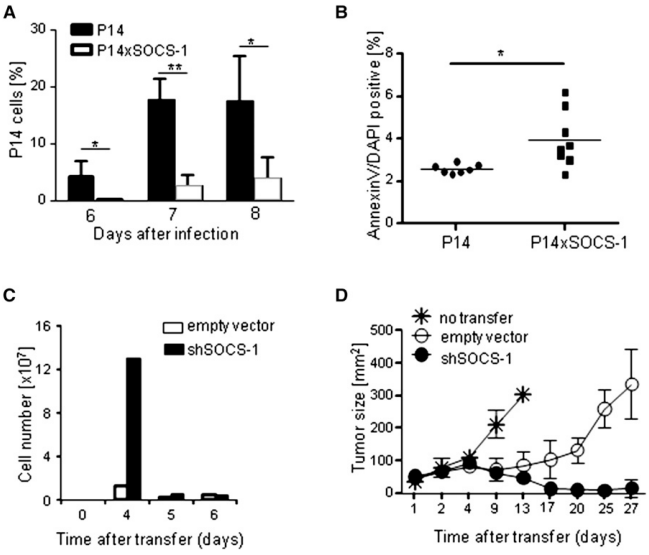

Figure 7. SOCS-1 Limits the CD8+ T Cell Response to Virus and Cancer

(A) TCR transgenic P14 CD8+ T cells overexpressing SOCS-1 (P14xSOCS-1) or not were adoptively transferred before LCMV WE infection. Data show the percentage of transferred cells in the lymphocyte gate at days 6, 7, and 8 post-infection as mean ± SEM.

(B) Apoptotic cells within P14 T cells 7 days after infection. Symbols represent single mice, and the line is the mean.

(C and D) TCR transgenic pmel CD8+ T cells were transduced with a retrovirus encoding for a scrambled control or shSOCS-1 mRNA and adoptively transferred into tumor-bearing mice. (C) Absolute numbers of donor CD8+ T cells were determined in spleen at days 4 to 6 after adoptive transfer and (D) tumor size of mice was monitored. Data are from one representative out of two independent experiments with two (C) to five mice (D) per group and displayed as mean ± SEM. Please also see Figure S5.

by miRNA-155, we measured SOCS-1 mRNA in WT and *Mir155*$^{-/-}$ CD8+ T cells, as well as in cells overexpressing miRNA-155 or scrambled control miRNA. We found that the amounts of SOCS-1 transcripts were inversely related to the cellular content of miRNA-155, with the highest concentration of SOCS-1 in *Mir155*$^{-/-}$ cells and the lowest in miRNA-155 transduced cells (Figure 6B). These results were further confirmed at the protein level, indicating that miRNA-155 is a critical regulator of SOCS-1 translation in CD8+ T cells (Figure 6C). To test whether the loss of miRNA-155 impaired γc chain cytokine signaling in CD8+ T cells by upregulating SOCS-1, we compared STAT5 phosphorylation in response to IL-2, IL-7, or IL-15 in WT and *Mir155*$^{-/-}$ cells. Stimulation of naive and effector CD8+ T cells isolated 8 days after LCMV infection resulted in a limited phosphorylation of STAT5 in miRNA-155 ablated cells, demonstrating an impaired cytokine signaling (Figure 6D). Diminished STAT5 phosphorylation was not due to differential expression of the cytokine receptor chains CD25, CD122, CD127, or CD132 (Figure S4). To further investigate whether the impaired cytokine signaling was dependent on the higher SOCS-1 concentration in *Mir155*$^{-/-}$ CD8+ T cells, we transduced WT and *Mir155*$^{-/-}$ CD8+ T cells with control or shSOCS-1 lentivirus. Although in vitro activation of T cells diminished the impact of miRNA-155 on cytokine signaling, we consistently detected a rescue of pSTAT5 generation in shSOCS-1 transfected *Mir155*$^{-/-}$ cells (Figure 6E). Interestingly, baseline pSTAT5 expression was already higher in WT than in *Mir155*$^{-/-}$ cells without additional IL-2 stimulation. The difference between WT and *Mir155*$^{-/-}$ cells was still apparent with intermediate but disappeared with high IL-2 concentrations, demonstrating that saturating amounts of IL-2 overcome the miRNA-155 and SOCS-1-dependent inhibition of cytokine signaling, as shown for regulatory T cells (Lu et al., 2009). Together, these results demonstrate a dynamic and differentiation-dependent regulation of SOCS-1 during the response to LCMV and suggest that *Mir155*$^{-/-}$ CD8+ T cells have impaired cytokine signaling due to increased SOCS-1.

SOCS-1 Restrains CD8+ T Cell Responses to Virus and Cancer

To test whether increased SOCS-1 expression recapitulated the impaired antigen-driven expansion of *Mir155*$^{-/-}$ CD8+ T cells, we adoptively transferred SOCS-1 transgenic or WT P14 CD8+ T cells into congenic mice prior to infection with LCMV WE strain. The expansion of SOCS-1 transgenic P14 T cells in blood and spleen was reduced compared to P14 WT cells (Figure 7A; data not shown). Whereas effector phenotype, granzyme B, and cytokine production were not impaired (Figures S5A–S5C), we detected enhanced apoptosis of SOCS-1-overexpressing cells (Figure 7B), thus phenocopying *Mir155*$^{-/-}$ CD8+ T cells (Figures 2 and 3; Figure S2). To test whether suppression of SOCS-1 could be therapeutically exploited to enhance the CD8+ T cell antitumor response, we adoptively transferred Pmel CD8+ T cells transduced with shSOCS-1 into tumor-bearing mice. SOCS-1 depletion by the construct was verified by immunoblot analysis (Figure S5D). An increased expansion of cells expressing shSOCS-1 was detected in the spleen on day 4 compared to control (Figure 7C), associated with profound tumor regression in mice that received shSOCS-1 transduced cells compared to untreated mice or mice treated with control cells (Figure 7D). Together, these results demonstrate that SOCS-1 is negatively regulating the effector CD8+ T cell response to virus and cancer and highlight the importance of SOCS-1 downregulation by miRNA-155 for efficient CD8+ T cell responses.

DISCUSSION

We have shown that miRNA-155 expression is essential for optimal CD8$^+$ T cell responses toward virus infection, vaccination, and cancer. Interestingly, the phenotype of *Mir155*$^{-/-}$ CD8$^+$ T cells was similar to Dicer-deficient CD8$^+$ T cells (Zhang and Bevan, 2010), suggesting that miRNA-155 is an important, likely nonredundant miRNA for CD8$^+$ effector T cells. Recently, we and others found differential miRNA-155 expression associated with discrete differentiation stages in CD8$^+$ T cells (Almanza et al., 2010; Salaun et al., 2011; Wu et al., 2007). Here, we showed that miRNA-155 is highly upregulated in effector CD8$^+$ T cells responding to viral infection but at intermediate concentration in memory cells. This dynamic modulation of miRNA-155 raises the question of the nature of the regulating factors that are involved. miRNA-155 is induced by NF-κB-dependent factors (Kluiver et al., 2007) and AP-1 downstream of B and T cell receptors (Haasch et al., 2002; Yin et al., 2008). Interestingly, we observed that miRNA-155 expression was proportional to the strength of TCR signaling, suggesting that miRNA-155 provides competitive fitness to the most avid antigen-specific CD8$^+$ T cells.

Whereas others reported a disadvantage of *Mir155*$^{-/-}$ naive CD8$^+$ T cells in bone-marrow chimeras (Lu et al., 2009), we found that expansion and long-term survival of naive *Mir155*$^{-/-}$ CD8$^+$ T cells transferred into lymphopenic hosts were not different from WT cells. miRNA-155 was crucial for effector CD8$^+$ T cell proliferation and survival during the peak of LCMV infection, but it did not influence cell survival upon antigen clearance. In contrast to the reported role of miRNA-155 in other immune cells, CD8$^+$ T cells were not affected in effector functions such as killing or cytokine production, and miRNA-155 deleted mice readily cleared low doses of LCMV. Interestingly, we detected an impaired generation of virus-specific central memory CD8$^+$ T cells because most *Mir155*$^{-/-}$ cells displayed a terminally differentiated phenotype. Whether this was due to a lack of CD4$^+$ T cell help (Janssen et al., 2003; Shedlock and Shen, 2003), lower cytokine signaling due to higher SOCS-1 expression, and/or additional miRNA-155 targets will be subject of further investigation. In contrast to low-dose LCMV infection, we found that virus-specific *Mir155*$^{-/-}$ effector cells disappeared during chronic LCMV infection by using high doses of clone 13. Under these conditions, WT CD8$^+$ T cells undergo progressive attrition and display an ''exhausted'' phenotype with impaired effector functions and high PD-1 expression (Jin et al., 2010; Mueller and Ahmed, 2009; Wherry et al., 2003). We hypothesize that miRNA-155 is key for the survival of effector CD8$^+$ T cells in conditions of long-term exposure to antigen and inflammation as found in chronic viral infections or cancer. Consistent with the impaired virus control, lack of miRNA-155 protected from LCMV-induced severe immunopathology, which is in line with reports demonstrating a pivotal role of miRNA-155 in autoimmune inflammation (Murugaiyan et al., 2011; O'Connell et al., 2010). With regard to T cell survival, miRNA-155 was shown to inhibit caspase 3 activity in Jurkat T cells (Ovcharenko et al., 2007) and FADD expression in macrophages (Tili et al., 2007). Whether the increased apoptosis of *Mir155*$^{-/-}$ CD8$^+$ T cells was due to such mechanisms remains to be determined.

In line with our results in virus infection, we demonstrate a central role for miRNA-155 in tumor-specific CD8$^+$ T cells. First, the efficient accumulation of CD8$^+$ T cells by vaccination with adjuvanted peptide required intrinsic miRNA-155 function, recapitulating our observations in LCMV infection. More importantly, *Mir155*$^{-/-}$ effector cells were severely impaired in their ability to curb tumor growth. Because these cells acquired full effector functions independently of miRNA-155, the different tumor control was likely due to defect in the magnitude of tumor-specific T cells or to higher susceptibility to the suppressive tumor-microenvironment (Klebanoff et al., 2011). Conversely, overexpression of miRNA-155 greatly increased tumor killing by WT CD8$^+$ T cells. This indicates that tumor-specific CD8$^+$ T cell activity is directly dependent on miRNA-155 expression.

The accumulation of effector CD8$^+$ T cells is influenced by the cytokine milieu; e.g., their initial expansion is promoted by γc cytokines, which signaling is regulated by SOCS-1 (Cornish et al., 2003). More specifically, CD8$^+$ effector cell accumulation in the LCMV response is reduced if cells lack γc signaling (Decaluwe et al., 2010). Interestingly, IL-2 was found to be critical for the maintenance of effector CD8$^+$ T cells in chronic LCMV infections (Bachmann et al., 2007). Here we show that miRNA-155 enhances cytokine signaling in CD8$^+$ T cells by targeting SOCS-1. Consequently, *Mir155*$^{-/-}$ naive and effector CD8$^+$ T cells failed to mount physiologic levels of pSTAT5 in response to γc cytokines. In line with in vitro experiments using CD4$^+$ T cells (Lu et al., 2009), we observed strong and transient in vivo downregulation of SOCS-1 mRNA in virus-specific CD8$^+$ T cells at day 6 of the response. Interestingly, this occurred in a partially miRNA-155 independent manner, whereas SOCS-1 protein concentrations of naive and effector CD8$^+$ T cells were found to be clearly dependent on miRNA-155. Moreover, we did not detect an impact of miRNA-155 on pSTAT5 in response to IL-2 in the early effector response in vivo as well as after in vitro priming (data not shown), whereas the effect was pronounced in naive and late effector cells. This suggests a miRNA-155 independent mechanism allowing full cytokine signaling early in the effector response, whereas miRNA-155 is modulating cytokine signaling in naive and late effector cells, promoting their accumulation and survival. Interestingly, at the peak of the response, effector CD8$^+$ T cells are reportedly dependent on IL-2 and IL-15 for sustained expansion (D'Souza and Lefrançois, 2003; Sanjabi et al., 2009). We were able to confirm a SOCS-1-dependent inhibition of cytokine signaling in *Mir155*$^{-/-}$ T cells by transduction with a shSOCS-1 lentivirus in in vitro experiments. Although the formal proof that loss of SOCS-1 in *Mir155*$^{-/-}$ CD8$^+$ T cells in vivo would restore their function remains to be shown, the observed rescue of STAT5 phosphorylation in *Mir155*$^{-/-}$ cells back to WT levels suggests that suppression of SOCS-1 in WT, but not *Mir155*$^{-/-}$ T cells, caused the differences in pSTAT5 signaling. This hypothesis was further supported by our observation that virus-specific, SOCS-1 transgenic CD8$^+$ T cells fully differentiated but failed to accumulate to normal numbers at the peak of the LCMV response, which mirrors the phenotype of *Mir155*$^{-/-}$ T cells and identifies SOCS-1 as an important regulator of CD8$^+$ T cell responses in vivo. Conversely, suppression of SOCS-1 in tumor-specific cells increased the accumulation of transferred cells and subsequently was highly therapeutic in limiting growth of established melanoma. Thus, although a

negative role for SOCS-1 in CD8$^+$ T cell responses has been suggested before (Chong et al., 2003; Cornish et al., 2003; Davey et al., 2005; Marine et al., 1999; Palmer and Restifo, 2009), we here demonstrate a cell-intrinsic role of SOCS-1 in responses to virus and cancer. Our results of the highly dynamic regulation of SOCS-1 expression in vivo and the strong impact of SOCS-1 alterations in responses to virus and tumor suggests that a major part of the effects caused in CD8$^+$ T cells by miRNA-155 deletion are due to increased SOCS-1 expression.

In summary, the results presented here identified a crucial cell-intrinsic role of miRNA-155 and its target SOCS-1 in effector CD8$^+$ T cells and demonstrated that this miRNA is required for an optimal CTL response to both virus and tumor. Moreover, miRNA-155 overexpression in tumor-specific CD8$^+$ T cells substantially increased their potency, thus providing strong evidence for a clinical potential in the context of therapeutic adoptive T cell transfer.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were from Harlan. *Mir155*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, TCR transgenic OT-I, and P14 mice bearing a transgenic TCR-specific for K^b/OVA$_{257–264}$ or LCMV D^b/GP$_{33–41}$ MHC-I/peptide complexes, respectively, were from Jackson and were bred under specific pathogen-free conditions. P14xSOCS-1 transgenic (Seki et al., 2007) and Pmel-1 transgenic mice expressing a TCR-specific for an H-2D^b-restricted CD8$^+$ T cells epitope from the murine melanoma tumor antigen gp100$_{25–33}$ or human gp100$_{25–33}$ have been described (Overwijk et al., 2003). All animal experiments were conducted with the approval of the Lemanic Animal Facility Network (RESAL), NCI (protocol SB-126) or NIAID (protocol LI-10) Animal Use and Care Committees.

Quantitative PCR

Total RNA from subsets was extracted with the miRNAVana kit (Ambion), and mature microRNAs (miRNA-155 and controls RNU44 and snoRNA202) were reverse transcribed with TaqMan RT MicroRNA Kit (Applied Biosystems) and amplified by using Universal Fast Start Rox Probe Master Mix (Roche) and microRNA assay kits in 384-well plates (Applied Biosystems) on an ABI Prism 7900 HT device (Applied Biosystems). RT-PCR for *Socs1* was performed with primers from Applied Biosystems. Gene expression was calculated relative to G6PDX.

In Vitro Priming with Dendritic Cells

T cells were purified from lymphoid tissues by using anti-CD8α beads (Miltenyi) and cocultured with peptide pulsed *Mir155*$^{-/-}$ DC. In competition experiments, equal numbers of WT and *Mir155*$^{-/-}$ OT-1 CD8$^+$ T cells were cocultured with WT DC.

Differential Stimulation of Human CD8$^+$ T Cells and Microarray

Human T cells transduced with NY-ESO-1 TCR variants (Schmid et al., 2010) were stimulated with 0.01 μg/ml (for miRNA-155 quantification) or 0.002 μg/ml (for BIC microarray) NY-ESO-1$_{157–165}$ multimer. BIC quantification was done by Miltenyi Biotec.

LCMV Infection

Mice were infected intravenously with 200 pfu of the WE strain or 2 × 10^6 pfu clone 13 of LCMV. Cell counts in blood were performed with TrueCount tubes according to the manufacturer's instructions (BD). Viral titrations were performed by a standard plaque assay.

Cytokine and Cytotoxicity Assays

Splenocytes were stimulated with indicated peptides or a mix of LCMV peptides (gp33, gp70, gp92, gp118, gp276, np166, np205, np235, np396) at 5 μM each or PMA (50 ng/mL) and Ionomycin (500 ng/mL) in the presence of GolgiStop (BD PharMingen) for analysis in flow cytometry. Cytolytic activity was assessed by using the chromium release assay with EL4 pulsed with 1 μM GP$_{33–41}$ peptide and measured with a TopCount reader (Canberra Packard).

Adoptive Cell Transfers

Before LCMV infection, CD8$^+$ T cells were purified by magnetic beads sorting (Miltenyi Biotec) and 1 × 10^6 purified CD8$^+$ T cells were injected into the tail vein. In the P14xSOCS-1 experiments, 3 × 10^4 cells were injected normalized to gp33 tetramer-positive populations. For vaccination and tumor challenge, OT-1cells were purified and 1 × 10^6 cells were injected intravenously 3 days before subdermal injection of 1 × 10^5 B16 melanoma cells expressing OVA. Seven days later, mice were vaccinated subcutaneously with 25 μg OVA$_{257–264}$ peptide and 50 μg CPG-ODN in PBS.

T Cell Transduction and Transfers into Tumor Bearing Mice

Pmel CD8$^+$ T cells were stimulated with anti-CD3 (2μg/ml) and anti-CD28 (1μg/ml) for 24 hr, transduced with retrovirus expressing miRNA-155 or scrambled miRNA, and expanded for 4 days. To knock down SOCS-1, we transduced pmel CD8$^+$ T cells 2 days after stimulation with lentivirus expressing shSOCS-1 or empty vector and selected in puromycin from days 4 to 7 prior to transfer into tumor-bearing hosts. We injected 10^7 cells (miRNA-155 experiments) or 10^6 (shSOCS-1 experiments) intravenously into mice bearing B16 tumor in conjunction with 2 × 10^7 pfu rvvhgp100 and IL-2 (6X6e4 cu). In shSOCS-1 experiments, mice were sublethally irradiated prior to cell transfer. Tumor volumes were plotted as the product of perpendicular diameters. For rescue of pSTAT5 in miRNA-155 cells, WT and *Mir155*$^{-/-}$ CD8$^+$ T cells were transduced with lentivirus encoding shSOCS-1 or control sh and cultured for 1 week with IL-2 or IL-15 (R&D systems, 20 ng/ml) before cytokine stimulation (vector sequences upon request).

Flow Cytometry and pSTAT5 Measurements

Cells were stained with antibodies of the indicated specificities (eBioscience) or H-2 D^b/peptide-loaded MHC. PD-1 PE-Cy7 antibody was from Biolegend. For detection of pSTAT5, cells were stimulated with indicated cytokines (R&D systems) for 18 min and fixed with 0.5% formaldehyde in PBS for 15 min. Upon washing in medium containing 10% FCS, cells were permeabilized in 80% methanol for 20 min, washed, and stained with pSTAT5 antibody (BD Biosciences) and additional markers (eBiosciences). Labeled cells were analyzed on LSR-II (Becton Dickinson).

Immunoblot

Cells were lysed (Cell Signaling Technologies) and immunoblotting was performed by using Bio-Rad TGX reagents and protocols on nitrocellulose paper, incubated with antibodies against SOCS-1 (Lifespan Biosciences [Figure 6C] or Imgenex [Figure S6]) and with appropriate HRP-conjugated secondary antibodies (Cell Signal Technologies). Blots were developed by using chemiluminescence (Pierce), gel images were captured with Gel Doc XRS (Bio-Rad), and densitometry was evaluated using Quantity One software (Bio-Rad).

Proliferation and Apoptosis Assay

Mice were injected intraperitoneally with 200 μg BrdU, and proliferation was measured in spleen cells 1 hr (P14xSOCS-1 experiments) or 4 h (miRNA-155 experiments) later by staining with anti-BrdU and anti-ki67$^+$ antibodies. Apoptotic cells were detected by staining splenocytes for AnnexinV upon 1 hr in vitro incubation at 37°C (all BD PharMingen).

Statistics

The two-tailed Student's t test was used to compare two groups; multiple groups' comparisons were performed with one-way ANOVA corrected with Bonferroni's multiple-comparison test factor. Statistical survival differences in the tumor experiments were analyzed with the log rank test. Statistical significance is displayed as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data are displayed as mean and SEM if not indicated otherwise.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.12.006.

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