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Immune responses of naive and memory CD8+ T cells

Imunitní odpovědi nezkušených a paměťových CD8+ T lymfocytů

Bachelor´s thesis

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Poďakovanie

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Abstract

Adaptive immune system plays a crucial role in effective pathogen clearance as well as establishment of immunological memory and its understanding is important for vaccine and drug development, besides cancer and autoimmune disease treatment. CD8+ T lymphocytes are able to efficiently kill infected cells and develop into antigen-specific memory cells, which are kept in a steady-state and demonstrate enhanced cytokine production and faster response upon reinfection, compared to naive T cells. Additionally, the pool of CD8+ memory T cells is more abundant, diversified and localizes to lymphoid as well as non-lymphoid tissues. On the other hand, proliferation rate, threshold of activation and CD28 costimulation independence are questionable. Even though the opposite was accepted for a long time, it seems that on a per cell basis, memory cells aren't superior to naive in these features and have decreased TCR sensitivity. Interestingly, in contrast to naive, memory CD8+ T cells can be activated independently of TCR, even in the absence of a cognate antigen, which emphasizes their increased sensitivity to inflammatory milieu and contribution to innate immune responses.

Keywords:

adaptive immunity, immune protection, immune response, CD8+ T cells, immunological memory

Abstrakt

Adaptívna zložka imunity hrá významnú úlohu v efektívnom odstránení patogénu, ako aj vo vzniku imunologickej pamäte, ktorej pochopenie je dôležité ako pre vývoj vakcín a liekov, tak pre výskum liečby rakoviny a autoimunitných ochorení. CD8+ cytotoxické T lymfocyty sú schopné účinne zneškodniť infikované bunky a dať vznik pamäťovým bunkám špecifickým pre daný antigen, ktoré sú pripravené na ďalšiu infekciu a vykazujú zvýšenú podukciu cytokínov a rýchlejšiu odpoveď, v porovnaní s neskúsenými bunkami. Navyše je populácia CD8+ pamäťových buniek väčšia, rôznorodejšia a je lokalizovaná ako do lymfoidných, tak do nelymfoidných tkanív. Na druhej strane, rýchlosť proliferácie, aktivačný prah a nezávislosť na CD28 kostimulácii sú otázne. Napriek tomu, že bol dlho prijímaný opak, ukazuje sa, že na úrovni bunky nie sú v týchto vlastnostiach pamäťové lymfocyty lepšie a majú zníženú citlivosť na T receptor bunky. Zaujímavé je, že na rozdiel od naivných, môžu byť pamäťové bunky aktivované nezávisle od T receptoru bunky aj v neprítomnosti antigénu, na ktorý sú špecifické. To zdôrazňuje ich zvýšenú citlivosť k zápalu a schopnosť prispieť k odpovedi sprostredkovanej vrodenou zložkou imunity.

Kľúčové slová:

adaptívna imunita, imunitná ochrana, imunitná odpoveď, CD8+ T lymfocyty, imunologická pamäť

Contents

Abbreviations

Ag	Antigen
ATAC seq	Assay for Transposase-Accessible Chromatin using sequencing
BA	Bystander activation
CCR5	C-C motif chemokine receptor 5
CDK	Cyclin dependent kinase
CTL	Cytotoxic T lymphocyte (CD8+ T cell)
CX3CR1	C-X3-C motif chemokine receptor 1
CXCR3	C-X-C motif chemokine receptor 3
GZM	Granzyme
H3	Histone 3
H3K4me3	trimethylation of 4^{th} lysine of histone 3
H3K27me3	trimethylation of 27^{th} lysine of histone 3
H3K9ac	acetylation of 9^{th} lysine of histone 3
ChIP seq	Chromatin Immunoprecipitation Sequencing
IFN I	Type one interferons
IFNγ	Interferon gamma
IL	Interleukin
IL-2Rα	Interleukin 2 receptor α
ITGA1	Integrin α1
LAT	Linker for activation of T cell
LCMV	Lymphocytic Choriomeningitis Virus
LM-OVA	*Listeria monocytogenes*-ovalbumin
MHC I	Class I Major Histocompatibility Complex
NK cell	Natural Killer cell
OT-I T cells	ovalbumin peptide SIINFEKL-specific T cells
PRF	Perforin
PTP	Protein Tyrosine Phoshatase
Rb	Retinoblastoma protein
SLO	Secondary Lymphoid Organs
T_{CM}	Central memory CD8+ T cells

TCR	T cell receptor
T_{EFF}	Effector CD8+ T cells
T_{EM}	Effector memory CD8+ T cells
T_{M}	Memory CD8+ T cells
T_{N}	Naive CD8+ T cells
TNFα	Tumor necrosis factor α
T_{PM}	Peripheral memory CD8+ T cells
T_{RM}	Tissue resident memory CD8+ T cells
T_{SCLM}	Stem cell-like memory CD8+ T cells
VV	Vaccinia Virus
ZAP70	ζ-chain-associated protein kinase

Introduction

Organisms are constantly exposed to various pathogens, such as bacteria, viruses or fungi. This leads to an evolutionary pressure on development of sophisticated resistance in the form of flexible immunity. Generally, mammalian immune system consists of two big branches acting side by side to fight infectious agents: innate and adaptive. Whereby innate immunity reacts immediately and non-specifically, the adaptive one is activated later in the course of infection and its response is antigen (Ag)-specific. In addition, once the pathogen is cleared, elements of adaptive immunity 'remember' the Ag and create a form of immunological memory, enabling them to respond more vigorously and rapidly upon repeated Ag exposure. Whereas humoral immunological memory is secured by memory B cells and circulating antibodies, cellular memory consists of both CD4+ and CD8+ memory T lymphocytes, capable of cytokine production and killing of the infected cell. Particularly CD8+ T cells are specialized to directly induce the death of cells infected by intracellular pathogen, thanks to recognition of non-self peptide presented on class I major histocompatibility complex molecule (MHC I).

This thesis aims to compare memory and naive CD8+ T cells (T_M and T_N, respectively) and their way of action in the context of acute infection. T_M cells rise from effector CD8+ T cells (T_{EFF}), which survived the contraction phase after pathogen clearance. From the earliest studies, they are considered superior to T_N in the overall response, exceeding in cytokine production, proliferation, speed of activation and reduced necessity of costimulation (reviewed in Dispirito and Shen, 2010b). Moreover, differences in migratory properties and size of T_M pool were observed (Blattman et al., 2002; Chtanova et al., 2009).

For a long time, these features were accepted without any demand of reexamination of T_M's superiority. Consequently, many authors cite papers published a long time ago, whose experimental setup was not that advanced and may have provided distorted results. However, several authors challenged these dogmas and show that the responses of T_M are not always supreme when measured on a per cell basis.

Additionally, T_M pool is more diversified than T_N pool. At first, two major populations of T_M cells with different properties were recognized: central memory (T_{CM}), which resemble more to T_N and effector memory (T_{EM}), which circulate in the blood (Sallusto et al., 1999). Later, stem-cell like memory (T_{SCLM}), tissue resident memory (T_{RM}), and peripheral memory (T_{PM}) cells were

identified (Gebhardt et al., 2009; Gerlach et al., 2016; Zhang et al., 2005). The revelation of this complexity contests the interpretation of acquired results.

Moreover, apart from pathogen induced activation, T_M were also observed to be non-specifically activated in an innate-like manner, in a phenomenon called bystander activation. For a long time, its contribution to immune response was neglected, but it gains more attention in recent years.

Thus, the difference between T_M and T_N response to pathogen attack in various contexts is very complex, many processes remain unclear and still offer space for further research.

1 TCR signalling of naive and memory CD8+ T cells

The activation of a CD8+ T cell depends on the interaction of its receptor (TCR) with a peptide bound to MHC I molecule, present on other cells. This contact leads to initiation of a signalling cascade, triggering acquisition of effector functions of a T cell.

For a long time, it was thought that T_M are more sensitive to Ag which results in their enhanced and more vigorous secondary response to cognate Ag and lower amount of Ag necessary for activation. However, this feature attributed to T_M may need to be reconsidered.

There are several elements of TCR signalling to reflect on. Interestingly, the signalling itself was found to be comparable in both T_N and T_M (Kersh et al., 2003). That led to a question, whether T_M are better in signal transduction when compared to T_N. Kersh et al. focused on the TCR in context of the membrane. After measuring the amount of GM1 glycosphingolipids, they claim that lipid rafts are more abundant and greater in size and solidity in T_M cells. These rafts also contained more phosphoproteins which could contribute to a more efficient signal transduction in Ag-experienced cells (Kersh et al., 2003). Additionally, Kersh et al. suggest that T_M don't have to undergo a process of desensitization to avoid autoimmunity since the TCR sensitivity itself is not affected due to the TCR independence of the proposed mechanism of transduction effectivity increase. However, the concept of lipid rafts is still not fully resolved and remains a controversial matter since low resolution limits the thorough study of the lateral order of the membrane (reviewed in Levental and Veatch, 2016).

A few years later, Tewari et al. came up with the study showing that T_M can be, in contrast to T_N, activated independently of LCK (a signalling molecule phosphorylating CD3ζ and ζ-chain-associated protein kinase (ZAP70) upon TCR engagement) and are still capable of producing

cytokines. The proposed explanation is the compensation of LCK functioning by another Src kinase, probably FYN (Tewari et al., 2006). Refering to Kersh et al., they hypothesize that the presence of phosphoproteins in lipid rafts may act as a tool to overcome LCK deficiency. Nevertheless, whereas the activation of T_N is measured by proliferation, the activation of T_M is detected by cytokine expression, in this study. Hence, they don't demonstrate diminished cytokine production in T_N compared to T_M in LCK deficient mice and more importantly, don't show enhanced proliferation of T_M. Also, more recent study suggets that T_M cells do need LCK activity, indeed, and additionally, shows differences of its activity in T_{EM} and T_{CM} (Moogk et al., 2016).

Moreover, Kersh et al. have previously shown that T_M are more effective in linker for activation of T cells (LAT) signalling, meaning that T_M cells may use altered TCR signalling pathway compared to T_N cells upon activation (Kersh et al., 2003). Nevertheless, later it was shown that LAT is expressed more in T_N (Cho et al., 2016) and its presence is indispensable for optimal response of both T_N and T_M cells (Ou-Yang et al., 2013).

What is more, the type of memory cell matters as well. It seems that T_{CM} are transducing the signal better than T_{EM} (Kersh et al., 2003). It's good to keep in mind that there are more types of memory cells that can react differently in different environment (reviewed in Martin and Badovinac, 2018).

In 2011, Kumar et al. linked higher TCR sensitivity of T_M to formation of TCR oligomers affirming that T_M build larger oligomers of TCRs which makes the signal transduction more efficient (Kumar et al., 2011). Again, referring to Kersh et al., they assume that larger lipid rafts formed in T_M's membrane allow the TCR oligomers to build.

Nevertheless, in 2014, an elegant paper focusing on several features of T_M cell activation-related events, came out (Mehlhop-Williams & Bevan, 2014). Performing low dose Ag stimulation in a non-inflammatory environment, they challenged previously declared closures and their results are bringing new insights into the TCR signal transduction and molecules involved. They claim that the expression of elements proximal to TCR is distinct in central memory CD8+ T cells, when compared to naive counterparts and that the signal they receive is duller. The proof of this blunted signal, i.e. decreased TCR sensitivity, is the reduced expression of TCR and cMYC (cell cycle positive regulator) seen in T_M, leading to decreased proliferation rates as well as the delay in cell cycle progression. In addition, they reveal a decreased capacity of T_M to activate ZAP70, one of the most important proximate TCR

signalling element, compared to T_N in response to low dose Ag stimulation, even though its overall expression was higher. Nevertheless, total expression and phosphorylation of Src family kinases was comparable in memory and naive CD8+ T cells (Cho et al., 2016; Mehlhop-Williams & Bevan, 2014).

Later, Cho et al. affirmed weakened phosphorylation of ZAP70 and added decreased ERK phosphorylation in T_M at early stages of infection and declare their decreased TCR sensitivity relative to T_N. Interestingly, this initial TCR desensitization was then backtracked by the presence of IL-2 (Cho et al., 2016).

1.1 Protein Tyrosine Phosphatases

Mehlhop-Williams and Bevan also observed an increased expression of several protein tyrosine phosphatases (PTPs) in T_M. PTPs are known to modulate TCR signalling, hence they speculated that it's the way of TCR desinsitization in T_M and eventual prevention of autoimmunity.

Subsequently, Cho et al. examined the PTPs' contribution to signal transduction regulation, in relation to TCR desensitization of memory cells, approving their increased expression in T_M (Cho et al., 2016). However, the only phosphatase proven to be effective in modulating TCR signalling was CD45, a phosphatase dephosphorylating activating Tyr^{394} or inhibitory Tyr^{505} on LCK, and cells expressing it to a lesser extent had higher Ag sensitivity. Hence, they propose a mechanism of self-reactivity prevention consisting in an increased expression of CD45 leading to LCK dephosphorylation and desensitization of TCR (to avoid self-reactivity), resulting in lowered activation threshold in T_M.

TCR sensitivity in Ag-experienced T cells is apparently influenced also by the appearance of a different antigen. It was found that just a presence of pro-inflammatory molecules was enough to modulate the TCR sensitivity not only in T_{EFF} CTLs but also in T_M, whereby it decreases to the initial level upon pathogen clearance (Richer et al., 2013). Authors of this paper also observed altered signalling in memory T cells exposed to pro-inflammatory molecules, which may be connected to bystander activation of T_M, a process discussed later in this thesis.

To sum up, although it's generally accepted that memory cells have higher TCR sensitivity and are able to transduce the TCR signal more efficiently, it may necessitate a reevaluation. Earlier studies affirm that the superior capacity can be a result of different cell membrane composition, i.e. the formation of lipid rafts and TCR oligomers strengthening the signal

transduction, or distinctions between T_M and T_N cells' signalling and utilization of different signalling molecules proximal to TCR.

Yet more recent studies contradict this dogma, claiming that TCR sensitivity in T_M cells is lower when compared to T_N, on a per cell basis, upon stimulation by low dose of Ag and in non-inflammatory conditions. Moreover, the avoidance of auto-reactivity in T_M cells is also not fully resolved. While in some earlier papers authors explain that it's the signal transduction capacity that is increased, not the TCR sensitivity itself, later studies suggest that PTPs, most probably CD45, play a role in decreasing the TCR sensitivity in T_M cells.

Moreover, different types of T_M cells with different properties have to be taken in consideration because what holds for T_{CM} doesn't necessarily have to be the case in T_{EM}, T_{PM} or T_{RM} since these cell types exhibit differences in several properties (Martin & Badovinac, 2018; Moogk et al., 2016).

Thus, the exact mechanism of TCR signalling nuances in T_N and T_M is still not completely clear and new scientific approaches could be beneficial and more precise in reexamination of previous results.

2 Activation threshold of naive and memory CD8+ T cells

In order to activate a CD8+ T cell and enhance its cytotoxic potential, an activation threshold must be reached, meaning that a certain amount of Ag has to be presented to the cell to detect the pathogen presence. Moreover, the TCR affinity is also important in modulating the threshold activating the T cell (Zehn et al., 2009).

T_M are traditionally considered to necessitate a smaller amount of Ag than do T_N for optimal activation (Curtsinger et al., 1998; Esser et al., 2003; Pihlgren et al., 1996). However, this is in contrast with a study where authors reported these two cell types to enter cell cycle simultaneously and their response to low doses of Ag is comparable (Zimmermann et al., 1999). Likewise, results from aforementioned paper by Mehlhop-Williams and Bevan confirm that T_N respond earlier compared to T_M, when exposed to reduced amount of Ag, in a non-inflammatory milieu. Both T_M and T_N received the signal, but only T_N were able to enter the cell cycle (Mehlhop-Williams & Bevan, 2014).

One of the differences in these studies is the use of exogenous cytokine interleukin 2 (IL-2) in earlier studies, which was later shown to be prerequisite for T_M expansion (Williams et al.,

2006). What's more, T_M apparently express IL-2 receptor (IL-2Rα) more rapidly than T_N (Pihlgren et al., 1996), which would explain why the activation threshold seems to be lower. In the absence of this costimulatory molecule, the responses were similar in both cell types (Cho et al., 2016; Curtsinger et al., 1998).

Higher activation threshold in T_M was reported by Carpenter and colleagues as well. They've performed experiments with *Mycobacterium tuberculosis* infection, observing T_N to require less Ag to start proliferating when compared to T_M, which affirms Mehlhop-Williams and Bevan's results in the model of bacterial infection (Carpenter et al., 2016).

However, when they compared activation of specific T_M and T_N with different TCR affinities, T_M cells with higher TCR affinity were able to outcompete T_N with lower TCR affinity (Carpenter et al., 2016). Therefore, they suggest that it's the affinity of T_M, which gives them the ability of a faster response and T_M are selected for clones with increased affinity to Ag as a compensation for low activation threshold. Nevertheless, that is questionable because even though it's apparently true that the final magnitude of expansion in part correlates with TCR affinity and cells with low affinity TCRs do not show altered cytokine production nor cytotoxicity (King et al., 2012; Zehn et al., 2009), T_N with low rather than high affinity primarily develop into memory precursors, suggesting the generation of T_M clones with lower affinity (Knudson et al., 2013; Solouki et al., 2020). What is more, in other experiments, where increased activation threshold of T_M was observed, anti-CD3 antibody and SIINFEKL-specific (OT-I) T cells were used (Cho et al., 2016; Mehlhop-Williams & Bevan, 2014), which excludes the possibility of different TCR affinity.

Hence, even though the minimal level of Ag needed for CD8+ T cell activation may have appeared to be clearly lower in memory CTLs, more recent studies show the opposite. Hypothetically, the activation threshold may be higher in T_M to prevent autoimmune reactivity, but extrinsic factors, such as inflammatory environment or presence of specific cytokines, decrease it. A series of experiments with minimized occurrence of extrinsic factors could bring new insights into the activation threshold differences on a per cell basis.

Moreover, TCR affinity apparently also contributes to activation threshold level and interestingly, low affinity T_N clones seem to preferentially develop into T_M.

3 Processes upon activation of naive and memory CD8+ T cells

3.1 Cytokine production

Memory CD8+ T cells are deemed to produce cytokines and other effector molecules more rapidly than Ag-inexperienced CTLs. This ability was confirmed in experiments with mouse models (Cho et al., 1999; Kersh et al., 2003; Pihlgren et al., 1996; Stock et al., 2006; Veiga-Fernandes et al., 2000; Zediak et al., 2011; Zimmermann et al., 1999), as well as human cells (Akondy et al., 2017; Araki et al., 2008; Fann et al., 2006). The majority of studies focuses on expression of cytokines interferon γ (IFNγ) and IL-2, along with cytotoxic molecules granzymes (GZM) and perforin (PRF).

Of note, it was shown that T_M are able to launch cytokine production almost immediately after infection (Barber et al., 2003; Liu & Whitton, 2005; Stock et al., 2006; Zediak et al., 2011). Interestingly, expression of tumor necrosis factor α (TNFα), another important inflammatory mediator, does not differ between these two cell types (Denton et al., 2011). Initial studies focusing on differences in naive, memory and effector cells' gene expression showed that the expression profile of T_M is intermediate between that of T_N and T_{EFF} and resembles more to that of T_{EFF}. This indicates that T_M retain some T_{EFF} properties and might persist in a steady-state with an enhanced ability to produce aforementioned molecules more rapidly when encountering cognate Ag (Holmes et al., 2005; Kaech et al., 2002; Peixoto et al., 2007).

3.1.1 Epigenetic regulations upon CD8+ T cell activation

Several types of epigenetic regulations and variable chromatin accessibility profile were observed to differ between T_N and T_M, which contributes to enhanced gene expression in T_M and their increased speed of pathogen clearance (Russ et al., 2014; Scharer et al., 2017; Scott-Browne et al., 2016).

In due course, we'll focus on differences of these post-transcriptional regulations in light of immune responses of T_N and T_M on a chromatin as well as DNA level.

3.1.1.1 Chromatin modifications

Changes on chromatin, mainly methylation and acetylation of histone 3 (H3), predominantly on its N-terminus, were shown to regulate expression of important, function-related genes (Denton et al., 2011; Juelich et al., 2009; Russ et al., 2014; Wang et al., 2008). Notably trimethylation of H3 on the 4th lysine (H3K4me3), acetylation of H3 on the 9th lysine (H3K9ac)

and trimethylation of 27th lysine (H3K27me3) (first two shown to act positively and the latter negatively (Wang et al., 2008)) are critical and differ between T_N and T_M cells. In correlation, distinct chromatin accessibility was observed too.

Starting with methylation, it is a general way of changing gene expression in all cells in the body by adding methyl groups to proteins or DNA. As already mentioned, H3K4me3 (permissive) and H3K27me3 (repressive), are important methylations determining distinct gene expression in T_N and T_M cells. Apparently, T_M demonstrate enrichment in permissive methylations and on the other hand, lower level of the repressive ones on H3 (Denton et al., 2011; Russ et al., 2014; Zediak et al., 2011). Of note, H3K27me3 seems to be more important because its loss induced transcription start even without H3K4me3 enrichment (Russ et al., 2014). What is more, effector T cells demonstrate decreased amount of nucleosomes near transcribed genes, notably *Gzmb*, and this state is maintained in T_M (Zediak et al., 2011). The fact that there are more permissive marks and fewer nucleosomes at these loci in memory cells could account for RNA Polymerase II recruitment and binding to DNA and an eventual increase of the inducibility of gene expression.

To be more concrete, *Ifng* promotor was observed to be enriched in permissive H3K4me3 and had reduced amount of H3K27me3 deposition in T_M. In contrast, T_N cells were H3K4me3^{low} and H3K27me3^{high} at the same locus (Denton et al., 2011; Russ et al., 2014; Zediak et al., 2011). In addition, higher amount of RNA Polymerase II was found at the *Ifng* transcription start site in T_M than in T_N (Zediak et al., 2011).

A similar pattern was noticed at the *Gzm* locus, even though it was not as evident as in the case of *Ifng* (Russ et al., 2014; Zediak et al., 2011). Other genes encoding effector molecules such as C-C motif chemokine ligand 3 and 5 (CCL3, CCL5), X-C motif chemokine ligand 1 (XCL1), together with Killer cell lectin-like receptor G1 (KLRG1), IL-2Rα and Blimp-1 were also found to have high H3K4me3 and low H3K27me3 deposition at their promotors in T_M and T_{EFF}, whereas it was the contrary in T_N (Russ et al., 2014).

Tnfa had similar H3K4me3^{high}H3K27me3^{low} pattern in all populations, in correlation with its rapid production after activation of both T_N and T_M cells (Denton et al., 2011; Russ et al., 2014).

Histone methylation variations were observed in human cells as well. Araki et al. found 434 genes that were expressed more in T_M than in T_N among which KLRG1, GZMA or PRF. This group also identified a category of 'poised' genes having increased amount of H3K4me3 whose

transcription is induced more rapidly in T_M upon activation and thus may account for fast transition from T_M to secondary effectors (Araki et al., 2009).

Similarly to methylation, acetylation influences gene expression as well. Its level was found to differ between naive, effector and memory CD8+ T cell populations. Experiments on both murine (Denton et al., 2011; DiSpirito & Shen, 2010a; Northrop et al., 2008) and human (Araki et al., 2009; Fann et al., 2006) cells prove that H3 is acetylated more in T_M than in T_N. Not only the genes that are expressed more in resting T_M than in resting T_N had higher extent of H3K9ac deposition, but also those that were expressed similarly to T_N in resting state, but more in activated state of T_M, were acetylated more in resting state of T_M (DiSpirito & Shen, 2010a; Fann et al., 2006). They also suggest that after activation, acetylation is acquired in effector cells and is then maintained in T_M, contributing to enhanced transcription of genes upon activation and steady-state maintenance (DiSpirito & Shen, 2010a; Gubser et al., 2013; Northrop et al., 2006). Of note, CD4+ T cells' presence is required for proper acetylation of CD8+ T cells' H3, since murine T_M cells, which didn't receive help from CD4+ T cells during their development, had decreased H3 acetylation at important loci compared to the helped ones. Consequently, their cytokine production was diminished (DiSpirito & Shen, 2010a; Northrop et al., 2008). Thus, the ability of T_M to substantially increase H3 acetylation and subsequent state of readiness for Ag encounter depends on CD4+ T cells' presence.

Additionally, the development of methods studying chromatin accessibility, such as Chromatin Immunoprecipitation Sequencing (ChIP seq) or Assay for Transposase-Accessible Chromatin using sequencing (ATAC seq) enabled the research of chromatin availability to transcription factors and nucleosome mapping and brought new insight into chromatin state of CTLs.

Obtained results reveal previously unidentified differences, indicating that T_M have distinct accessibility profile compared to T_N and it resembles more to T_{EFF} than T_N (Scharer et al., 2017; Scott-Browne et al., 2016). Notably, genes whose expression was downregulated in T_M maintained an open chromatin state after acute infection, including those encoding effector molecules such as GZMA, GZMB and IFNγ along with transcription factor Eomes and adhesion molecule integrin α1 (ITGA1) (Scharer et al., 2017).

3.1.1.2 DNA modifications

Apart from chromatin, methylation also occurs on the DNA itself, usually acting in a repressive manner (reviewed in Moore, Le and Fan, 2013). Several studies showed differences between T_N and T_M cells on this level as well.

After observing T_M to contain 10 to 20-fold more IFNγ mRNA and to start its production several times faster upon activation, compared to T_N, Kersh et al. looked at its methylation at promotor sites. The *Ifng* promotor in both T_N and T_M was methylated and it even had a similar number of methylations per strand (2.7 in T_M and 2.8 in T_N). However, the percentage of DNA which remained unmethylated differed: in T_N, it was only 2%, whereas in T_M it was 23%. For reference only, the methylation of *Ifng* locus in T_{EFF} was almost none (Kersh et al., 2006). Therefore, T_M display decreased DNA methylation compared to naive, but it's methylated to a bigger extent when compared to T_{EFF}. In addition, the demethylation present in T_M seems to be independent of cell cycle progression and DNA synthesis (Kersh et al., 2006).

Similar situation, although to a lesser extent, occurred at the locus encoding IL-2: it was methylated in T_N, almost unmethylated in T_{EFF} and only a little in T_M (Kersh et al., 2006). Later, other authors claimed the same, adding that the demethylation occurring during the T_N to T_{EFF} transition, persists in T_M cells and *Gzmb* and *Prf1* loci have reduced methylation as well (Northrop et al., 2006; Youngblood et al., 2017).

Interestingly, CD4+ T cells' support is needed for demethylation of *Il-2* promoter in T_M, since they were incapable of sustained *Il-2* demethylation in CD4 depleted mice. In contrast, this was not the case of *Ifng* promoter, which wasn't influenced by CD4 deficiency (Northrop et al., 2006).

In brief, T_M seem to display superior capacity to produce effector and cytotoxic molecules, mainly IFNγ, IL-2, GZMB and PRF upon activation, when compared to T_N. Histone and DNA methylation; histone acetylation and chromatin accessibility alterations of CD8+ T cells' genes represent an important way to modulate gene expression throughout their life. In contrast to T_N, T_M cells were shown to be kept in a steady-state, ready to start transcription immediately after Ag encounter due to an elevated amount of permissive marks acquired during T_N to T_{EFF} transition in resting state and more accessible chromatin state. Interestingly, these processes seem to be dependent on CD4+ T cell help, which only emphasizes that the immune system is fully functional only when operating as a whole.

3.2 Proliferation

When it comes to proliferation capacity of these two cell types, it's still controversial whether T_M are able to expand more rapidly and more vigorously than T_N. Enhanced proliferation capacity of T_M cells was observed in several experiments (Cho et al., 1999; Grayson et al., 2002; Veiga-Fernandes et al., 2000). Veiga-Fernandes and Cho and colleagues both used Ag-specific transgenic TCR (Tg TCR) $RAG2^{-/-}$ mice, to generate naive and memory cells. These cells were then transferred to immunized hosts and proliferation and differentiation of T_N and T_M was compared (Cho et al., 1999; Veiga-Fernandes et al., 2000). Here, T_M were shown to progress through the cell cycle more rapidly. These experiments are being problematic, however, because they don't represent naturally occurring T cells and their activity nor the Ag and inflammatory environment. Nevertheless, Grayson and colleagues used Lymphocytic choriomeningitis virus (LCMV)-specific cells and observed faster proliferation of T_M compared to T_N as well as their reduced contraction (Grayson et al., 2002). This model is generally used to imitate natural infection.

On the contrary, some groups argue that memory CTLs do not demonstrate more pronounced proliferation rate than naive CTLs (Martin et al., 2012; Masopust et al., 2006; Mehlhop-Williams & Bevan, 2014; Stock et al., 2006; Zimmermann et al., 1999). Masopust et al. and Stock et al. both observed T_N and T_M to be equal in proliferation speed after LCMV epitope or vaccinia virus (VV) infection and herpes simplex virus infection, respectively (Masopust et al., 2006; Stock et al., 2006). Thus, one of the major factors causing contradictory results could be the infection model and inflammatory milieu in which the proliferation was measured. Alternatively, immunized mice may also fight infection by other types of immune memory, e.g. pre-existing memory B cells or serological antibodies.

Hence, in order to define responses of naive and memory CD8+ T cells on an intrinsic level, Martin and colleagues aimed to characterize their activity in one host to exclude any extrinsic factor possibly impacting the immune response (Martin et al., 2012). They observed that one T_N has higher proliferative and memory generation potential than one T_M on a per cell basis, regardless of Ag type and dose and localization of the infection or cells, in a non-inflammatory environment. However, during systemic inflammation, they report equal number of responding T_N and T_M. The rate of apoptosis was excluded as a potential cause of this phenomenon in this study. As an explanation, they propose that it’s the speed in which they acquire effector

phenotype, not the expansion magnitude, that makes T_M faster in pathogen clearance. This could theoretically be the case, considering the previously mentioned steady-state in which T_M are maintained.

Succeeding studies comparing proliferation capacity of T_N versus T_M CD8+ cells observed diminished proliferation capacity of T_M compared to T_N as well. Mehlhop-Williams and Bevan reported decreased proliferation rate of T_M cells when Ag load was limited and in the absence of adjuvants, i.g. without inflammation, which confirmed previous experiments by Zimmerman et al. (Mehlhop-Williams & Bevan, 2014; Zimmermann et al., 1999). Similarily, experiments with *Mycobacterium tuberculosis* affirm that CD8+ T cells were able to expand more efficiently during primary infection than during the secondary and secondary effector CD8+ T cells were outcompeted in number by day 15 by primary effectors (Carpenter et al., 2016).

The magnitude of proliferation essenetially depends on the speed of progression from G0/G1 phase to S phase of the cell cycle. Both naive and memory cells are in the state of quiescence, which is terminated by antigenic TCR stimulation. Generally, the progression into the cell cycle depends on the formation of cyclin and cyclin dependent kinase (CDK) complexes in G0, which relocate from the cytoplasm to the nucleus, in order to phosphorylate retinoblastoma protein (Rb). Subsequently, in G1 state, Rb is phosporylated by cyclin E-CDK2 complex, dissociates from transcription factor E2F and the genes necessary for progression to S phase are transcribed (reviewed in Satyanarayana and Kaldis 2009). In G0/G1 phase of cell cycle, cyclins D2 and D3 and CDK4 together with CDK6 are the most abundant (Latner et al., 2004; Satyanarayana & Kaldis, 2009).

Surprisingly, T_M cells were shown to be in a different cell cycle arrest than T_N (Latner et al., 2004; Veiga-Fernandes & Rocha, 2004). In addition, they express significantly higher levels of cyclin D2 and D3 as well as CDKs which are bound together in pre-formed complexes (Allam et al., 2009; Latner et al., 2004; Veiga-Fernandes & Rocha, 2004). Interestingly, these complexes persist in the cytoplasm and don't migrate to the nucleus to phosphorylate Rb and to eventually launch cell division (Veiga-Fernandes & Rocha, 2004). Together with higher cyclin D-CDK concentrations in quiescent state, T_M were observed to upregulate the expression of cyclins and CDKs encoding genes upon Ag encounter and to proceed into the cell cycle more rapidly (Allam et al., 2009; Latner et al., 2004).

Intriguingly, Allam and colleagues showed that quiescent T_M were able to change their cell cycle state and revert into that characteristic of T_N when cultured *in vitro* in media. These reverted

cells remained in naive-like state after adoptive transfer into naive murine hosts. However, cultures that contained DCs or stimulation with anti-CD27 antibody prevented the reverse of T_M into T_N. Moreover, according to their observances, TNFR superfamily proteins contribute to memory-like cell cycle state and T_M hyper-responsiveness to Ag exposure (Allam et al., 2009). Later, Eberlein et al. observed the rebound of aged T_M to naive-like state, but with slightly different phenotype, similar to T_{SCLM} *in vivo*, confirming changes of T_M's phenotype and properties in time (Eberlein et al., 2016). Thus, not only intrinsic mechanisms but also extrinsic factors keep T_M in a state of readiness.

However, T_M express higher levels of $p27^{Kip}$, which is a cell cycle regulator binding to cyclin D-CDK complex (Mehlhop-Williams & Bevan, 2014; Veiga-Fernandes & Rocha, 2004). Conflicting interpretations of its amount in T_M were presented.

Veiga-Fernandez and Rocha consider $p27^{Kip}$ to stabilize cyclin D-CDK6 and inhibit cyclin E-CDK2. They report it to bind preferentially to cyclin E-CDK2 complex in T_N, in contrast to T_M, where it binds favorably to cyclin D-CDK6. Moreover, the observed ratios of $p27^{Kip}$ and CDK6 present in T_M were thought to be exclusive to dividing cells (reviewed in Sherr and Roberts 1999). Thus, they propose a type of cell cycle arrest specific for T_M which keeps them ready for faster response to secondary Ag encounter, in correlation with already mentioned gene accessibility and expression similar to T_{EFF}.

In contrast, Mehlhop-Williams deem $p27^{Kip}$ an inhibitor of cyclin D-CDK4/6 complex. To affirm their hypothesis, Mehlhop-Williams et al. performed experiments, which indicate inhibitory activity of $p27^{Kip}$ in T cells (Mehlhop-Williams & Bevan, 2014). Firstly, they looked at Rb phosphorylation and observed increased amount of phosphorylated Rb only in T_N in response to low Ag dose in a non-inflammatory milieu. In addition, increase in phosphorylated Rb correlated with $p27^{Kip}$ downeregulation in this experiment. Secondly, they measured the expression of another cell cycle regulator, which is engaged in $p27^{Kip}$ degradation, cMYC. Interestingly, its expression was observed in both cell types after administration of a high load of peptide, but was detected primarily in T_N in response to limited Ag load. Therefore, the expression of cell cycle effectors seems to be expressed later or not at all in T_M cells in response to low dose of Ag in a non-inflammatory milieu, which indicates that T_M need stronger TCR stimulation in order to start the expansion.

In fact, $p27^{Kip}$ acts either positively or negatively on cell cycle progression, depending on CDKs to which it binds and whether it is phosphorylated or not. Its role in inhibiting cyclin E-CDK2

complex was confirmed, but the capacity to activate and assemble CDK4/6 with cyclin D remains in question (reviewed in Choi and Anders, 2014 and Tigan et al., 2016). However, studies of $p27^{Kip}$ activity in CD8+ T cells reported it to limit the proliferative capacity of T_M, which was renewed in $p27^{Kip}$ depleted mice, so it presumably does have inhibitory activity in CTLs, indeed (Jatzek et al., 2013).

Nevertheless, experiments using adoptive transfer of Tg TCR cells may be an issue when simulating naturally occurring proliferation of naive and memory CD8+ T cells. The estimated number of naive Ag-specific precursor ranges on average from 100 to 600 cells in lymphoid tissues per mouse, depending on the pathogen (Blattman et al., 2002; Obar et al., 2008). Even though the number can be slightly higher when considering cell loss during experimental procedure, presence of naive cells outside the lymphoid tissue and cross-reactivity, it would still be less than 10^5, which is the amount of naive Tg TCR cells usually transferred into recipient mice. In contrast, a memory pool is estimated to be 1,000-fold greater (Blattman et al., 2002). Importantly, high frequency of Tg TCR precursor cells was shown to influence their phenotype and function and limit their expansion abilities, along with memory generation potential upon Ag stimulation (Badovinac et al., 2007). Therefore, one has to take in consideration that experiments using adoptive transfer of Tg TCR cells may give misrepresenting results and don't mimic natural progression of the infection.

To this end, whether T_M promote enhanced proliferation capacity compared to T_N remains doubtful. Feasibly, T_M may not actually proliferate better on a per cell basis, but inflammatory milieu and individual infection history may influence their expansion capacity. Plus, they still seem to demonstrate superior cytokine production.

Analyses of cell cycle progression helped to better understand the state in which naive and memory CD8+ T cells are, but the research is still quite limited by general knowledge of cell cycle regulation. More recent papers report diminished proliferative response of T_M to low dose of Ag, without inflammation, challenging the idea of their decreased activation threshold, using more advanced methods and infection models.

Additionally, the experimental setup matters as well, owing to the assumption that cell amounts generally used in adoptive transfer may alter CTL's functioning. Thus, a well-defined experimental setting using viral and bacterial infections as well as precise methodology could bring new insights into expansion ability of naive and different types of memory CTLs.

4 Location and trafficking of naive and memory CD8+ T cells

An important thing to consider when comparing CD8+ T_N and T_M cell responses is their location and migratory properties. It is well established that T_N are not present in non-lymphoid tissue and recirculate from blood through lymph vessels to secondary lymphoid organs (SLO) via high endothelial vessels in SLO. In contrast, T_{EFF} and T_M are able to enter non-lymphoid tissues too. This notion was challenged by Cose and colleagues, who report the presence of significant populations of T cells phenotypically and functionally naive, in organs other than SLO (Cose et al., 2006). Their results were later negated since the perfusion of organs was shown to fail in removing blood-borne lymphocytes effectively because of their size and tissue resident T cells might have been mixed with those from the blood (reviewed in Masopust and Soerens, 2019). Hence, the presence of T_N outside the lymphoid tissues remains doubtful.

With regard to memory CD8+ T cells, they are generally divided into two main subsets: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}), which are found in SLO, similarly to T_N, and in periphery, respectively (Sallusto et al., 1999). These cells either express receptors for SLO homing and effectively differentiate into T_{EFF} upon stimulation (T_{CM}) or are rapidly producing effector molecules (T_{EM}) (Sallusto et al., 1999).

However, the T_{CM} and T_{EM} paradigm was recently challenged and the diversity of T_M was shown to be more complicated. Gerlach et al. identified another subset based on C-X3-C motif chemokine receptor 1 (CX3CR1) expression. They discriminate 3 T_M types: $CX3CR1^{HIGH}$, $CX3CR1^{LOW}$ and CX3CR1 intermediate ($CX3CR1^{INT}$) (Gerlach et al., 2016). Accordingly, $CX3CR1^{HIGH}$ represents T_{EM}, $CX3CR1^{LOW}$ represents T_{CM} and $CX3CR1^{INT}$ characterizes a new subset, peripheral memory CD8+ T cells (T_{PM}). Intriguingly, T_{PM} are mainly migratory, but can enter lymph nodes in a CD69L-independent manner. Moreover, they can differentiate into $CX3CR1^{LOW}$ subset, but not *vice versa*. Importantly, T_{PM} and not T_{EM} are the dominant tissue surveying type since non-lymphoid tissues were almost completely devoid of $CX3CR1^{HI}$ cells.

Additionally, another important subset of T_M CD8+ T cells was identified: tissue resident memory T cells (T_{RM}) (Gebhardt et al., 2009). These cells are occupying mainly pathogen contact sites but also SLO and are sessile, but able to expand and perform cytolysis similarly to T_{CM} or T_{EM} cells and contribute to secondary immune response (Behr et al., 2020; Ge et al., 2019; Gebhardt et al., 2009; Masopust et al., 2001; Schenkel, Fraser, & Masopust, 2014). They patrol the tissue and can be activated immediately at the site of pathogen entry. Therefore, compared

to T_N, T_M pool comprises different subsets occupying different locations, which increases their chances to encounter cognate Ag and initiate the response more quickly (Fig.1).

As to differences in migratory capacity, it links up with location and number of T_N and T_M. It has been known for a long time that upon Ag encounter in SLO, T_N are activated, become T_{EFF} and gain access to the site of infection, navigated by chemokines (reviewed in Samji & Khanna, 2017). On the other hand, T_M are patrolling both lymphoid and non-lymphoid tissues, so they‘re positioned closer to the site of infection and their migratory pathway is shorter.

Moreover, it was observed that only T_M can recruit from the blood and spleen to inflamed tissue independently of TCR stimulation at the initial state of infection, leading to local increase of T_M pool in size (Chtanova et al., 2009; Kedl & Mescher, 1998; Masopust et al., 2001; Nolz & Harty, 2014; Schenkel et al., 2013; Wakim et al., 2008). This is probably related to bystander activation, which will be discussed in detail in a subsequent chapter.

Intriguingly, T_M were observed to be faster in migration than T_N early after infection and the initial increase in speed was Ag-independent (Chtanova et al., 2009). Jeffrey et al. also observed that T_M are expressing 2-O glycans, which bind to P- and E-selectin. Their formation depends on Glucosaminyl (N-Acetyl) transferase 1 (GNTC1) whose locus was found to have open chromatin in T_M, but not in T_N, hence it's kept in steady-state in T_M (Nolz & Harty, 2014). Interestingly, the expression of GNTC1, followed by expression of 2-O glycans was triggered by IL-15. In contrast, this cytokine was unable to do the same in T_N, which needed TCR stimulation to achieve 2-O glycans expression (Nolz & Harty, 2014).

Notably, it was recently shown that T_{CM} express 2-O glycans to a higher extent compared to T_{EM}, indicating that T_{CM} and not T_{EM}, as initially thought, are the subset migrating to non-lymphoid tissues upon infection (Osborn et al., 2017). This observation contributed even more to questioning the aforementioned paradigm of T_{CM} and T_{EM} division of memory pool and may change the way of looking at memory cells‘ responses to pathogen invasion.

Therefore, location and migratory properties of CTLs surely play role in effectivity of responses of T_N and T_M. Whereas T_M are divided into multiple subsets occupying lymphoid and non-lymphoid tissues to screen the organism for pathogens, T_N are destined to blood-lymph-SLO circulation. Considering their greater Ag-specific precursor pool (Blattman et al., 2002), presence in non-lymphoid organs and non-specifically induced migration following pathogen entry, T_M are surely more potent to encounter the pathogen and initiate the immune response.

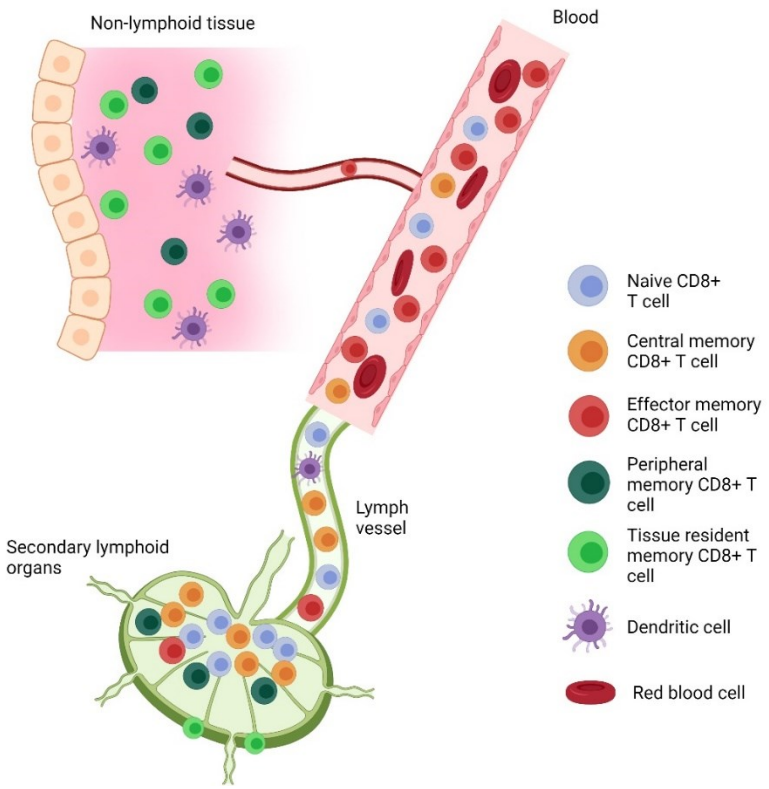

Fig.1 A scheme of distribution of T_N, T_{CM}, T_{EM}, T_{PM} and T_{RM} CD8+ cells in the absence of infection. While T_N circulate in blood-lymph vessel-SLO circuit, different subtypes of T_M are either residing in non-lymphoid tissues, surveying the non-lymphoid tissues, circulating in blood or following the same pathway as T_N (T_{RM}, T_{PM}, T_{EM} and T_{CM}, respectively). Created with BioRender.com.

5 Innate-like features of memory CD8+ T cells

5.1 Bystander activation of memory CD8+ T cells

Bystander activation (BA) is the process of T cell activation in an innate-like manner, without TCR engagement and in the absence of cognate Ag. This ability seems to be exclusive to memory CD8+ T cells since it was not observed in the naive ones (Chu et al., 2013; Soudja et al., 2012) and was found to be present during both murine (Chu et al., 2013; Goplen et al., 2016; Judge et al., 2002; Kambayashi et al., 2003; Kohlmeier et al., 2010; Liu et al., 2002; Maurice et al., 2019; Soudja et al., 2012) and human (Kim et al., 2018; Meresse et al., 2004; Odumade et al., 2012) infections.

This type of activation is dependent on pro-inflammatory cytokines such as IL-15, IL-18, IL-12 and type I IFNs, which often act synergistically (Chu et al., 2013; Freeman et al., 2012; Ge et al., 2019; Goplen et al., 2016; Kambayashi et al., 2003; Kim et al., 2018; Kohlmeier et al., 2010; Liu et al., 2002; Meresse et al., 2004; Soudja et al., 2012). All the latter are expressed after

inflammasome activation or IFNα/β receptor stimulation, hence their production is dependent on the activity of innate immune cells (Kambayashi et al., 2003; Soudja et al., 2012). The activation of a vast number of different T_M is achieved probably by enhanced expression of cytokine receptors on their surface (Ge et al., 2019; Judge et al., 2002; Kambayashi et al., 2003; Martin et al., 2017). Kambayashi and colleagues also hypothesized that the absence of receptors on T_N cells and on some T_M is the reason of their unresponsiveness to BA (Kambayashi et al., 2003).

After infection by certain pathogen, bystander activated preexisting T_M cells which are specific for other Ag start to proliferate and produce effector molecules IFNγ and GZMB, which enhances their cytolytic capacity and may even lead to degranulation (Fig.2 (B)) (Kambayashi et al., 2003; Liu et al., 2002; Maurice et al., 2019; Odumade et al., 2012; Soudja et al., 2012). Interestingly, BA of preexisting peripheral blood T_M didn't elicit their decrease in number after pathogen clearance in human cells (Odumade et al., 2012). In contrast, attrition of unspecific T_M during BA was reported earlier in murine cells (Kim & Welsh, 2004; McNally et al., 2001). Hence, we still have to be careful when drawing conclusions about human immune system functioning from the data obtained from murine immune responses. The question of decline of T_M haven't been fully resolved, however. Possibly, it's the way of removing previous T_M and regulating the overall size of T_M pool, in order to clear the space for new T_M generated during ongoing infection.

Later it was found that the activation of T_M cells can be achieved in an NKG2D-dependent manner too (Chu et al., 2013; Kim et al., 2018). NKG2D is an activating receptor predominantly found on natural killer (NK) cells, whose killing mechanism contributes to innate immunity. Upon infection, in response to aforementioned pro-inflammatory cytokines, T_M start to express NKG2D, or in human cells also Nkp30 (another NK cell receptor) (Chu et al., 2013; Kim et al., 2018). This signalling is followed by an enhanced NK cell-like cytolytic activity in T_M specific for unrelated Ag (Fig.2(A)) (Chu et al., 2013; Kim et al., 2018; Maurice et al., 2019).

Authors propose various explanations of this type of redundancy, where T_M exert in essence the same function as NK cells. Firstly, T_M don't express inhibitory receptors of NK cells, hence when infected or tumor cell expresses inhibitory ligands blocking NK cell activation, T_M can act as a substitute (Chu et al., 2013). Secondly, memory T cell pool is much larger than that of NK cell, so the pathogen elimination is faster and more effective (Soudja et al., 2012). Thirdly, BA can play a role in cancer elimination: since TCR is not required, the T cell exhaustion, which is

TCR mediated, is prevented. Moreover, the NKG2D signalling is cytolytic and IFNγ production stimulates macrophage activity (Maurice et al., 2019).

In addition, it was recently observed that bystander activated T_M are not present at the site of infection, but are recruited there from uninfected tissue via C-X-C motif chemokine receptor 3 (CXCR3)-dependent chemoattraction or upregulation of C-C motif chemokine receptor 5 (CCR5), which implies that BA is an active process capable to induce migration of T_M (Crosby et al., 2014; Maurice et al., 2019; Schenkel et al., 2013; Seo et al., 2021).

Interestingly, Goplen et al. found out that IL-12 activates TCR pathway to induce bystander activation of T_M and IFNγ production by these cells was decreased when self-MHC interaction was diminished. However, they claim to be unable to conclude direct relation between TCR and IL12-R signalling (Goplen et al., 2016). However, Chu et al. reported more efficient NKG2D-dependent killing when TCR signalling was engaged (Chu et al., 2013). Therefore, despite the actual TCR-independence of bystander activation of T_M, it seems that TCR signal may increase cytotoxic capacity of bystander activated T_M.

Even though the most probable purpose of BA is the support of innate immune mechanisms at early stages of infection, immunopathological outcome was reported along with it. In experiments with *Leishmania major* infection, LCMV-specific memory CD8+ T cells recruited to the site of infection, were activated in an NKG2D-dependent manner, but instead of limiting pathogen load, their activity resulted in immunopathology promotion (Crosby et al., 2014). Similarly, NKG2D-mediated cytolysis by intraepithelial CD8+ T cells induced by increased expression of IL-15 was immunopathological in patients suffering from celiac disease and vitiligo (Jacquemin et al., 2020; Meresse et al., 2004). Likewise, during hepatitis A virus infection, bystander activated T_M CD8+ T cells destroyed uninfected hepatocytes apart from the infected ones, which lead to severe damage of healthy liver tissue (Kim et al., 2018).

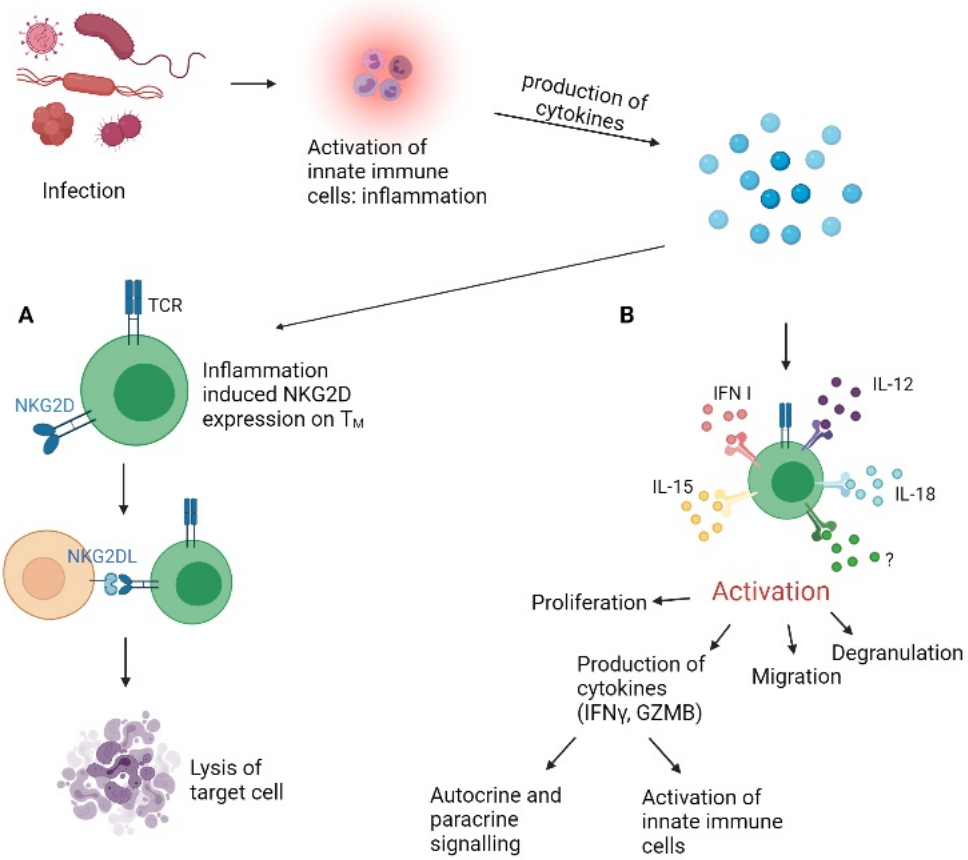

Fig.2 Two types of BA of CD8+ T_M cell. (A) Inflammation induced NKG2D expression resulting in T_M activation and acquisition of NK cell-like killing ability. **(B)** Activation of T_M by cytokines resulting in acquisition of effector functions and proliferation. Created with BioRender.com.

5.2 Sentinel function of T_{RM} cells

Apart from BA, another innate-like feature of T_M was observed. Aforementioned T_{RM} settled in non-lymphoid tissues were observed to 'sound the alarm' when confronted with antigen (Schenkel et al., 2013). T_{RM} were reported to produce chemokines and cytokines (such as IFNγ and IL-2) mediating recruitment to infected area and cytolytic activity of T_{EFF} cells, unspecific resting T_M cells and innate immune cells in response to presence of cognate Ag (Ariotti et al., 2014; Ge et al., 2019; Schenkel et al., 2013; Schenkel, Fraser, Beura, et al., 2014). Intriguingly, T_{RM} produced chemokines in higher concentrations in comparison to innate immune cells and the response triggered by T_{RM} activation was visible on the tissue level (Ariotti et al., 2014; Schenkel et al., 2013). Apparently, it's the IFNγ produced by T_{RM} what stimulates neighboring tissue to control the danger (Ariotti et al., 2014).

Therefore, not only T_M can be bystander activated in an innate-like manner, but they comprise a subset that can produce cytokines and activate the surrounding tissue itself, upon cognate Ag encounter.

To sum up, an important and exclusive feature of T_M cells is their ability to be activated by unrelated Ag in a TCR independent manner, only by presence of proinflammatory cytokines and in a NKG2D-dependent manner similar to NK cells. Moreover, a subset of memory cells residing in organs can alarm the neighboring tissue, so it initiates inflammation, in response to cognate Ag presence.

These features are generally attributed to innate part of the immune system, but apparently, adaptive branch also plays its role in making the early response more effective by means of T_M, which perform an innate-like activity at the beginning of infection. The evolutionary reason of this phenomenon, why it‘s attributed to memory T cells and how it is advantageous for the host remains in question. Possibly, large pool of T_M could increase the potential of an individual to respond to any infection in a very fast manner by alarming other cells or act directly on pathogen, gaining time for Ag-specific response. It would mean, that the more T_M the individual has, i.e., the more infections he has overcome, the more resistant he is to the subsequent ones. If this was the case, T_M wouldn’t only participate in adaptive immune memory, but also, to some extent, in trained immunity, which consists of reprogramming of innate immune cells and modifying their secondary response (reviewed in Netea et al., 2020).

Alternatively, strengthening of the innate immunity may be the way to compensate for downsizing the naive pool since, in contrast to T_M, T_N decrease in number with age (Thome et al., 2016). Additionally, as previously discussed, it could contribute to antitumor response in case of NK cell inhibition or T_{EFF} exhaustion.

Fortunately, bystander activation of CD8+ T cells is recently getting more attention in immunological research, so its true potential may be uncovered in the near future.

6 CD28/B7 costimulation in naive and memory CD8+ T cells

CD28/B7 costimulation is one of the most important signal 2 providing interaction and plays a crucial role in activation, proliferation and IL-2 production in response of CTLs to Ag (reviewed in Beyersdorf, Kerkau and Hünig, 2015). CD28 is a receptor found on T cells, which binds to its ligands B7.1 (CD80) or B7.2 (CD86) present on APCs. Since long time ago, these costimulatory

molecules weren't considered a necessity for memory CD8+ T cell activation, but are necessary for activation of their naive counterparts (reviewed in van der Heide and Homann, 2016).

First papers affirming CD28/B7 costimulation-independent signalling in T_M appeared at the turn of the 20th century (Bachmann et al., 1999; Kim et al., 1999; Suresh et al., 2001). One of these publications declares that T_N cells don't need higher amount of Ag to be activated but to achieve that on a maximal level, they need a costimulation through CD28. T_M, on the other hand, don't need it that much, thus there may be another costimulation existing, in order to activate them (Bachmann et al., 1999). These authors also claim that in T_N, the interactions of CD8 coreceptor with LCK are less frequent than in T_M and suggest it as an explanation, why T_M don't need CD28 mediated costimulation strictly: the signal is strengthened by the help of LCK.

Others consider a surface molecule 41BB to be compensating the role of CD28 in T_M (Bertram et al., 2004). However, there are experimental imperfections in this study, such as measuring the responses of memory cells 21 days post infection, which is a time insufficient for full establishment of memory.

Since then, this paradigm of CD28/B7 independence was commonly alleged as one of many features, thanks to which T_M prevail over T_N in immune response and the explanation of this independence was the existence of an unknown alternative mechanism.

Nonetheless, the situation may not be that clear-cut. There are publications that say the contrary and challenge this conclusion, arguing that in the majority of published papers, the experiments are performed *in vitro* which doesn't reflect the real environment.

After experiments on different viral models, it seems that CD8+ T_M cells do need CD28 costimulation to get properly activated as well and this signalling is apparently important for their proliferation and cell cycle progression (Arens et al., 2011; Borowski et al., 2007; Fuse et al., 2008). Borowski et al. also shows that these cells need CD28 mediated signalling to properly control the viral replication, mainly at the beginning of the infection. Interestingly, this costimulation doesn't influence the number of Ag-specific T_M (Arens et al., 2011; Borowski et al., 2007; Fuse et al., 2008). Thus, the absence of this signalling doesn't decrease nor increase the generation of CD8+ T cell memory. This is, in contrast with what Mittücker et al. says, because in their experiments, the frequency of cells specific to Ag was reduced in CD28 deficient mice (Mittrücker et al., 2001).

Hence, CD28/B7 costimulation appears to be necessary in T_M. In CD28 deficient mice or mice with impairment of this signalling, the ability to 'remember' the Ag was disturbed (Fuse et al., 2008; Grujic et al., 2010; Mittrücker et al., 2001). Fuse et al. also proved that the absence of this costimulation results in perturbed production of IL-2 by T_M.

Recently, one study used two models of inhibition of CD28 costimulation (tamoxifen inducible depletion of CD28 and anti-CD28 blocking antibody) to resolve its importance for T_M activation (Fröhlich et al., 2016). Their results suggest that CD28/B7 costimulation does influence the primary and secondary responses as well as it impairs the clonal expansion of both cell types. Interestingly, when CD28 was inhibited at the peak of primary infection (i.e. activated T_N), generated T_M didn't show detrimented recall response, which would mean that with time a certain compensation or adaptation to deficiency occurs. Moreover, naive cells deficient in CD28 have proliferation capacity dependent on this signalling, but the acquisition of effector functions was faster (in contrast to exogenous blockade of CD28 signalling). Similar situation was observed in T_M. This group also added experiments, where they looked at the generation and maintenance of immunological memory in CTLs in association with CD28. In this case, the impact of the deficiency was not observed, which is in correlation with previous studies.

What may be objectionable in this study, in context of comparison to other ones, is the infection strategy used. It was the classical *Listeria monocytogenes*-ovalbumin (LM-OVA) model, which is a bacterial infection. Other studies were done on different viral infections. Therefore, it would be convenient to perform such experiment using a viral model, preferably comparing LCMV with a less virulent virus, e.g. influenza.

There are several reasons possibly causing these contradictory results. Firstly, it may be the use of distinct viral strains. It seems that in the LCMV infection model (Bachmann et al., 1999; Kim et al., 1999; Suresh et al., 2001), the response is independent of CD28 mediated costimulation, but models using Influenza, *Listeria* or Vaccinia virus show the dependence (Arens et al., 2011; Borowski et al., 2007; Fuse et al., 2008; Grujic et al., 2010; Mittrücker et al., 2001). Secondly, it could be the way of inhibiting this signalling. In experiments with blocked receptor or ligand, the independence was observed, but it was the contrary in the results of groups using deficiencies in those. Other causes might be the Ag load differences or distinct results obtained in *in vitro* and *in vivo* experiments. One also has to keep in mind that the immune system is redundant and the absence of one molecule can be compensated by another, mainly in mice with inbred deficiencies.

Recently, a study focused on human CD4+ naive and memory T cells using genomic profiling and ATACseq to determine TCR and CD28 sensitivity of these cells, revealing increased sensitivity of CD4+ T_M to CD28 costimulation, compared to CD4+ T_N. The authors also observed the expression of several genes involved in cell cycle progression, proliferation and DNA replication to be TCR signal dependent in naive and CD28 signal dependent in memory CD4+ T cells (Glinos et al., 2020). Using a similar approach to study differences of T_N and T_M CD8+ cells in TCR and CD28 sensitivity upon infection could help to unveil the strength of CD28/B7 costimulation, if any, necessary for a proper response.

In summary, the independence of CD28 costimulation in memory CD8+ T cells was generally accepted for many years. However, this claim should be reconsidered because the majority of early studies, which determined this conclusion, used CD4+ T cells and experiments *in vitro*. What is more, different groups used different methods and models, which doesn't help in finding the final answer. The enigma of the need of interaction between CD28 and B7 in T_M cells is still not definitively resolved. The latest publications lean more towards the dependence over independence of the costimulation. Their observations can result from technical and methodological advances and overall progress that came with time. Therefore, the use of new approaches could be helpful in finally answering the question of CD28/B7 signalling requirement in T_M.

Conclusion

To conclude, T_M do seem to respond differently to Ag encounter, when compared to T_N. It appears that T_M have increased activation threshold and reduced proliferation capacity in non-inflammatory conditions, on a per cell basis. It was shown that T_M express CD45 more than T_N, which could be the way of TCR desensitization in order to prevent autoimmunity. Lower TCR affinty may also play role in modulation of activation threshold of T_M, but doesn't explain why they expanded less in experiments using anti-CD3 antibody or OT-I T cells (Cho et al., 2016; Mehlhop-Williams & Bevan, 2014).

On the other hand, T_M acquire effector functions more rapidly than T_N, which probably results from their epigenetic state, which consists of lower deposition of repressive epigenetic marks and inversely, higher deposition of the permissive ones at key effector genes. What's more, T_M, but not T_N, can be activated independently of TCR, in an innate-like manner. This means that T_M are more sensitive to innate signals, such as pro-inflammatory cytokines and NKG2D engagement, which might compensate for their lower TCR sensitivity.

Hypothetically, upon pathogen invasion, T_N predominantly respond to TCR stimulation, whereas T_M respond less sensitively to TCR, but are more sensitive to inflammatory signals induced by pathogen infiltration. The epigenetic state of T_M enables their faster immune response and acquisition of effector functions, compared to T_N (Fig. 3).

In contrast to T_N, T_M can be fully activated in a bystander manner in the presence of other than cognate Ag, which underlines the importance of inflammation in their activation and their sensitivity to pro-inflammatory molecules as well as their innate-like properties and contribution to pathogen control at early stages of the immune response.

Moreover, the size, location and diversity of memory CD8+ T cell population is important as well. Whereas hundreds of Ag-specific T_N circulate only between blood and SLO, thousands of Ag-experienced T_M occupy blood, SLO and peripheral tissues, which increases their chance to detect the pathogen.

A deep understanding of T_M activity is important for the development of vaccines and drugs, along with treatment of cancer and autoimmune diseases. The future research should focus on reevaluation of T_M properties using state-of-the-art methods and performing experiments in a well-defined setting. The undetected diversity, several metabolic properties, molecular

pathways and signalling mechanisms of T_M, in which they do or do not differ from T_N remain incompletely understood and open the door for the following research.

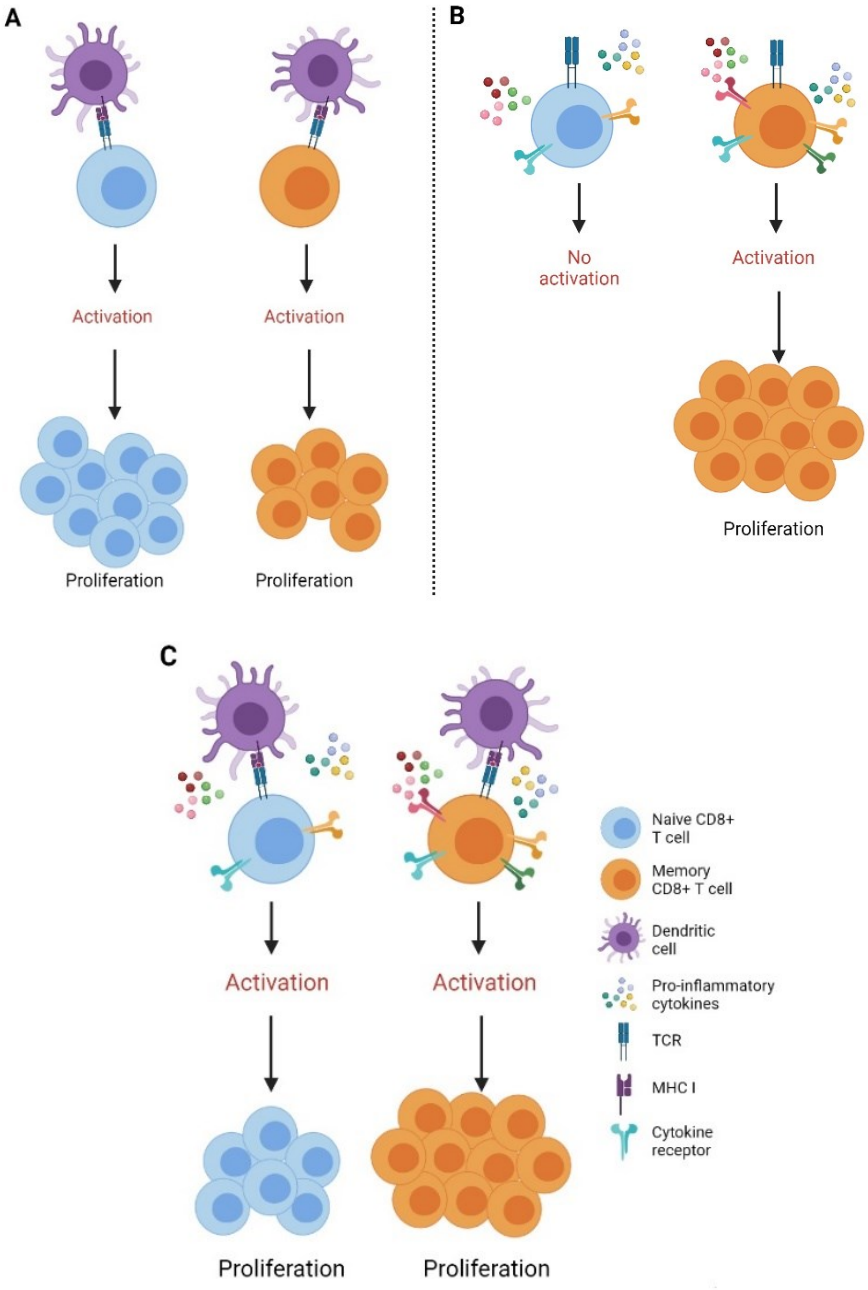

Fig. 3 Hypothesis of T_M and T_N activation accomplishment. It seems that T_N have lower TCR-mediated activation threshold and proliferate more than T_M on a per cell basis, in non-inflammatory conditions (A). However, T_M can be activated independently of TCR by innate signals only (B) and thus compensate their decreased TCR sensitivity by increased sensitivity to inflammation, which enables them to respond to Ag faster than T_N, when the infection occurs (C). Created with BioRender.com

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