universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„*In vitro* development of tissue resident memory T cells from circulating precursor lymphocytes“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie
Betreut von / Supervisor:	Univ.-Prof.Mag. Dr. Pavel Kovarik

The present master thesis was conducted at the Instituto de Medicina Molecular in Lisbon under direct supervision of **Dr. Marc Veldhoen**.

Die vorliegende Masterarbeit wurde am Instituto de Medicina Moleculat in Lissabon unter direkter Supervision von **Dr. Marc Veldhoen** durchgeführt

Acknowledgements

I would like to thank all people who directly and indirectly contributed to this work. Having a supportive and lovely environment to share many moments with, is one of the most important things. Inside and outside the lab.

Therefore, the first person to thank is my supervisor and PI Dr. Marc Veldhoen. First, for giving me the opportunity to undertake my thesis in his research group. But also, for his support and patience and Dutch sense of humour. Marc and his broad repertoire of multidisciplinary knowledge, ranging from Immunology to the Brexit-situation, was always highly appreciated.

Warm gratitude to Cristina Ferreira for all her gentle scientific inputs and for supporting my 2-weeks phase, substituting coffee with Mate tea and filling the lab with an unusual scent. Next, to the person who hated my mate-phase the most: Marta Sofia. Without the supportive work of Marta, the lab would not run so smoothly, and it wouldn't be such a lovely place to work. Thank you, Marta Sofia, for delighting your colleague's days, being a "cat-crazy-lady" and funnily clumsy. But most of all: thank you, for becoming my friend. While Marta clearly loved me from the first second on, it took a few more days to win Patricia Campos' heart. Nevertheless, she soon realised how much she likes me. Not as much as shopping and not as much as her "beautiful little Eimeria" but there is a place in her heart for me and, with no doubt, vice-versa.

Speaking of big hearts: I want to thank Leandro Barros, the best and most lovable bench-partner. Leandro is not only a bright PhD student, but has fantastic online stalking skills and is a living lexicon of modern pop culture and I can't wait until we meet each other again.

Furthermore, many thanks to Roos Haszewski and her Labrador spirit animal which makes her always being excited, supportive, friendly and made me laugh uncountable times. Ein großes DANKE to Birte Blankenhaus, the greatest mostly-vegan-no-sugar-eating-tiramisu-loving-plastic-avoiding-minimalist who impressed me not only with her knowledge of Immunology, but also by being a wonderful human being and a caring friend! Speaking of caring, I want to thank Sylvia Ariotti who stepped in as a mentor when I needed one (and became secretly super protective). With her tremendous brightness, her independent and lovely soul, Silvia turned into a role-model and a friend I will always ask for advice. Another advise giver, I want to thank, is Pedro Papotto, for making me smile every time he entered our lab, for checking up on my climbing "skills" and over-talking anything. And finally, thanks, to all other lab members and iMM neighbours who made my time at this institute to such an incredible one.

Additionally, I want to thank all the people who supported me outside the lab. I am an extremely lucky girl to have the best, loveliest and most supporting parents on this planet. Thank you, Mama and Paps, without you I wouldn't be where I am now (literally but also metaphorically). I want to thank my amazing friends in Vienna for supporting my departure and hurrahing my coming-home. Thanks to all the wonderful people I met in Lisbon: my party-friends, my flatmates and the special soul I met on my second day in Portugal.

It was a great year. **Muito obrigada!**

Abstract (English)

Tissue resident memory T cells (Trm) are a recently identified, functionally distinct subset of memory T lymphocytes that reside in various tissues of the body. Unlike other memory T cell populations, Trm cells are non-circulating and persist in their respective niches for years. While heterogeneity is reported in different niches, the co-expression of the cell surface markers CD69 and CD103, which are associated with tissue-retention, are reliable markers for CD8+ Trm cells at epithelial sites. Over the last years, their key role in immune surveillance and defence became widely acknowledged. Being antigen-specific, they show specific immune-responses and act quick upon viral and bacterial re-infection. Furthermore, Trm cells recruit other immune cells to the site of infection and were shown to have anti-tumoral behaviour in solid cancers. This make them interesting targets for novel immunotherapy and vaccination approaches. Thus, Trm cells development is becoming an important research topic in basic research. Yet, the signals that drive Trm cell development and are responsible for their maintenance, are not fully understood.

We analysed the expression of CD103 and CD69 upon T cell activation in a murine model and show that CD103 expression is highly variable among circulating T cell subtypes. Moreover, by defining and setting-up an *in vitro* culture system, we tested extrinsic factors that affect Trm cell differentiation from naive pre-cursor T cells. Different *in vitro* conditions and interactions between cell-types were assessed and the expression of Trm cell surface markers CD103 and CD69 was analysed via multi-colour flow cytometry. By this, we aimed to study complex cellular interactions in a reductionist way. In the following studies we could show that expression of CD69 was highly dependent on T cell receptor mediated signalling. Furthermore, TGF-β can drive naive precursor T cells into presenting a Trm cell-like phenotype by upregulating CD103. When CD8+ T cell interactions were tested in co-culturing studies with dendritic cells, the impact of the microenvironment could be even further highlighted.

Abstract (Deutsch)

Tissue resident memory T cells, zu Deutsch Gewebsresidente Gedächtnis-T-Zellen (Trm), sind eine erst kürzlich identifizierte, funktionell unterschiedliche Untergruppe von Gedächtnis-T-Lymphozyten, die in vielen Geweben und Organen unseres Körpers zu finden ist. In den meisten Geweben lassen sich Trm-Zellen durch die Co-Expression der Zelloberflächenmarker CD69 und CD103 identifizieren. Beide Oberflächenproteine sind für die Verankerung im Gewebe zuständig. Im Gegensatz zu anderen Gedächtnis-T-Zellen, zirkulieren Trm-Zellen nämlich nicht und verbleiben jahrelang in ihren jeweiligen Nischen. Dort tragen sie eine wichtige Funktion zur Immunüberwachung und -abwehr bei. Nachdem sie bereits ein bestimmtes Antigen erkannt haben, zeigen sie eine spezifische Immunantwort und reagieren daher schnell auf virale und bakterielle Neuinfektionen. Darüber hinaus rekrutieren Trm-Zellen andere Immunzellen zur Infektionsstelle und zeigen ein anti-tumorales Verhalten bei manchen Krebsarten. All das macht Trm- Zellen interessant für neuartige Immuntherapie- und Impfansätze. Jedoch sind viele Mechanismen und molekulare Regulationen bezüglicher ihrer Entstehung und Aufrechterhaltung, nicht vollständig verstanden.

In den folgenden Studien analysierten wir die Expression von CD69 und CD103 während CD8+ T-Zell-Aktivierung in dem Modellorganismus Maus. Wir konnten zeigen, dass CD103 unter den verschiedenen T-Zell Subtypen unterschiedlich stark exprimiert wird. Darüber hinaus haben wir in verschiedenen *in vitro* Ansätzen den Einfluss von extrinsischen Faktoren getestet, die die Zelldifferenzierung von Trm- Zellen beeinflussen. Unterschiedliche Bedingungen und Wechselwirkungen zwischen Zelltypen wurden getestet und die Expression der Trm-Zelloberflächenmarker CD103 und CD69 mittels Mehrfarben-Durchflusszytometrie analysiert. Auf diese Weise wollten wir komplexe zelluläre Wechselwirkungen auf einfachere Weise untersuchen. Wir konnten zeigen, dass die Expression von CD69 stark von T-Zell-Rezeptor-vermittelten Signalen abhängt. Darüber hinaus kann TGF-β naive CD8+ Vorläufer-T-Zellen durch Hochregulieren von CD103 dazu bringen, einen Trm-ähnlichen Phänotypen anzunehmen. Als CD8+ T-Zell-Wechselwirkungen in Co-Kultivierungsstudien mit dendritischen Zellen und regulatorischen T-Zellen (Tregs) getestet wurden, konnte ein potentieller externer Einfluss gezeigt werden.

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1. Introduction

1.1. T cell mediated immunity

The human body has evolved a complex system to prevent disease, diminish infections and to maintain a healthy homeostasis. Our immune system provides an elaborated combination of different types of immune cells that interact and react upon infection. At the same time, it is designed to keep a balance between enduring and reacting. The human immune system is often described as a “multi-layered” defence mechanism, supported by the innate and the adaptive immune system, working in a complementary manner. Apart from the body’s protective surfaces, that function as physiological barriers against invading pathogens, cells of the innate immune system provide a first line of defence. Using specialised receptors to recognize foreign molecules, innate immune cells distinguish between self and non-self and respond accordingly. Phagocytotic cells recognize, ingest and destroy different pathogens. In addition, the innate immune system can support local inflammation. Activated macrophages release pro-inflammatory cytokines, such as TNF-α and IL-6, which recruit other cells to the site of infection. Although innate immune cells lack the establishment of a specific long-term memory to prevent re-infection, they have a major role in initiating an adaptive immune response. With their unique ability to create a long-lived memory against a specific pathogen, cells of the adaptive immune system can thereby generate a more efficient response upon re-exposure [1,2].

T lymphocytes are part of the adaptive immune system; they undertake important tasks in immune management, surveillance and establish a memory compartment. Committed T cell precursor cells enter the thymus where they mature and undergo selection until they are released to the periphery as either CD8+ or CD4+ T cells. CD8 and CD4 are co-receptors involved in T cell activation and can be used to distinguish between subsets of helper T cells (CD4) and cytotoxic T cells (CD8) with respective characteristics each [3]. Once matured in the thymus, naive T cells circulate between blood and secondary lymphatic organs until they encounter cognate antigen via their T cell receptor (TCR). Each lymphocyte bears multiple copies of the same specific TCR on their surface, determining antigen-specificity. Due to somatic recombination and random TCR chain rearrangement during T cell development, an enormous diversity of antigen binding receptor is created. While the majority of T cells express αβ T cell receptors (named after the variable α and β chains of the receptor), a small amount of T cells bear γδ TCRs [1,4].

1.1.1. T- cell activation

The diverse and specific functions of CD4+ and CD8+ T cells depend on their successful activation and differentiation. The first contact with antigen is often referred to as T cell priming, in order to distinguish from later T cell responses of effector and memory T cells. Antigen-presenting cells, such as dendritic cells, interact with T cells through the surface molecules

MHCI and MHCII (major histocompatibility complex) with the TCR of CD8+ and CD4+ T cells, respectively [5].

CD4+ and CD8+ T cells differ in the process of activation and their following immune response. First, while class II MHC are expressed exclusively on professional antigen-presenting cells, such as dendritic cells and macrophages, class I MHC are expressed by all nucleated cells of the host. Antigens that originate intranuclearly (e.g. from a viral or cell-own origin) will be presented by MHC I molecules. Those class I MHC interact specifically with the CD8 co-receptor of cytotoxic T cells. Contrary, extracellular antigens derived from pathogens are presented via class II MHC complexes to activate CD4+ T helper cells. Additionally, via cross priming, some dendritic cells can acquire exogenous antigens from infected cells and present them on their MHC I complex and present to CD8+ T cells. [6–8].
Dendritic cells function as a link between the innate and the adaptive immune system. They recognize pathogen-derived particles or damaged cells and undergo a programme of maturation, which allows them to provide all signals required to activate and differentiate naïve T cells. During this process of activation, they upregulate the expression of MHC molecules and begin to express co-stimulatory molecule. In order to become fully activated, naive T cells do not only need activation via their TCR but also co-stimulatory signals, often referred to as signal 2 and 3 [5,1,9]. CD28 receptor binding by the costimulatory proteins CD80 and CD86 on APCs, is the best characterized second signal to stimulate cell proliferation. CD28, being exposed on all naive T cells, binds to induced co-stimulatory ligands (mainly CD80/CD86) on activated dendritic cells. This assures that T cells are only responding to a foreign molecule. If co-stimulatory signals are missing, the cell will become anergic. For example, blocking CD28 costimulatory signalling is a therapeutic approach to treat certain inflammatory autoimmune diseases [7,10,11].

Upon successful activation, T cells proliferate and differentiate into a large number of effector cells. The combination of different cytokines, signal 3, regulates the differentiation into effector sub-types [10,12]. Depending on which lineage specific cytokines are present, CD4 T cells will differentiate into 5 main subsets: Th1, Th2, Th17, Tfh and (induced) Tregs. Each subset has its specific way of enhancing immune activity and to promote pathogen clearance [13]. Activated naive CD8+ T cells will differentiate into effector CD8 cytotoxic T cells, which main role is killing target cells. They migrate to site of infection, produce inflammatory cytokines, such as IFN-γ and TNF-α and cytotoxic molecules, such as perforin and granzymes to induce apoptosis of the infected target cell [14,15]. Since cytotoxic T cells are so destructive, it is thought they require even more enhanced co-stimulation to drive their differentiation [1,16]. CD4+ T cell help is often required. IL-2, produced by activated CD4+ T cells, induces cell expansion and promotes CD8+ T cell effector formation. However, it has been shown that CD4+ T cell help is not always necessary [17]. Another third signal can be the proinflammatory cytokine IL-12, which is produced by macrophages and dendritic cells during infection. IL-12 It induces toxicity of activated CD8+

T cells and the production of IFN-γ [16].

Effector T cells are usually short lived. After the process of cell expansion, differentiation into various subtypes and subsequent clearance of the pathogen, most effector T cells die. This step is often called contraction phase and can results in memory formation. During the response, a heterogenous pool of memory T cells are formed that ensure life-long immune protection against re-infection [18]. Even though memory T cells are formed upon the initial response to a specific pathogen, they can persist without repeated exposure to the same antigen. Upon antigen reencounter they respond in a much rapid and effective way, under which the principle of vaccination lies [1,19]. The formation of memory T cells depends on a differential transcriptional expression and seems to be highly influenced by extrinsic factors, such as the cytokines IL-15 and IL-7, produced by APCs and stromal cells [18,20]. IL-15 and IL-2 are structurally very similar and share a common binding site subunit. Both cytokines presumably direct CD8+ memory differentiation into their subtypes [21]. Therefore, it seems very likely that CD4+ T cell help also plays an important role in generating functional CD8+ memory T cells, not only during priming. Memory CD8+ T cells that developed independent of CD4+ T cell help, show a reduced responsiveness to reinfection [22,23]. Furthermore, IL-10, known as an immunosuppressive cytokine produced by CD4+ Tregs, has been shown to promote CD8+ T cell memory development [24]. However, the full mechanisms behind these interactions is not completely understood and might be different between memory T cell subtypes.

1.1.2. Memory T cell subpopulations

Memory T cells can be categorized according to their migration and effector potential. Central memory T cells (Tcm) are predominantly found in SLOs and circulate between blood and lymph. Effector memory cells (Tem) on the other hand migrate between blood and peripheral non-lymphoid tissues [18,19]. Additionally, a non-circulatory memory subset, named tissue resident memory T cells (Trm) was identified more recently. Trm cells are a distinct subset of memory T cells that reside in non-lymphoid organs where they make important contributions to immune surveillance, which will be discussed in detail later [20].

Due to their expression of specific surface proteins, different memory subtypes can be identified. Memory and effector lymphocytes are distinguished from naive cells by the high expression of cell-adhesion molecule CD44. CD44 is the receptor for hyaluronic acid, commonly expressed by cells of the peripheral tissues, marking migration potential of memory and effector CD8 T cells [25]. Additionally, CD62L (L-selectin) is used to differentiate between Tem and Tcm subsets. Since CD62L is upregulated on lymphocytes that interact with endothelial cells in lymph nodes, it is highly expressed on Tcm cells. Whereas Tem cells are commonly defined as CD62L-. Similarly, the chemokine receptor CCR7 is expressed on Tcm cells and enables them to home to lymphoid tissues [18]. However, it must be said that plasticity among all subsets is often observed. This means, the fate of a subset might not be uniformly defined and depends on extrinsic signals [26]. As an example, some of the mechanisms for CD8+ memory T cell

homeostasis include the cytokines IL-7 and IL-15 that have been shown to maintain the numbers of cells by upregulating anti-apoptotic proteins [16].

1.2. Tissue resident memory T cells

Circulating immune cells constantly search for possible antigens throughout the body's fluids. Today we know they are complemented by a variety of non-circulatory lymphocytes in tissues and organs. These tissue resident populations of different innate-like or adaptive lymphocyte display an important part of immune surveillance and homeostasis in tissues and organs of humans. They include invariant natural killer T cells (iNKT), innate lymphocytes (ILCs), mucosal-associated invariant T cells (MAIT), γδ T cells as part of intraepithelial lymphocytes (IELs) and Trm cells[27]. In the following part I will focus on tissue resident memory T cells, mainly CD8+ Trm cells.

The discovery and appreciation of tissue resident memory T cells started around 2001 when researchers defined a different set of memory cells residing in tissues after infection [28]. Before, memory T cell populations were classically divided in Tem and Tcm cells, however this new subset showed a distinct phenotype. Moreover, it showed a long-lived, self-renewing characteristics and provided enhanced immunity against local infection [29] . Soon, Trm cells were widely acknowledged as a distinct memory cell population and extensively characterized [30]. A key hallmark is their ability to reside in tissues, showing little to no recirculation. They are numerically predominant at epithelial barrier sites of various tissues, such as the skin, the intestine, female reproductive organ and lungs. At these possible entry sites for pathogens, Trm cells seem to play a key-role in local immune surveillance and immune protection. But also, in non-mucosal tissues like the brain, liver or heart, populations of Trm cells have been found, both in humans and in mice [20,28,31,32]. They are primed to prevent re-infection by pathogens and react in a specific and fast effector-like manner. Their specificity and location make them favourable targets for novel vaccinations against viruses that effect sensitive epithelial sites, like the lung [33]. By expressing Granzyme B for cytotoxicity and secreting interferon (IFN)-γ and tumour necrosis factor (TNF)-α, Trm cells can not only directly defend against pathogen invasion, but also recruit other cells of the adaptive and the innate immune system to the site of infection [34,35]. On top of that, they were found being able to infiltrate soluble carcinomas and showing important roles in anti-tumour immunity by activating immune-mediated tumour destruction [33,36].

Resident memory T cells are located in many different tissues, interacting with their given environment. Hence, Trm cells have been identified as phenotypically a rather heterogenous population [27,31]. Not only according to their location and cellular behaviour but also on a transcriptional level, they showed to be slightly different in various tissues. However, in the last couple of years intensive research identified a shared phenotype and reliable Trm cell surface marker proteins [18]. Both in human and in mice, CD103 and CD69 are the most common surface

markers used to distinguish Trm cells from other memory T cell subtypes in most of the tissues. Notably, a CD103+CD69+ phenotype is not an uniform marker for all Trm populations, but CD103 and CD69 seem to be consistent marker for CD8+ Trm cells in at least the skin and the gut [32]. Moreover, CD103+ Trm populations of murine skin, gut and lung show a similar transcriptional phenotype. This suggests that there is a molecular pattern regarding their development and maintenance [37]. Both receptors, which will be discussed in detail later in this report, are strongly connected to maintaining tissue placement, residency and retention [20].

1.3. Trm cell surface receptors CD69 and CD103

1.3.1. CD69

During inflammation CD69, a C-type lectin protein, is briefly expressed on activated lymphocytes. In SLOs, CD69 is rapidly upregulated upon TCR stimulation but decreases shortly after. It is not found in circulating lymphocytes in the periphery and therefore often used as an "early-activation" marker [18,38]. As mentioned before, naive T cells constantly re-circulate between periphery and lymphoid organs. This dynamic process of entering and exiting SLOs is essential for immune surveillance. During the process of cell priming however, retention in the lymphoid organ is needed to guarantee a successful process of T cell activation [38]. The quick upregulation of CD69 on recently activated effector cells is thought to slow the egress in SLOs. There, it works as an antagonist of S1P1, the sphingosine-1-phosphate receptor on T cells, a key molecule for lymphoid egress. CD69 complexes with the receptor and thereby shortly inhibits its mediated signalling. In that way, T cells in SLOs can become properly activated and develop into their respective subtypes before they exit into their destined periphery [32]. Interestingly, constant CD69 expression is only observed in tissue resident lymphocytes. As an example, the almost entire IEL compartment and lymphocytes of the lamina propria show high CD69 expression [39,40]. Furthermore, when CD69 is knocked out CD8+ T cells showed difficulties to be maintained in skin and lungs [34,41]. CD8 T cells can express CD69 also without the presence of cognate antigen. It has been shown that other signals, such as IL-33 and IFN, induce an upregulation of CD69 as well [34,42]. As being highly upregulated on mucosal sites, CD69 is considered not only as an activation marker but also linked to regulation of immune responses. There, it is thought that the expression of CD69 in the gut is indirectly dependent on the intestinal microbiota [38]. However, it is not fully understood what exact mechanisms regulate continuous CD69 expression and which role it plays in Trm cell formation and tissue positioning. While CD69 is expressed on a majority of Trm compartments, there are some tissues (such as the female reproductive organ) where CD69- Trm cells have been identified [20,40,43]. Also, it has been shown that CD69 is beneficial for successful skin Trm cell formation, but not crucial [41]. This highlights the dynamics of tissue-depending signals and the demand for multiple retention markers.

1.3.2. CD103

A second hallmark of tissue residency is the production of the heterodimeric cell surface receptor, integrin αE(CD103)β7. Whereas CD103 strictly refers only to one subunit, it addresses herein the full receptor protein. CD103 is frequently expressed on tissue-associated lymphocytes, such as IELs and their memory subsets. But also, dendritic cells and Tregs show elevated CD103 production [44,45]. However, particularly Trm cells have been found to constantly highly express CD103. In fact, CD8+CD103+ Trm cells have been identified in the small intestine, in the skin, lung and in vaginal mucosa of mice and humans. And when CD103+ Trm cells of different tissues were compared, they exhibited a similar transcriptome [37]. The main ligand of CD103 is the cell-adhesion protein E-cadherin. In tissues, the transmembrane protein E-cadherin is expressed on the cell surface of epithelial cells to facilitate cell-cell adhesion and create tight junctions. Thus, it seems very likely that by expressing CD103 on the cells' surface, a physical interaction with the epithelium and other surrounded cells is enabled. Also, in terms of Trm cell positioning in the tissue, their placement and retention is achieved by binding and tethering E-Cadherin [41,44–46]. Emphasising the role of CD103 in Trm cell development, a deficient production leads to a reduced number of viable Trm cells in tissues [32,41]. However, it seems like loss of CD103 doesn't affect formation or migration of Trm to tissues but their retention, at least in the small intestine [42].

A nowadays well characterised key driver for CD103 expression is the cytokine TGF-β. TGF-β is secreted by many different cells, especially in the epithelial tissue and therefore highly present in the environment of Trm cells. Studies have shown, that interfering with TGF-β signalling has severe effects on Trm cell development. Lymphocytes with a deficient TGF-β receptor on their surface, are impaired in CD103 expression and tissue retention is reduced [47,41].

In perspective of cancer immunology, analysis of the microenvironment of solid tumours have shown that tumour infiltrating Trm cells are often CD8+CD103+. These cells appear to have a higher killing efficiency and higher cytotoxic activity and are therefore targets of anti-tumour research [36,44]. Nevertheless, CD103 is not a universal Trm cell marker. Like CD69, there are different reports about functional CD8+CD103- Trm subsets. For example, CD103- Trm populations were found in the skin, the lamina propria and in the brain, where they show a different gene expression profile than CD103+ Trm cells [48]. Nevertheless, the majority of Trm cells in most of murine and human tissues show constant and high CD103 expression, which is why it commonly used as an identification marker for CD8+ Trm cells.

1.4. Transcription factors linked with the Trm cell phenotype

The up and down regulation of specific transcription factors guide developmental pathways of immune cells. Lineage-specific genes are repressed or activated, and cells adapt to their

surrounding [18,49]. Trm cells are not only phenotypically different from their circulating relatives, but also show a transcriptionally different signature. In general, transcription factors that are higher expressed in circulating memory T cells, are strongly downregulated in Trm cells. Genes linked to tissue retention are upregulated. And since Trm cells can still rapidly exert effector functions, transcription factors involved in effector differentiation are maintained [50]. For example, the transcription factors Blimp-1 and its homologue Hobit were identified as regulators of Trm cell differentiation and formation. Deletion of both results in an impaired Trm cell maintenance in the skin [51]. Blimp-1 and Hobit are thought to be key regulators for tissue retention mechanisms in Trm cells. Both transcription interfere with intracellular exit pathways, for example through CCR7 or S1PR1, by blocking their expression and indirectly supporting tissue retention [51,52]. Two transcription factors, which downregulation has been strongly linked to Trm cell formation, are the T-box proteins T-bet and Eomes. Supressing the T-box transcription factors T-bet and Eomes has shown to be crucial for a stable Trm cell phenotype, in both human and mice [32].

1.4.1. T-bet and Eomes

T-bet (encoded by *Tbx21*) and Eomes (*Eomesodermin*) are T-box transcription factors and both involved in processes during T cell development and priming [53,54]. While they share regulation of some similar genes, they have opposing functions in effector-to-memory differentiation. T-bet is highly expressed in short-lived effector T cells, whereas high Eomes production is linked to memory T cell formation. An overexpression of both Eomes or T-bet in CD8+ T cells negatively impacts Trm cell differentiation in skin and lung [20,46,50].

T-bet has important regulatory functions during effector differentiation. In CD4+ T cells T-bet is induced by inflammatory cytokines and drives Th1 cell differentiation. In the development of CD8+ effector T cells, T-bet expression promotes their cytotoxicity and differentiation, induced by high levels of the proinflammatory cytokine IL-12 [55]. On the contrary, IL-12 seems to have the opposite effects on Eomes. On activated CD8+ T cells, IL-12 was shown to supress Eomes expression [56]. Usually, Eomes is highly expressed in long-lived memory CD8+ T cells. In particular, it plays an important role during the development and homeostasis of central memory T cells [57].

Like T-bet, Eomes is downregulated during Trm cell development, although to a much greater extend. While the Trm cells still have a low level of T-bet, Eomes expression is nearly completely shut down [46]. Therefore, it is believed that downregulation of both T-box transcription factors mediates Trm cell survival and tissue maintenance. Nevertheless, Trm cells can act specifically and rapidly upon reinfection. Hence, it is thought that the dynamic regulation of Eomes and T-bet, amongst others, is mediating their effector functions. For example, both transcription factors are involved in expressing effector molecules like Granzyme B and IFN-γ, which are upregulated upon their re-activation [50,55]. Both T-bet and Eomes induce expression of the IL-15 receptor (IL-15Rα). The low level of T-bet expression allows Trm cells to keep and regulate production of the IL-15 receptor [46].

On a regulatory level, it has been shown that TGF-β can control the transcriptional suppression of the T-box transcription factor T-bet. When T-bet and Eomes are overexpressed in effector CD8+ T cells, a reduced level of TGF-β receptor expression was observed, which resulted in impaired CD103 expression [46]. Moreover, on a molecular level, it has been shown that T-bet and Smad3 bind the same TF binding site at the CD103 promoter in a competitive way. While T-bet seems to prevents its expression, Smad3 binding is required for a TGFβ-dependent CD103 expression [58,59].

Besides Hobit, Blimp-1, Eomes and T-bet, there have been other transcription factor identified to be involved in Trm cell regulation. As an example, AhR (short for aryl hydrocarbon receptor) is involved in sensing signals from the environment, highly expressed on IELs, was observed to be upregulated in Trm of skin, lung and intestine [50,60]. Runx3, involved during CD8+ thymic development, is also thought to have important regulatory functions of Trm cell differentiation, by downregulating T-bet and inducing Hobit and Blimp, for instance [61].

This shows that the transcriptional regulation of Trm cells is highly dynamic and needs further detailed identification. For example, which tissue specific signals and cytokines drive gene expression. Trm cells obtain a specific transcriptional program, that is necessary to adapt to their environment and maintain their unique phenotype. How it is regulated on a molecular level, is still not fully understood.

1.5. Development of Trm

How and when Trm cells are generated, is one of the major questions in fundamental Trm cell research. There are different hypotheses for the typical path of naive-to-memory (into Tcm and Tem subtypes) differentiation [18,31]. Up until now, it remains known whether a committed precursor derived from one developmental path exists or whether there are multiple ways to develop into respective memory subtypes.

In case of Trm cells, it was shown that they can be classically formed in the course of T cell priming after infection [29]. It has been also shown that CD8+ KLRG- effector T cells can give raise to CD8+CD103+ Trm cells in multiple tissues [41,62]. Therefore, it is strongly believed that Trm cells, in the same way as other memory subsets, arise after the effector-phase and seed to non-lymphatic tissues upon tissue-specific stimulation [30,34] . Thereby, it seems like local antigen presentation and inflammation have an enhanced effect of Trm cell formation [63]. When antigen-specific effector T cells migrate to nonlymphoid tissues, exposure to local cytokines besides TGF-β, such as IFN-γ, IL-33 and IL-15 have shown to orchestrate Trm cell recruitment and differentiation [46,64,65]. But it is suspected that this can happen in an antigen-independent matter. In fact, it is debated that effector CD8+ T cells can migrate to non-lymphoid tissues and differentiate into Trm cells without inflammatory signals or a secondary local antigen recognition [30,42,66]. It was shown that CD8+ T cells, which were activated *in vitro* into effector cells and transferred to naive mice, successfully formed Trm cells in skin and liver. In this case, constant

antigen presentation or inflammation in the organ was not essential [42,43,65]. Therefore, their development might have different pathways, seems to be diverse and depending on the circumstances encountered. The importance of certain specific homing-effectors, are not excluded [30,67]. It must be noted, while their specificity and longevity is promising for therapeutically approaches, their development and tissue-specific induction needs to be further investigated, in order to design novel vaccines.

1.6. Role of the microenvironment in Trm cell development

Given the many different niches of the body, Trm cells are composing a phenotypically heterogenous population, not all of them similarly well understood. While tissue derived TGF-β, inflammatory signals and IL-15 signals are required for CD103+CD69+ Trm cells in the intestine, there may be other regulators in different tissues. Development and maintenance of Trm cells depends on signals from the microenvironment that are distinct from the ones for circulating CD8+ T cells [40,41]. Notably, the microenvironment is tissue specific and consist of different cell populations and composition. Therefore, the secretion of molecules and chemicals are similarly different. For example, in the intestine almost all IELs express the chemokine receptor CCR9 and a high number of CD8+CD103+ Trm cells are found. On the other hand, there are CD103- and/or CD69- Trm populations observed in the brain, where a different environment is present. In some tissues specific chemokines are only present when there is local inflammation. This is just one example to emphasize the specific conditions in tissues that influence Trm cells [20,42,67].

Apart from stromal cells, other immune cells are seeded in epithelial barrier sites and play important roles of the local immune network. As major antigen-presenting cells macrophages and dendritic cells parole tissues for pathogens and regulate T cell responses [68,69]. Although there is detailed information about regulation and activation of particular cell types, their complex interplays are not fully decoded. Especially when it comes to Trm cells, there are many open questions regarding the cellular interactions with other cells.

Regardless, one important connector seems to be TGF-β. TGF-β1 is secreted by many different cells of hemopoietic and non-hematopoietic origin. It can be produced by epithelial cells, such as keratinocytes in the skin, but also by immune cells, like macrophages. The effects of TGF-β1 are known to have a huge variety, being both pro- and anti-inflammatory. Therefore, precise TGF-β1 regulation is critical on many levels [70]. When TGF-β1 is produced it is usually secreted and complexed to polypeptides that prevent it to bind to its receptors. A way of regulating TGF-β1 induced effects is by controlling its availability. The latent TGF-β1 complex can be enzymically transferred into an activated form by tissue specific mechanisms. For example TGF-β1 is enzymatically activated by αvβ8 integrins on the surface of other cells [71]. It has been shown, that besides epithelial cells, some dendritic cells express that integrin and thereby provide active TGF-β1 to other cells [72]. In that way, TGF-β1 mediated regulatory functions such

as CD103 expression in naive CD8+ T cells can be induced [70,73]. Another tissue migrating immune cell that might be found sharing the environment with Trm cells are CD4+ Tregs. Tregs are well-known to play a crucial role in immune suppression and auto-immunity by the expression of suppressive cytokines like TGF-β1 and IL-10. Especially at mucosal barrier sites Tregs have local anti-inflammatory effects. As a CD4+CD25+ subpopulation, Tregs are identified by the master transcription factor Foxp3 [6,49]. Tregs are important to reduce an excessive immune response and can support the formation of functional CD8 memory cells[74].

1.7. Aim of the project

Trm cells are important players of a sophisticated immune system and take on significant roles in immune surveillance and long-lived cellular immunity. While some of their phenotypes and characteristics are described, detailed knowledge of Trm cell development remains unknown. This is especially the case for potential cellular partners and tissue factors that guide the Trm cell developmental process. The aim of this project, independently conducted as a master thesis, was to analyse the development of a Trm cell-like phenotype in an *in vitro* system. To achieve this we determined the regulation of the Trm cell surface markers CD69 and CD103 during T cell activation. Secondly, we tested the influence of some extrinsic factors on the expression of these markers *in vitro*.

2. Materials and methods

2.1. Animal work

2.1.1. Mice

Female and male C57BL/6 mice were obtained from Charles Rivers. All mice used, between the ages of 5 to 12 weeks, were bred and housed under pathogen-free conditions by the rodent facility of the iMM Lisboa. All animal work was conducted under strict compliances to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA), monitored by the institution's animal ethics committee, under the licence held by Dr. Marc Veldhoen. Animal work was conducted under supervision and with kind help from Marta Sofia Baptista.

2.1.2. T cell activation with anti-CD3

For antigen-independent T cell activation, selected mice were injected intraperitoneally with 12.5 µg of anti-CD3ε (145-2C11, diluted in PBS) at a volume of 200 µl per mouse [75,76]. Depending on the experiment, mice were sacrificed 24, 48, 72 or 96 hours later.

2.1.3. CD103-Cre reporter mouse

Using the Cre/LoxP reporter system to generate reporter mouse models is widely used for cell lineage tracing experiments [77]. For our experiments, a transgenic mouse-line was developed in which the fluorescent protein eYFP (short for enhanced yellow fluorescent protein) is expressed only in cells that once expressed CD103. The eYFP gene was inserted into the ROSA26 locus together with a LoxP-flanked “Stop” sequence upstream. The Cre recombinase enzyme under the control of the CD103 promotor was knocked in (BAC transgene generated in the T. Sparwasser lab, Hannover) (illustrated in Figure 1). In theory, when a cell expresses CD103 it also produces the Cre enzyme. The enzyme specifically targets and removes the LoxP flanked “stop” region, allowing only those cells to express eYFP. In other words, a cell that has expressed CD103 will also express the fluorescent protein, which can be traced by flow cytometry. When a cell downregulates CD103, eYFP is still being produced. But to distinguish CD103 expressing cells from those that downregulated the protein, CD103 can be additionally stained for with fluorochrome labelled antibodies. Functional reporter mice with enhanced fluorescent signals, are a powerful tool to study expression patterns in different organs and can help to understand lineage development [77].

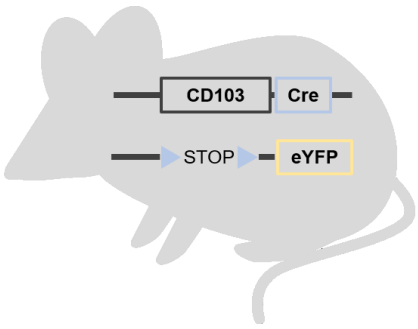

Figure 1: Illustration of the CD103-Cre reporter mouse model that was used in the experiments

2.2. Isolation of lymphocytes

After mice were sacrificed, spleen and lymph nodes (inguinal, mesenteric and axillary) were harvested and processed into single cell suspensions. This was done by carefully mashing the organs through a 50µm strainer (Sysmex®) to separate lymphocytes from the tissue into a 50ml Falcon tube. In case of spleen, the lymphocytes of one mouse were collected and incubated for 3 minutes in 3 mL ACK Lysis buffer (Ammonium-Chloride-Potassium, by Thermo Fischer®) to lyse red blood cells that might be interfering with later flow cytometry analyses. Lymph nodes were mashed and flushed through a cell strainer with PBS. In order to reduce cell death, all work was performed on ice. The cell suspension was washed with PBS, centrifuged (500xg, 5 minutes), the supernatant discarded. Cell pellets were re-suspended in 500µl - 1,5ml (depending on sample number and size of pellet) and processed for further experiments.

2.3. AUTOMACS™ cell purification

To obtain a monoculture for *in vitro* cultivation, CD8+ lymphocytes were isolated by the principle of MACS (magnetic-activated cell sorting). For the positive selection of CD8+ T cell the autoMACS™ purification system by Miltenyi Biotec was used according to the manufacturer's instructions. The magnetic MicroBeads (Miltenyi Biotech) are conjugated to a monoclonal antibody, e.g. α-APC. When added to the sample solution the antibody will specifically bind to its respective target. The cell suspension is then taken up by the autoMACS™ isolator, separated by the magnetic separation column and eluted [78].

As an example for CD8 selection, the suspension of isolated lymphocytes from spleen and/or lymph nodes was incubated with the fluorochrome-conjugated antibody APC α-mouse CD8a (BioLegend) in a dilution of 1:500. Thereby, all CD8+ lymphocytes are tagged with the fluorochrome APC. 500µl lymphocyte suspension and 1µl of antibody were mixed and incubated in the dark for 10 minutes at 4°C. After incubation a following washing step is removing unbound antibody (500xg, 5 min) and the cell pellet was resuspended in a 500µl – 1ml AutoMACS running buffer (1x PBS, 0.5% FBS, 0.4% EDTA). 2 µl of the manufacturer's magnetic beads coated with the anti-fluorochrome antibody α-APC were added to the suspension, carefully mixed and incubated 20 minutes at 4°C. After incubation the suspension was transferred into a new tube through a 40µm strainer and used for purification on the autoMACS™ magnetic cell sorter. The program "possel" (positive selection) isolates in the end CD8+ lymphocytes from the rest of the cell suspension.

Since the magnetic beads will specifically bind to APC, in principle any conjugated receptor can be used. Hence, the same procedure was done to obtain a single suspension of CD25+ (with APC anti-mouse CD25; BioLegend; 1:300) or CD19+ (APC anti-mouse CD19; BioLegend; 1:300) lymphocytes. If a higher number of cells was desired a second run of the negative fraction was performed.

After cell isolation the positive selected cell suspension was centrifuged (500g, 5min) and re-suspended in 2 ml of complete IMDM and counted using a 1:5 Trypan Blue mix and the Neubauer counting chambers.

2.4. Cell culture

To set-up *in vitro* cell culture experiments, lymphocytes were isolated and purified as described in 2.2 and 2.3 under sterile conditions. Prior to cell plating, wells of a flat 96-well plate were coated with 1 µg/ml anti-CD3 (clone 145-2C11, BioXcell) and 3 µg/ml anti-CD28 (clone 37.51, BioXcell) in 80µl 1xPBS per well, then incubated over night at 4°C. The antibodies become immobile on tissue plate, enabling to crosslink the T-cell and co-receptor and stimulate TCR mediated signalling in subsequently plated lymphocytes. Stimulation with anti-CD3 in combination with anti-CD28 enables effective T cell proliferation and expansion, as previously described [79]. $2x10^5$ CD8+ T cells were plated in complete Iscove's Modified Dulbecco's Media (IMDM, by Gibco® supplemented with $2x10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, $5x10^{-5}$ M β-Mercaptoethanol, and 5% FBS) and indicated cytokines.

2.4.1. Defining *in vitro*-conditions for cultivation tests

For the *in vitro* studies, a cytokine-supplement for the culture medium was defined. IL-2 is a known survival and growth factor for T cells in general and commonly added to lymphocyte in culture. IL-15 has beneficial effects on memory T cell formation and the IL-15 receptor is present on Trm cells[19,80]. Therefore, the cytokine IL-15 was supplemented at a concentration of 10ng/µL to the culture medium. It was confirmed that IL-15 had neither an significant negative nor positive effect on T cell viability over IL-2 (see **Supplementary Figure 1**). For the following experiments, our cultivation media contained 10ng/µl IL-15. Additionally when indicated, TGF-β at a concentration of 0.5 ng/µl was added to the culture medium (IMDM, by Gibco® supplemented with $2x10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, $5x10^{-5}$ M β-Mercaptoethanol, and 5% FBS). *Ex vivo* single or co-cultured cells were incubated at 37°C in a 5% CO_2 incubator at constant oxygen levels.

2.5. Generation of bone marrow derived dendritic cells

The hind legs of sacrificed C57BL6/J mice were cut at the tip of the hip joint and fur and skin was removed. Tibia and femur of hint legs were placed in a sterile petri dish filled with PBS and residual muscle tissue was gently removed, using a pointed tweezer and a razor blade. Naked tibias and femurs were carefully cut, right above the knee-joint. The leg pieces were transferred to a new sterile petri dish, filled with 5-8 ml PBS (containing 50 U/ml penicillin, 50 mg/ml streptomycin). Then, a 5ml syringe and needle (25G) was used to pushed in the bone marrow of the leg pieces and to flush the red bone marrow out. This was repeated until the red bone marrow was completely flushed out and the bones appeared white. The bones were discarded. The bone marrow was resuspended in PBS, collected and transferred through a 100µM cell

strainer (to retain residual tissue or bone pieces) to a sterile 15ml Falcon tube. The cell suspension was spun down (450g, 5min), supernatant was discarded and the cell pellet dissolved in ACK buffer to lyse red blood cells. After incubating for 2-3 minutes the suspension was washed with PBS again (450g, 5min) and finally resuspended in 4 ml IMDM.
In a 96 flat bottom well plate $1x10^5$ precursor cells were plated á 200 µl per well and incubated with 20ng/ml GM-CSF. The cytokine GM-CSF, short for granulocyte macrophage colony-stimulating factor functions as that stimulates progenitor cells to differentiate into macrophages and dendritic cells (APCs) *in vitro*[81]. Cells were incubated for min 6 days at 37°C until fully differentiated. At day 3 half of the media was changed and 24 hours before harvesting the full media (+GM-CSF) was changed. When gently changing the media, non-adherent cells are removed to promote differentiation of adherent APCs on the bottom of the tissue culture plate. After 6-7 days the bone marrow derived APCs were used for co-culture experiments, where $1x10^5$-$2x10^5$ isolated and purified (via autoMACS™) CD8+ T or CD25+ T cells were cultured in the same tissue culture plate. BMDCs and CD8+ T cells were co-cultivated in a dilution of approximately 1:2, as in $5x10^4$ BMDCs : $1x10^5$ CD8 T cells

2.6. Flow cytometry

2.6.1. Surface staining

Single cell suspensions were prepared either directly from the lymphatic organs (spleen, lymph nodes) or from cultured cells. Samples, usually still in the 96-well plate, were centrifuged (500g, 2 min, 4°C) and washed with 200 µl of PBS before incubation with respective dilutions of antibodies for the surface markers (***Supplementary Table 1***). A master-mix in ice cold PBS for the surface staining was prepared for 50µL volume per sample. To each sample 50µl of respective master-mix was added and incubated for 10 minutes at 4°C in the dark. The samples were washed with 150µl PBS (500g, 2 min, 4°C). In the case of a surface-only staining, the samples were incubated with 100µl eBioscience IC Fixation Buffer (Invitrogen™) for 20 minutes at RT (in the dark) and then washed with another 100µl of Permeabilization buffer (eBioscience) for 3 minutes (500g, 4°C). A final washing step with 200µl PBS was performed and finally samples were resuspended PBS.

2.6.2. Intranuclear staining

In case of intranuclear staining, the samples were washed, fixed and permeabilized according to the Foxp3 Transcription Factor Staining Kit by eBioscience™ (Thermo Fisher). After washing of the surface staining step, the cell samples were incubated for 20 min at RT (in the dark) with the eBioscience™ Foxp3 fixing solution, after which samples were washed with 150µl Permeabilization Buffer. 50µl of a master-mix containing respective antibodies against intranuclear proteins, prepared in Permeabilization Buffer were added to the samples and incubated for 15-30 minutes at RT. Cells were washed with Permeabilization Buffer and PBS (500g, 3min, 4°C) and finally resuspended in 200µl PBS.

As single cell suspensions the samples were acquired on a FORTESSA or a FORTESSA X20 cytometer (BD Biosciences). Single staining samples for compensations for each sample set-up were done along with the sample probes and compensation was properly performed using the provided BD software (BD FACSDiva^{TM}) compensation tools. All lymphocyte populations were firstly gated based on their physiological characteristics (using the forward and sight scatter), doublets were excluded in forward scatter area/height plots and dead cells were excluded using a LIVE/DEAD fixable viability dye (Invitrogen, see Appendix). All results presented in this study were afterwards carefully analysed using the software FlowJo X.

2.7. Graphs and statistical analysis

All graphs that are illustrated in section 3, Results, were created with the software GraphPad Prism 6 and illustrations were created in Power Point 2016. For statistical tests and interpretation (significance by unpaired students t-tests) of obtained data, GraphPad was use.

3. Results

3.1. Analysing CD69 regulation upon T cell stimulation *in vivo* and *in vitro*

3.1.1. An early-activation marker observed in culture

CD69 is commonly used as an early-activation marker for T cells, since its expression mediates retention in SLOs, shortly after T cell activation. Moreover, CD69 is usually not expressed on circulating lymphocytes but upregulated in different tissue residing lymphocyte populations and thereby it is linked to maintenance and organ retention [32,40].

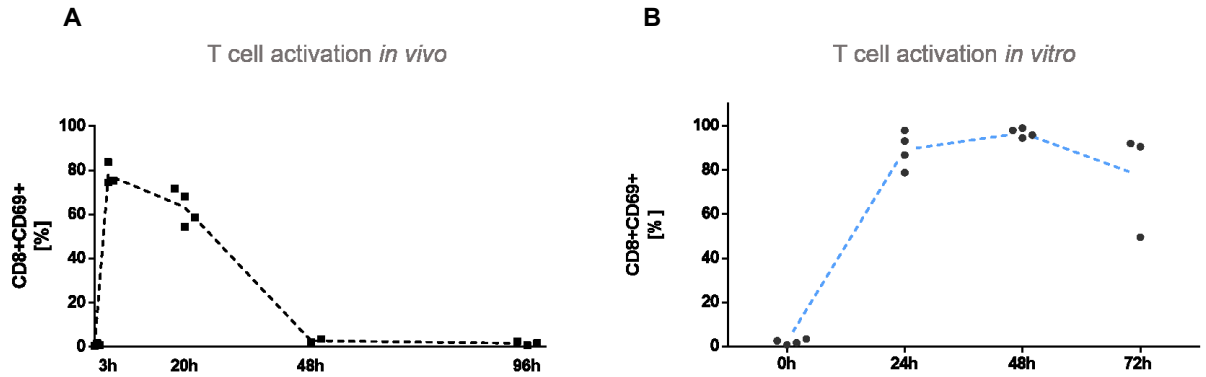

Figure 2: ***Expression of CD69 upon T cell activation in vivo and in vitro***
A) C57BL6 mice were injected i.p. with anti-CD3 and proportions of CD8+CD69+ T cells at indicated time-points after injection was assessed. Lymphocytes were isolated from spleen and lymph nodes, stained and analysed by flow cytometry. Doublets were excluded and gated on live cells, TCRβ+ CD8+ and CD69+ populations. Experiments were performed individually (n ≥ 2) **B)** autoMACS-enriched naive $CD8^+$ T cells were cultured on plates coated with anti-CD3/anti-CD28. At indicated time points the level of CD69+ expression was assessed by flow cytometry. Experiments were performed individually (n ≥ 3)

In order to investigate its role during the process of Trm cell differentiation, we firstly assessed the expression kinetics of CD69 on CD8+ T cells upon T cell activation *in vivo*. As visible in the expression curve **in Figure 2 A**, CD69 expression is upregulated within a few hours upon *in vivo* T cell activation. On average 1% (±0.44, n=4) of CD8+ T cells (from spleen and LNs) express CD69 at the steady state. Shortly after T cell activation, after 3 hours, 78% (± 4.2, n=3) of CD8+ T cells express CD69. The level decreases quickly again. 48 hours after activation, less than 3% (2,9 ±0.7, n=2) of CD8+ T cells express CD69. As well as after 96 hours, where expression levels on CD8+ T cells are around 1% (0.7 ± 0.8, n=3).

Next, we wanted to transfer our findings to an *in vitro* system, in which we can control most parameters. Naive CD8+ lymphocytes were isolated and activated *in vitro* by plate-bound anti-CD3/anti-CD28. CD8+ T cells were cultured up to three days and the level of CD8+CD69+ T cells was determined by flow cytometry each day. Directly after isolation, 2% (± 1.0, n=4) of naive CD8+ T cells expressed CD69, as shown in **Figure 2 B**. 24 hours after cultivation and receiving TCR stimulation, the level of CD69+ cells increased to 89% (±7.2, n=4). Expression

levels *in vitro* remain high until 48h of cultivation (97% ± 1.7, n=4). After 72 hours of cultivation a slight reduction was observed, where averagely 77% (± 19,7, n=3) of TCRβ+CD8+ cells expressed CD69.

When the expression kinetics of *in vivo* and *in vitro* activation-experiments are directly compared, CD8+ T cells can display CD69 for a longer period of time *in vitro*. While *in vivo* stimulation results in rapid CD69 expression and subsequent swift down regulation, *in vitro* stimulation shows high levels of CD69 up until 72 hours of cultivation.

3.1.2. CD69 expression is dependent on TCR stimulation

The aim of this experiment was to analyse factors that could drive CD69 expression.
Since CD69 is generally referred to an “activation marker”, regulation via the T cell receptor was a prime hypothesis. Since upon T cell activation, effector T cells leave the SLOs and arrive via the circulation at the place of infection, we wished to assess if effector CD8+ T cells would re-express CD69 upon encountering antigen in the tissues.
To test if TCR/CD28 stimulation results in re-expression of CD69 in vitro, we re-activated previously stimulated CD8 T lymphocytes. Therefore, lymphocytes were first activated in vivo by administration of anti-CD3 i.p. and isolated 96 hours later. At this time-point, CD69 expression is indistinguishable from naive CD8 T cells (**Figure 2A**). However, at this time-point CD8+ T cells show high CD44 expression (78% ±7.8, n=2), which indicates their migratory effector-like potential (shown in **Supplementary Figure 3**)[25]. **Figure 3** shows the expression of CD69 on *in vitro* re-activated CD8+ T cells, receiving a second TCR/CD28 stimulation or not.
Our findings show a significantly higher presence of CD8+CD69+ lymphocytes, when effector CD8 T cells receive a second activation signal compared to those that do not. At 48 hours of cultivation, on average 7% (±5.7, n=3) of CD8+ T cells that did not receive a second stimulatory signal express CD69, whereas those re-stimulated show 72% (±7.1, n=3) CD69 expression. When effector CD8+ T cells were cultivated for 48 hours, without additional signals, their expression level of CD69 remains on average lower than 15% (highest at 24 hours: 12.3±7.8, n=3).

Taken together, CD69 is quickly upregulated upon activation *in vitro*, in both naive and recently activated effector T cells. Culture of effector CD8+ T cells in an IL-15 containing growth media, without additional TCR/CD28 stimulation, is not sufficient to express CD69.

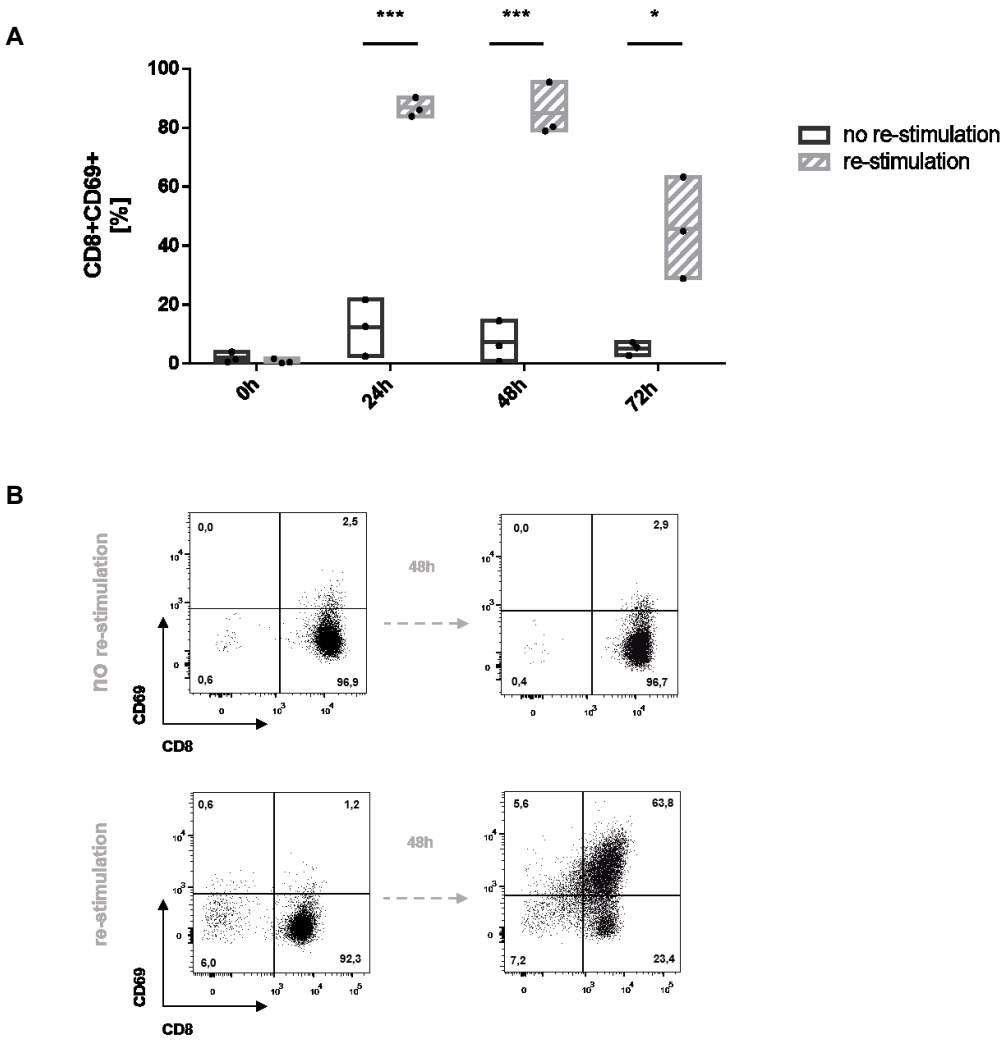

Figure 3: Regulation of CD69 expression upon in vitro re-stimulation of activated CD8+ T cells

C57BL6/J mice were injected with anti-CD3 i.p. CD8+ T cells were MACS enriched 96 hours later and cultured for indicated periods of time. Cells were cultured in presence or absence of anti-CD3/anti-CD28 re-stimulation, as indicated **A)** At indicated time-points CD69 expression levels were assessed by flow cytometry. **B)** Representative flow cytometry plots of freshly isolated CD8+ T cells (left) and after 48 hours of culture with plate-bound anti-CD3/anti-CD28(right). Experiments were performed individually (n=3). Statistical significance was determined via unpaired t-test (*P-value < 0.05, ***P-value < 0.001, no indication = not significant).

3.2. Analysis of CD103 expression in murine CD8 T

CD103 is frequently expressed by tissue-associated lymphocytes, such as IELs in the gut, and has been used as a hallmark for Trm cells in many tissues. CD103 expression is thought to be important for Trm cell development and tissue retention [19,41,43,45]. Hence, in the following experiments we wanted to analyse CD103 expression in naive CD8+ T cells and upon activation. Furthermore, we tested the use of a CD103-Cre reporter mouse that could provide more insights in the regulation of CD103 during Trm cell development.

3.2.1. CD8 T cell subtypes show differential CD103 production

The expression of CD103 was assessed in different naive T cell subtypes. Lymphocytes from spleen and lymph nodes were isolated and phenotyped using the T cell surface markers CD4, CD8, CD44 and CD62L. By their differential expression, naive cells can be distinguished from memory T cells, which can have an effector-like or an central-memory-like phenotype, as illustrated in **Figure 4** [19,43,18]. First, T cell sub-types were distinguished by the expression of the co-receptors CD4 and CD8. Subsequently, CD8+ or CD4+ T cells were sub-divided in naive T cells (CD62L+CD44^{lo}), Tcm (CD62+CD44^{hi}) and Tem (CD26L-CD44^{hi}) and the respective expression level of CD103 was compared. A significant difference of CD103 expression is

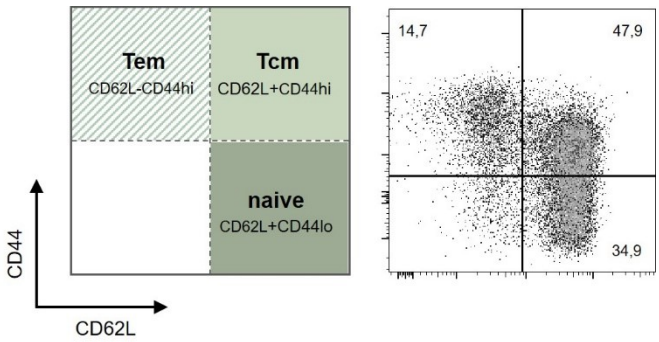

Figure 4: ***Illustration of memory T cell subtypes differentiation via flow cytometry***

Gating of CD8+ T cells to differentiate between naive CD8 T cells (CD62L+CD44^{lo}), Tcm (CD62+CD44^{hi}) and Tem (CD26L-CD44^{hi}) as an illustration (left) and an exemplary flow cytometry plot of steady-state murine lymphocytes of spleen (right)

observed between CD4+ and CD8+ T cells. As shown in **Figure 5A**, few CD4+ T cells express CD103, whereas a marked number of CD8+ T cells express CD103. This was observed when gated on total CD4 or CD8 T lymphocytes of both spleen and lymph nodes. More than half of CD8+ T cells (53.56 ± 3.6, n=5) were found to express CD103. Importantly, the CD103 distribution among CD8 T cell subtypes was distinct. Within the CD8+CD103+ lymphocyte population, most derived from the CD44^{lo} naive compartment. In lymph nodes and spleen on average 69% (±1.8, n=10) of all naive CD8 T cells express CD103. In contrast, within the CD8+CD44^{hi} memory T lymphocytes compartment where only 7% (± 1.4, n=8) of the Tcm cells and 15% (± 1.9, n=6) of the Tem cells express CD103 (see **Figure 5 B**). In summary, these results show differential expression of CD103 in circulating CD8+ and CD4+ T cells. A

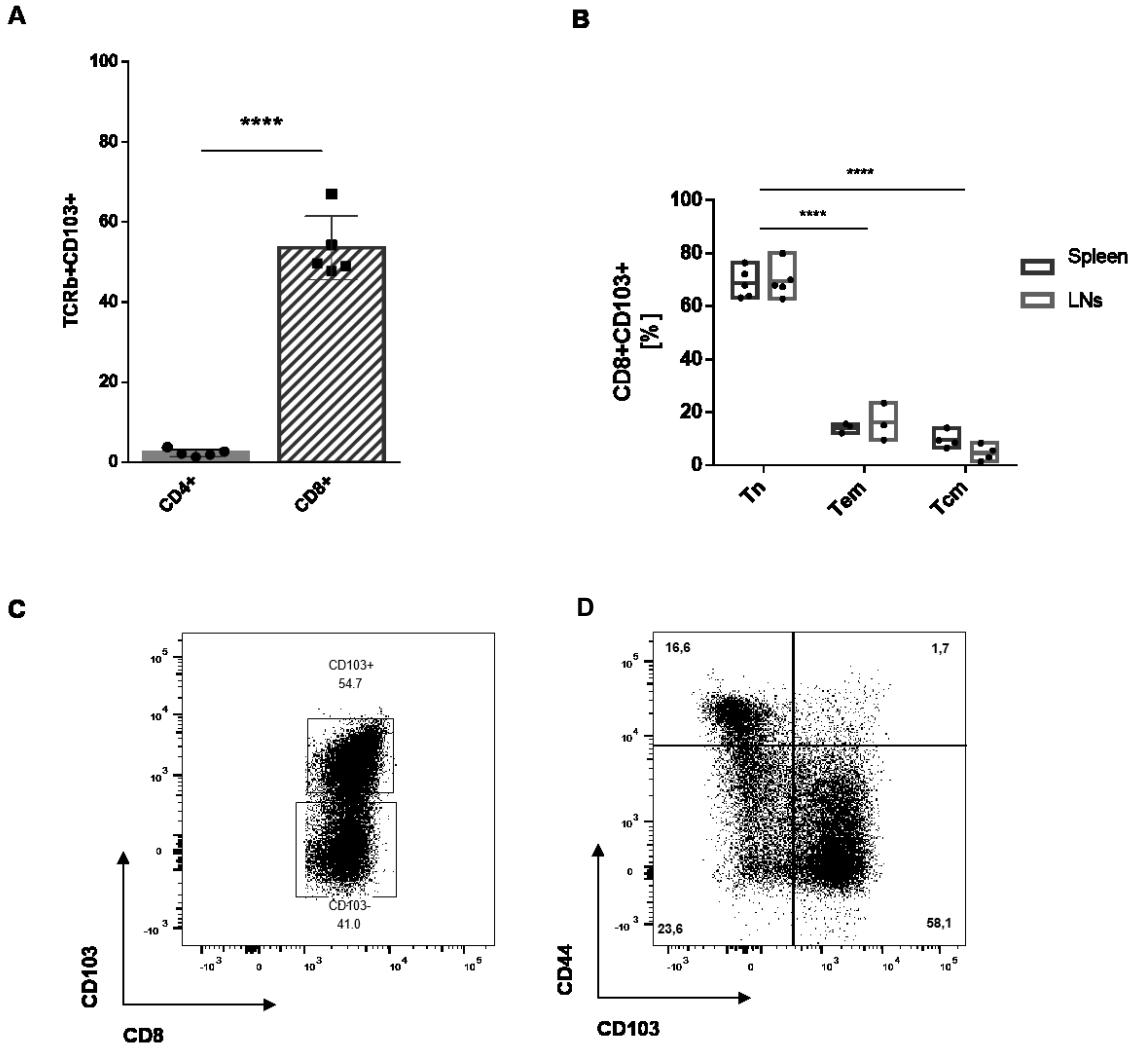

Figure 5: Differential expression of CD103 in T cell subtypes

CD4 and CD8 T cells isolated from spleens or lymph nodes of C57BL6 mice were assessed for the expression of CD103. **A)** expression levels of CD103 in CD4 and CD8 T cells. **B)** Expression of CD103 on CD8+ subsets, naive ($CD44^{lo}CD62L+$) Tcm ($CD44^{hi}CD62L+$) and Tem ($CD44^{hi}CD62-$) cells **C)** representative flow cytometry plot showing CD103 expression on total CD8+ T cells. **D)** Representative flow cytometry plot showing CD44 and CD103 expression on CD8+ T cells. For the analysis, T cells were gated as described above to exclude cell debris and doublets. Results were derived from 3-5 biological repeats (n ≥ 3), statistical significance was calculated via unpaired student t-tests (****P-value < 0.0001), bars indicate calculated mean and standard error.

substantial proportion of CD8+ T cells express CD103, whereas CD4+ T cells do not. The expression of CD103 on circulating CD8 T cells was largely within the naïve T cell compartment.

3.2.2. CD103 is downregulated in SLOs upon T cell activation

Having assessed CD103 expressions at steady-state level, we subsequently determined the expression kinetics of CD103 upon T cell activation.

Although at steady state, a majority of naive CD8+ T cells express CD103, we observed that upon T cell activation CD103 expression is downregulated. Our kinetic analysis shows that 48 hours after anti-CD3 induced T cell activation, less than 15% (12.6% ± 6.8, n=6) of CD8+ T cells isolated from spleens express CD103 (**Figure 6A**). Moreover, four days (96 hours) after T cell

activation only 10% (±1.9, n=3) of CD8+ T cells remain CD103+. 7 days after activation, higher levels of CD103 expression are observed again, although still at reduced proportions compared to non-stimulated controls. CD103-expression proportions and kinetics for CD8+ T cells in LN and spleen are similar.

As an activation control, we additionally monitored CD44 levels upon T cell activation *in vivo*. Elevated levels could be observed in both CD4+ and CD8+ T cell compartments, with the highest level of expression after 96hours, according to their activation state (**Supplementary Figure 2**) [19,82]. CD44^{hi} CD8+ T cells did not express CD103. These results highlight that when CD8 T cells are activated their expression of CD103 decreases.

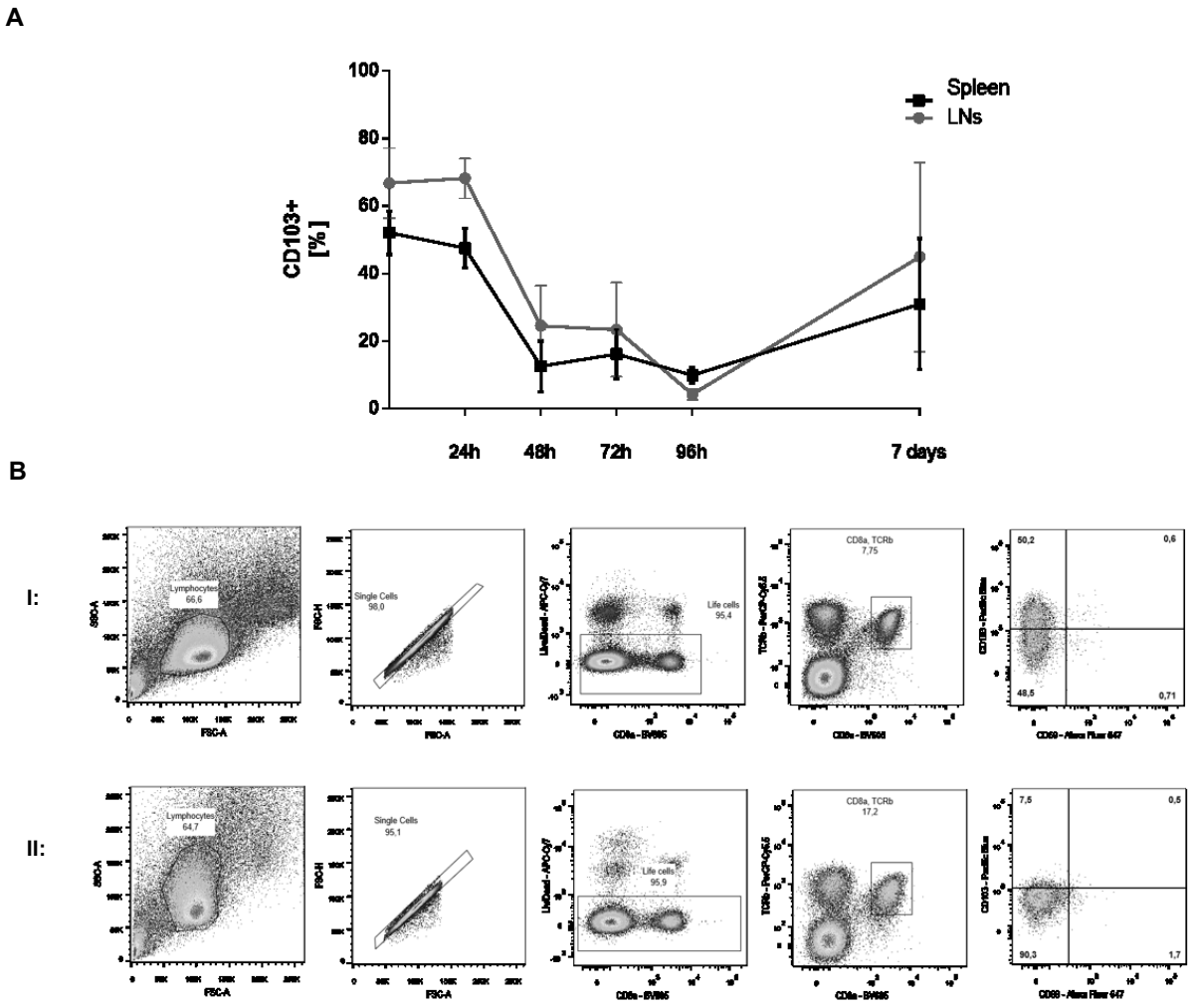

Figure 6: CD103 expression kinetics of CD8+ T cells upon T cell activation of lymph nodes and spleen

A) C57BL6/J mice were injected i.p with anti-CD3 and proportions of CD8+CD103+ T cells were assessed at indicated time-points after injection. Lymphocytes were isolated from spleen and LNs and their respective CD103 expression was assessed by flow cytometry. **B)** representative flow cytometry gating strategy I) at steady-state and II) 96h after *in vivo* activation. First gate excludes irrelevant cell population and cell debris. Doublets were discriminated, it was gated on live, CD8+TCRb+ populations and CD103 expression was assessed, for each sample. Strategy is representative for all measurements of this experiment. Experiments were performed individually (n ≥ 3), error bars indicate their standard deviation.

3.3. Development of CD8+CD103+ T cells

While the role of CD103 expression for a successful Trm cell development and retention is appreciated, their generation is not fully understood. Trm cells may develop from KLRG1- effector precursor cells and upregulate CD103 once they enter the tissues [41]. However, the existence of a potential precursor T cell population of which Trm cells might be generated remains uncertain [20,31]. A powerful way to determine product and precursor relationships is the use of lineage reporter mouse lines [77]. Therefore, we tested if a CD103-Cre mouse (kindly provided by Dr.Tim Sparwasser) could be potentially used to identify Trm cell precursors. The CD103-Cre mouse was crossed to incorporate the Rosa26 locus targeted with the eYFP gene, preceded by a transcriptional stop flanked by two LoxP sites (for reference see 6.2). The CD103-Cre R26-eYFP reporter mouse would allow the detection of all cells that once expressed CD103 by their permanent expression of eYFP (see section 2.1.3). We analysed lymphocytes of different organs of a CD103-Cre reporter mouse-line for their expression of CD103 and eYFP in T cell populations.

3.3.1. Analysing a CD103-cre lineage reporter mouse-line

To assess the potential use of the CD103-Cre lineage reporter mouse line, we aimed to distinguish between cells that express CD103 from cells that downregulated CD103 but once expressed CD103, via staining with anti-CD103 antibodies. Furthermore, this would simultaneously enable us to evaluate the strength of the reporter line by assessing the overlap between acute CD103 expression (antibody staining) and Cre-mediated eYFP expression (**Figure 7A**). Two six weeks old CD103-Cre reporter mice, derived from two individual litters, were analysed for the presence of eYFP reporter protein and CD103 in several organs (thymus, lymph nodes, spleen and IELs). **Figure 7B** shows a representative flow cytometry plot of a

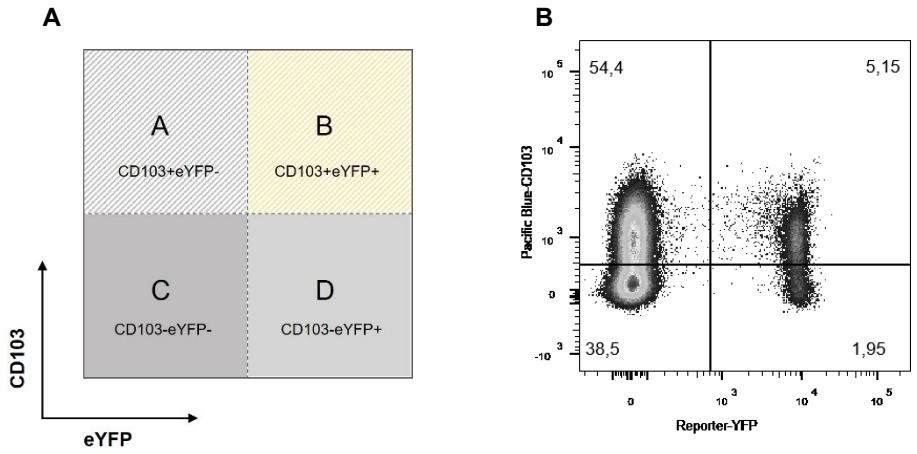

Figure 7: Analysis of CD103-Cre reporter mouse by flow cytometry

CD8+ T cells were isolated from lymph nodes, spleen, thymus and intestine of 3 male CD103-Cre reporter mice (Gt(ROSA)26Sortm1(EYFP)Cos/J) to assess the expression of the eYFP-reporter protein. **A)** Illustration of gating quadrants and respective expression profile. **B)** representative flow cytometry plot of CD8+ T cells isolated from lymph nodes of a CD103-Cre reporter mouse. Plots are representative for biological repeats (n=3).

lymph node sample of one of the two mice. The fluorescent signal of the reporter (eYFP) is exhibited on the x-axis and the signal of the antibody against the CD103 protein on the y-axis. A small population of CD8+ T cells showed an overlapping eYFP and CD103 expression. However, the majority of cells did not express the eYFP protein. As described previously (compare **Figure 5 C**), more than half of CD8+ T cells express CD103 at steady-state. A consistent CD103 expression level was observed in CD8+ T cells of LNs of the reporter mice. However, a large part of the CD103+ compartment did not show an eYFP signal. Similar results were observed in both mice and all organs we sampled. The data strongly suggest that the Cre activity from the CD103 promotor was not sufficiently efficient to accurately mark all T cells that express CD103. Therefore, we abandoned the use of this mouse-line from further lineage tracing experiments.

3.4. CD103 regulation of CD8+ T cells *in vitro*

As observed before, the majority of naive CD8 T cells express CD103. After CD8+ T cells become activated, obtain effector functions and leave SLOs into periphery, CD103 is downregulated (**Figure 6** A). It is thought that CD8+ effector T cells, exposed to specific signals derived from the respective tissue, differentiate into Trm cells and re-express CD103 [34,64]. TGF-β1, expressed by many cells in tissues, has been frequently associated as a key driver for CD103+ expression [37,41,46,83]. In order to develop an *in vitro* system that may recapitulate the generation of CD103+ Trm cells, we tested the effect of TGF-β1 on CD103 expression of naive and effector CD8+ T cells The aim was to assess whether TGF-β1 could regulate the generation of CD8+CD103+ cells and could maintain a stable CD103 expression, also *in vitro*.

3.4.1. Exogenous TGF-β1 induces CD103 expression

CD8+ T cells were isolated and enriched via autoMACS selection from the SLOs of mice and cultured. The cells received TCR/CD28 stimulation and as an additional signal either TGF-β1 at a concentration of 0.5 ng/µl at the start of culture, or not. CD103 protein expression was assessed 24, 48 and 72 hours after culture by flow cytometry. **Figure 8A** shows that CD103 levels remained constant (around 55%) when CD8+ T cells do not receive additional TGF-β1 stimulation, up until 3 days of culture. However, when TGF-β1 is added, the proportion of CD8+CD103+ cells increase. At 48 hours of culture 90% (±1,9, n=3) of CD8+ T cells express CD103 upon TGF-β1 stimulation, whereas the level of control cells remained around 54% (±7,0, n=3). In line with expectations, we observed that TGF-β1 had a positive effect on the proportion of CD103 expressing CD8+ T cells in culture. Next, we sourced activated T cells isolated from lymphocytes 96hrs after anti-CD3 injection. At this time-point, previous kinetic studies showed a low level of CD103 expression making this the ideal time point to obtain CD103-negative CD8+ effector T cells (**Figure 6**).

The isolated CD8+ T effector cells were re-stimulated with anti-CD3/CD28 and cultured for 72 hours with CD103 levels analysed each day. **Figure 8B** shows proportions of CD8+CD103+ T cell populations that either received exogenous TGF-β1 or not. Similar to cultures using naive CD8 T lymphocytes, TGF-β1 had a positive effect on the expression of CD103, with the initial starting level of CD103-expressing CD8+ T cells below 10% (8.7 ± 1.3, n=3). For example, after 48 hours of culture, effector CD8+ T cells show a CD103 expression of 36% (±10.8, n=3), when stimulated with TGF-β1. Whereas the level of CD103 on unstimulated effector CD8+ T remained similar to the starting level (11% ± 9.8, n=3). However, this difference is not significant. Compared to the culture starting with naive CD8+ T cells, TGF-β1 could not induce CD103 expression at a similar level. Also, a constant level of CD103 expression could not be maintained. In both *in vitro* experiments, naive and re-stimulated CD8+T cells, the proportion of CD103 expressing CD8+ T cells is reduced after 72 hours of culture.
Overall, our results indicate a positive impact of TGF-β on CD103 expression *in vitro*, especially on naive CD8+ T cells

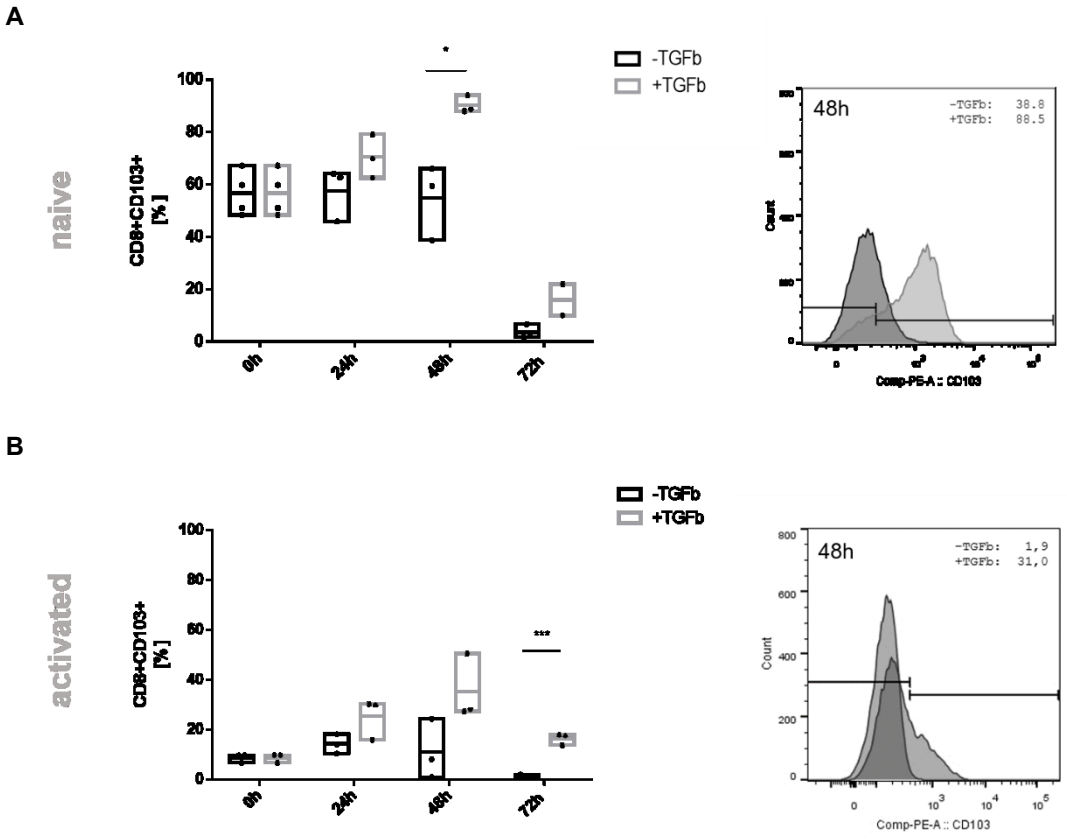

Figure 8: Effects of TGF-β1 on CD103 expression on CD8+ T cells in vitro

A) autoMACS enriched CD8+ Tcells of naive C57BL6/J mice were cultivated, *in vitro* activated and either received TGF-β at a starting concentration of 0.5 ng/µL, or not. The level of CD103 at indicated time of cultivation was assessed by flow cytometry. **B)** C57BL6/J mice were i.p injected and activated via anti-CD3 and CD8+ T cells were autoMACS 96h later. CD8+ T cells were cultivated, *in vitro* re-activated and received TGF- or not. Representative histograms (right) illustrate CD103 expression at 48h of TGF-β1 stimulated (light grey) or unstimulated (dark grey) CD8+ T cells. Experiments were performed individually (n=3). Bars indicate calculated mean standard error and. Statistical significance was determined via unpaired students t-test (*P-value<0.05, ***P-value<0.001, no indication=not significant).

3.5. Expression analysis of transcription Eomes during CD8 T cell activation

For the successful generation of CD8+ Trm cells, studies have shown that it is necessary to repress two transcription factors, T-bet and Eomes. While T-bet expression remains at a low level, Trm cells of different tissues have been found to completely abolish Eomes expression. The suppression of both transcription markers has been linked to tissue-maintenance and longevity [46,50]. On a molecular level, suppression of T-bet enables CD103 expression, possibly regulated in a TGF-β dependent manner [45]. With the following experiments we wanted to determine whether Eomes repression could be linked to TGF-β1 in a similar way and whether changes in CD103 expression would be predictable for changes in Eomes expression during Trm cell development.

Therefore, we first assessed Eomes expression upon CD8+ T cell activation *in vivo*. As

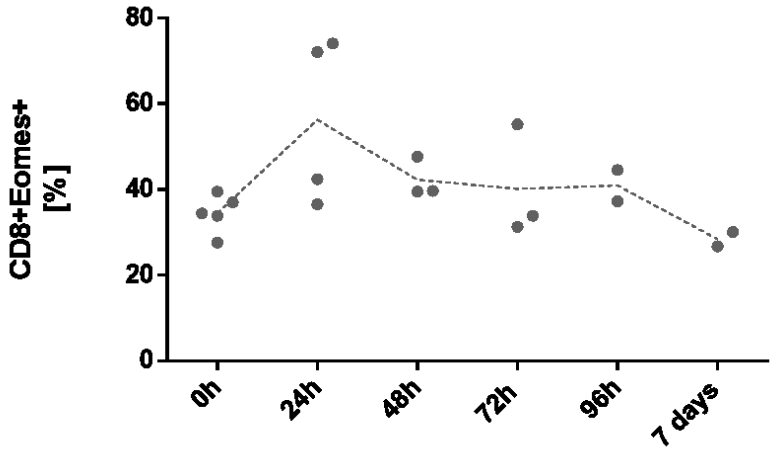

Figure 9: Expression of Eomes in total CD8+ T cells upon in vivo activation

C57BL6 mice were injected with anti-CD3 i.p. and at indicated time-points lymphocytes were isolated from lymph nodes. Intranuclear Eomes expression of total CD8+ T cells was assessed by flow cytometry. For the analysis, doublets were excluded, gated on live cells, TCRβ+ CD8+ and Eomes+ population were assessed. Expression kinetic illustrated as dotted line at calculated mean of indicated time points. Experiments were performed individually (n ≥ 2)

described previously, upon T cell activation levels of CD103 expression change on CD8+ T cells in SLOs markedly, showing a drop in CD103 expression after 24 hours of T cell activation (**Figure 6**). When the Eomes expression on total CD8+ T cells was assessed upon T cell activation, we could not observe similar changes in Eomes expression. The mean expression level of Eomes on total CD8+ T lymphocytes over time appears similar to none-stimulated controls, as the expression kinetics in **Figure 9** illustrate. On average 34% (± 4.0, n=5) of all CD8+ T cells display Eomes expression at steady-state. Upon activation, at time-point 24 hours after T cell activation, there is a slight increase in Eomes expressing CD8+ T cells observed (56% ±19.9, n=4). However, this is back to baseline expression levels after 48 hours.

Next, to question whether expression kinetics are different in CD103 expressing T cells compared to not CD103 expressing T cells, we assessed Eomes expression on exclusively

CD103+ CD8+ T cell populations. Naive CD8+ T cells expressing CD103 show a significantly (*P <0.05, n= 5) lower Eomes expression than CD103-CD8+ T cells. As shown in **Figure 10A**, around 10% (± 0.6, n=6) of naive CD8+CD103+ T cells express Eomes (*indicated as CD103+*). The majority (56% ± 2.8, n=6) of Eomes expression was assessed in CD8+ that did not express CD103 (*indicated as CD103-*). Upon T cell activation Eomes expression remains low on CD8+CD103+ T cells (**Figure 10 B**). 24 hours after T cell activation 28% (±5.6, n=4) of CD8+CD103+ T cell populations express Eomes. Whereas at 96h after T cell activation low Eomes expression was assessed (1.8% ± 0.4, n=3).

Next, we wanted to test whether Eomes expression could be repressed by TGF-β1 *in vitro*. When naive CD8+ T cells were *in vitro* activated and received exogenous TGF-β stimulation, their expression levels were significantly lower than the controls which did not receive TGF-β. After 48 hours of cultivation, we observed less Eomes expression on CD8+ T cells that received TGF-β1 (30% ± 2.4, n= 3), compared to controls (61% ±7.3, n=3), as illustrated in **Figure 11**.

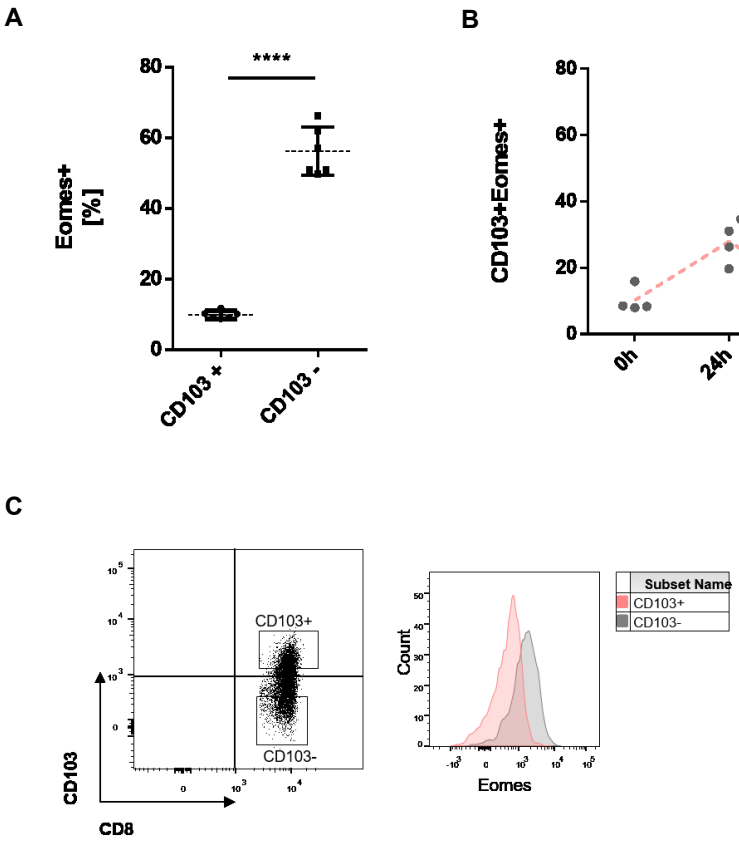

Figure 10: Differential Eomes expression in naive CD8+CD103+ T cells and upon activation

C57BL6 mice were injected with anti-CD3 i.p. and at indicated time-points lymphocytes were isolated from lymph nodes. **A)** Eomes expression levels in CD8+CD103+ T cells and CD8+CD103- T cells of naive CD8+ and **B)** Eomes levels of CD103+ CD8+ T cell populations upon T cell activation, were analysed by flow cytometry Experiments were performed in individuals (n≥2), calculated mean is indicated as dashed line and standard deviation as error bars. Statistical significance was determined via unpaired students t-test (****p-value<0.0001) **C)** representative flow cytometry gating strategy to determine CD103+ and CD103- populations of naive CD8+ T cells and assess respective Eomes expression levels in CD8+CD103+ (rose) and CD8+CD103- (grey) T cell populations as histogram. Flow cytometry plots are representative for all biological repeats.

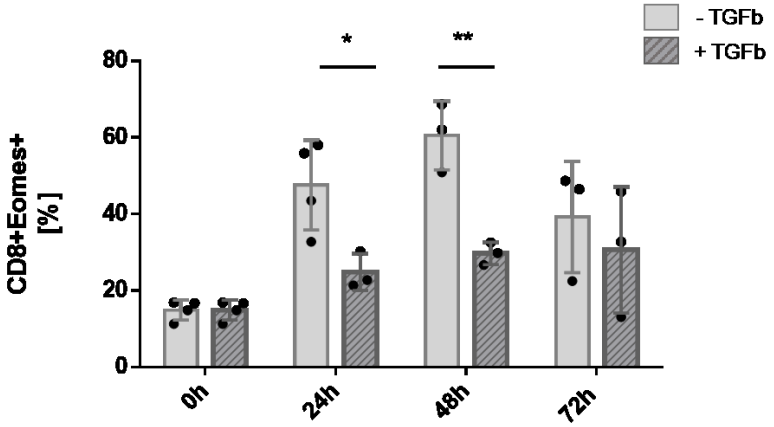

Figure 11: Levels of Eomes expression of in vitro cultivated naive T cells

CD8+ T cells of naive C57BL6/J mice, enriched by autoMACS, were cultivated, *in vitro* activated and either received TGF-β at a starting concentration of 0.5 ng/µl, or not. The level of Eomes expression in total CD8+ T cells at indicated time of cultivation was assessed by flow cytometry. For analysis, gating strategy was applied as in Figure 9. Experiments were performed in individuals (n≥3). Bars indicate calculated mean and standard deviation as error bars. Statistical significance was determined via unpaired students t-test (*p-value<0.05, **p-value<0.01)

In summary, we observed differences in Eomes expression kinetics of CD8 T expressing CD103, compared to CD8+ T cells that do not express CD103 upon T cell activation. CD103+ CD8+ T cell populations show significantly lower levels of Eomes expression at steady state and upon T cell activation CD8+CD103+ T cells remain low Eomes expressions. *In vitro* CD8+ T cells express significantly lower levels of Eomes when receiving exogenous TGF-β1. This indicates an inverse relationship of Eomes and CD103 expression enhanced by TGF-β1 stimulation.

3.6. Testing the influence of the microenvironment on Trm cell development *in vitro*

In order to develop an *in vitro* system to study Trm development, the major aim of this project was to test which molecular cues could drive CD8 T cells to a CD103+CD69+ tissue resident phenotype. In both naive and effector CD8+ T cells we observed that TCR/CD28 stimulation and exogenous TGF-β1 could each contribute to a Trm cell phenotype, inducing CD69 or CD103 expression respectively.

The microenvironment contains antigens, cytokines, chemokines and other tissue-specific signals provided by the surrounding cells, thereby affecting the development of Trm cells [67]. Trm cells must adapt to specific niches and interact with the local environment, including other immune cells [18,40]. While *in vitro* cultivating naive CD8+ and effector CD8+ T cells with individual extrinsic factors in controlled concentrations allowed us to monitor specific effects, in the following experiments we aimed to do so in a more complex way. By co-culturing with other immune cells, we wished to test if Trm cell development *in vitro* could be enhanced, more

closely mimicking the *in vivo* situation.

3.6.1. Analysis of a BMDC-CD8 co-culture

While dendritic cells as major antigen-presenting cells are required during the process of T cell priming, comparably less is known about their role in Trm cell formation. To evaluate a possible supportive role of antigen presenting cells during Trm cell development, we co-cultured BMDCs with naive CD8+ T cells and observed the simultaneous expression of Trm markers CD69 and CD103.

Naive CD8+ T cells were activated *in vitro* by plate-bound anti-CD3 and cultured with BMDCs, thereby receiving a co-stimulatory signal. As control, naive CD8+ T cells were cultured receiving *in vitro* activation by anti-CD3/anti-CD28. We observed similar over all expression levels of CD103 and CD69 on CD8+ T cells cultured with BMDCs, compared to the controls. After 48 hours of co-culture 79% (± 12.0, n=3) of CD8+ T cells display CD103 expression, while the controls show a population of 90% (±2.7, n=3) CD8+CD103+ T cells (**Figure 12A**). At the same time of culture, the level of CD103+CD69+ T cells is 69% (±18.4, n=3) in co-cultures of CD8+ T cells with BMDCs, compared to 64% (±10, n=3) in controls (**Figure 12B**). Furthermore, we observed a trend of slightly more expression of CD103 and CD69 when T cells were co-cultured with BMDCs, after 72h (42.5% compared to 16%; n=2), however, more repeats need to be performed to confirm this.

Figure 12B illustrates the gating used to obtain these data. In summary, our findings suggest that BMDCs provide necessary co-stimulation signals *in vitro* and can induce a similar phenotype as CD8+ T cells activated by anti-CD3/anti-CD28. However which additional cellular regulatory effect dendritic cells have on Trm cell formation, needs to be further investigated.

3.6.2. Tregs can substitute for exogenous TGF-β1

Tregs are a special subtype of CD4+ T cells, that are associated with a suppressive and regulatory role during immune responses and are able to support circulating CD8 memory T cell formation [49]. Moreover, they produce high levels of TGF-β1, which is thought to be critical for their own development and function, but could possibly have an effect on CD8 T cells as well [84,85]. A role during Trm cell formation, however, is unknown. Therefore, we wished to test if Tregs could contribute to CD8+ Trm cell formation. Having explored the supportive role of BMDCs on CD8+ T cell activation, we subsequently investigated triple cultures of BMDC, Treg and CD8+ T cells, after which the expression of CD103 and CD69 was assessed on CD8+ T cells.

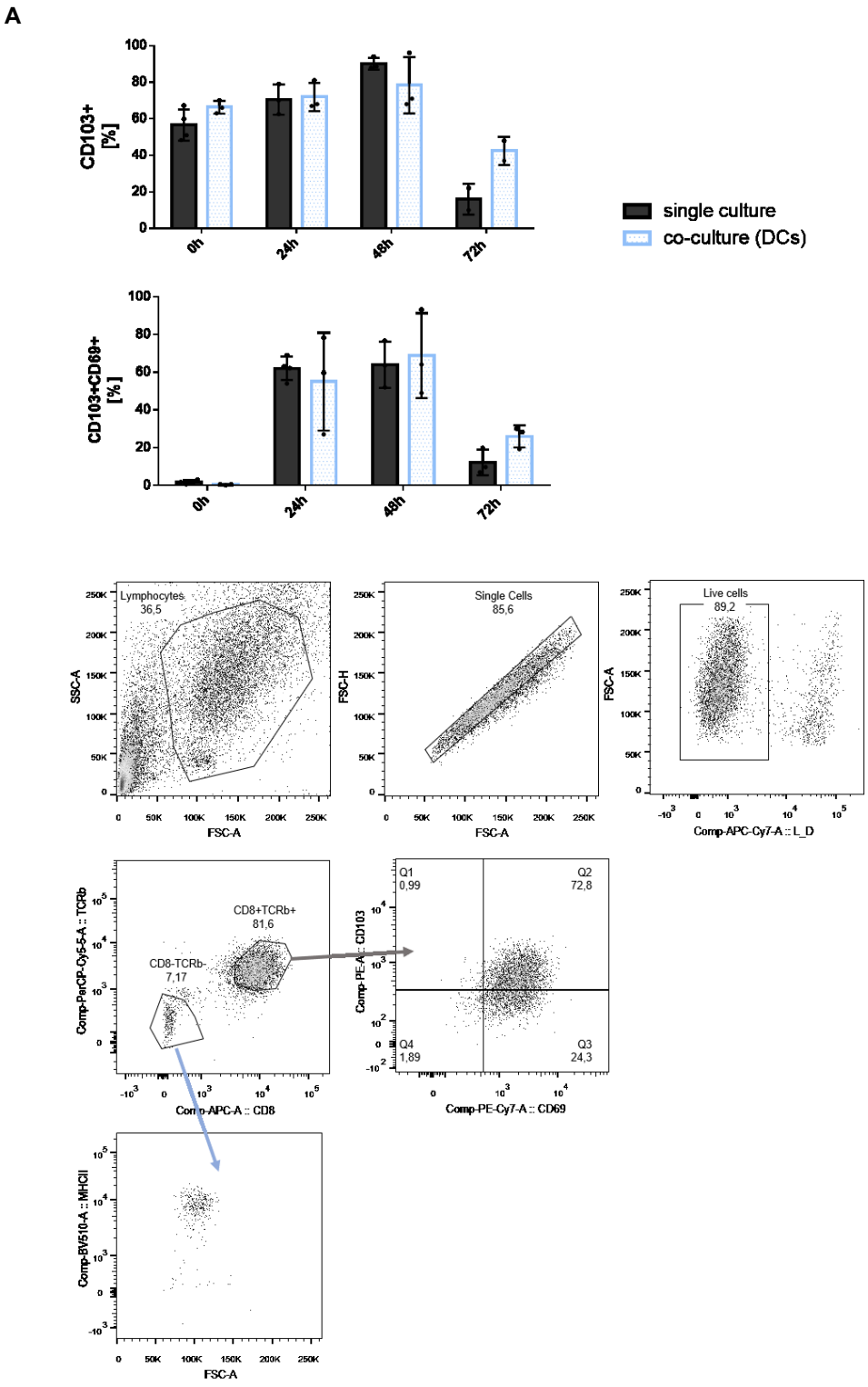

Figure 12: Analysis of the effect of co-cultured BMDCs and CD8+ T cells on CD103+CD69+ expression

CD8+ T cells, isolated from naive C57BL6/J mice and autoMACS enriched *in vitro* by anti-CD3 and co-cultured with BMDCs at a concentration of 2:1, receiving TGF-β1 (0.5 ng/µl). Control CD8+ T cells were in vitro activated by anti-CD3/CD28 and not co-cultured, receiving TGF-β1. **A)** Expression of CD103 and **B)** co-expression of CD103+CD69+ was assessed by flow cytometry. Bars indicate calculated mean and standard error. Experiments were performed individually (n ≥ 2). **C)** representative gating strategy of a co-cultured CD8+ T cells and BMDCs, at 48h after culturing. Cell Debris and doublets were excluded, live CD8+ T cells were gated on TCRb+ and CD8+, CD103+ were plottet against CD69+ T cells and CD8- populations were plotted against MHCII+. Flow cytometry plots are representative for individual all biological repeats.

Since different cell compositions could have an effect on Trm cell development, we cultured the three cell types in different proportions. Different cell compositions did not result in significantly different levels of CD103 and CD69 expressing CD8 T cells over time. Therefore, a ratio of 1:1:1 for future co-culturing experiments was used in subsequent experiments (**Figure 13**).

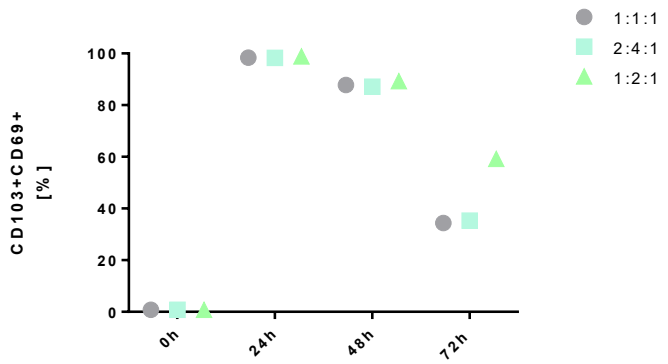

Figure 13: Levels of CD103+CD69+ CD8 T cells co-cultivated with BMDCs and Tregs in different proportions

CD8+ T cells and CD4+CD25+ T cells from C57BL6 mice were autoMACS enriched *in vitro* activated and co-cultured with BMDCs. The three cell types were cultured in different cell proportions. BMDCs, Tregs and CD8+ T cells in either 1:1:1, 2:4:1 or 1:2:1 and received TGF-β1 stimulation (0.5 ng/µl). Proportions of CD8+CD103+CD69+ T cells were assessed by flow cytometry following a gating strategy as described in Experiment was performed once (n=1), symbols indicate different compositions.

Next, we investigated the role of Tregs as TGF-β producing cells on the expression of Trm marker *in vitro*. We hypothesized a positive effect in relation to the ability of Tregs to produce TGF-β1. Therefore, naive CD8+ T cells were co-cultured with BMDCs and Tregs. The cells were *in vitro* activated but did not receive exogenous TGF-β1 signal. As a control, the same naive CD8+ T cells were single cultured, receiving *in vitro* activation plus exogenous TGF-β signal. We compared level of CD103 and CD69 expressing CD8+ T cells at indicated time-points of culturing. **Figure 14** shows the proportion of CD103+CD69+ CD8+ T cells in a triple co-cultivation over time, compared to single cultured CD8+ T cells. The control, naive CD8+ T cells are *in vitro* activated but not receiving exogenous TGF-β1, shows the highest level of CD103+CD69+ expression is assessed after 24 hours of cultivation (40% ±15, n=3), however when co-cultivated with Tregs it reaches 70% (±16, n=2). After 48 hours of culturing, 77% significantly higher than the control (14%±8.7, n=3). This suggests that adding Tregs *in vitro* has a supporting effect on CD103+CD69+ expression on CD8+ T cells. When naive CD8+ T cells (±9.3, n=2) of CD8+ cultured with BMDCs and Tregs express CD103 and CD69, which is are *in vitro* activated and receive TGF-β signal, they do express CD103+ and CD69+ (**Supplementary 4**). We observed that CD103+CD69+ expression levels in a co-culture with BMDCs and Tregs are at similar levels as the ones induced by TGF-β1 on CD8+ T *cells in vitro*. To determine the effect of other antigen presenting cells in combination with Tregs, we additionally co-cultured CD8+ T cells, Tregs and B cells instead of BMDCs. This resulted in a lower

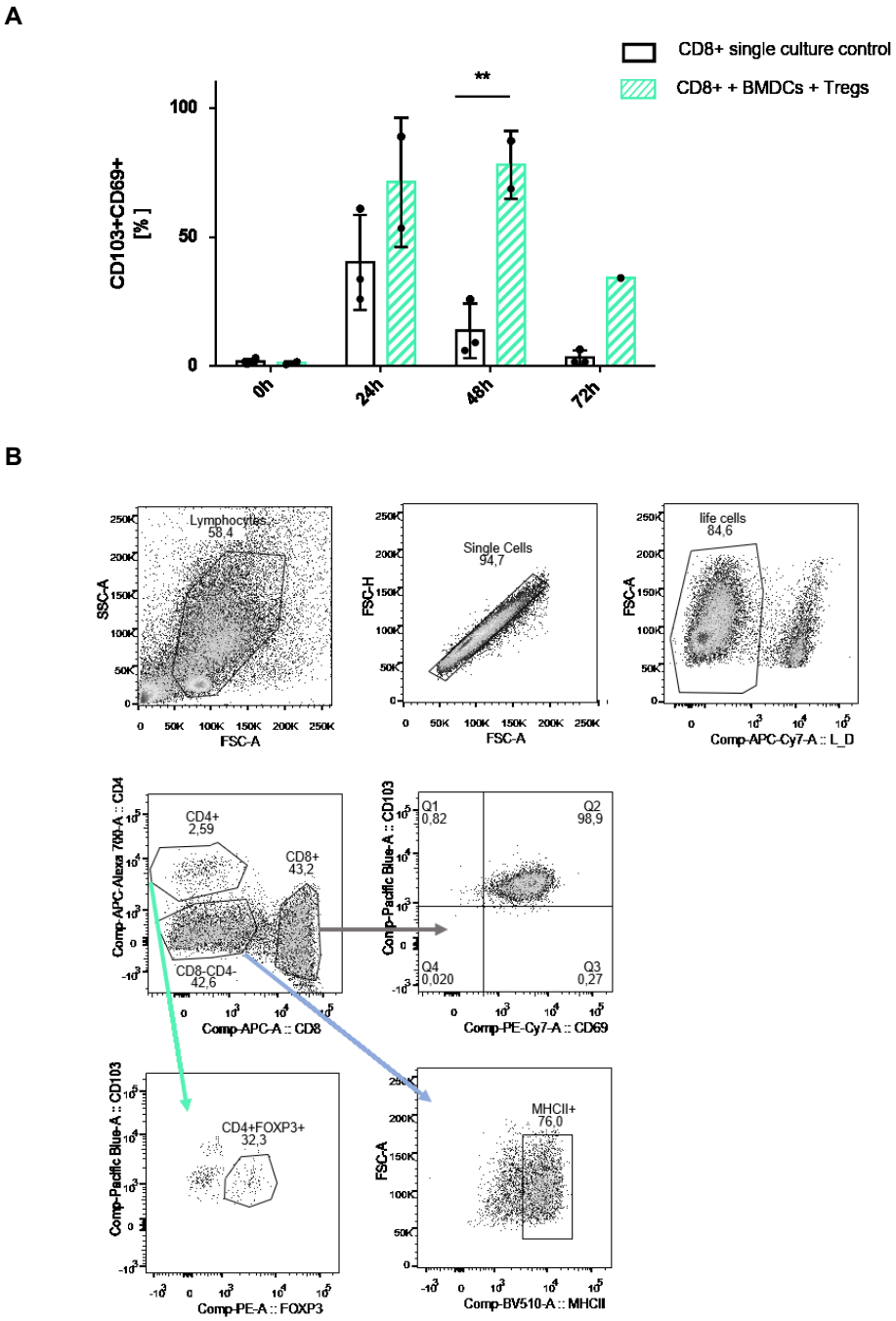

Figure 14: Level of CD103+CD69+ CD8+ T cells co-cultured with BMDCs and Tregs

Expression analysis of Trm surface markers on CD8+ T cells when co-cultured with BMDCs and Tregs, not receiving exogenous TGF-β1. **A)** naive CD8+ and CD25+ T cells are isolated from naive C57BL6/J mice, autoMACS enriched, activated *in vitro* by anti-CD3 and co-cultured with BMDCs at same concentration. As control naive CD8+ T cells were activated *in vitro* and single cultured. Expression of both CD103 and CD69 on T cells was assessed by flow cytometry. Gating as described below. Bars show calculated mean and snandard error. Experiments were performed individually (n ≥ 2). Statistical significance was determined via unpaired t-test (**P-value < 0.01, no indication = not significant). **B)** representative gating strategy of co-cultured CD8+ T cells, BMDCs and Tregs, after 48h. Cell Debris, doublets and dead cells were excluded, CD8+ T cells were plottet against CD69+ and CD103+, CD4+ T cells were analysed for FOXP3 expression and CD8-CD4- cells were plotted for MHCII+. Flow cytometry plots are representative for biological repeats.

proportion of CD103+CD69+ CD8+ T cells (**Supplementary Figure 5**).
Taken together, these results suggest a positive tendency on the expression of Trm cell marker when CD8+ T cells are co-cultivated with BMDCs and Tregs *in vitro*. Even though CD8+ T cells did not receive exogenous TGF-β1 stimulation, they show high proportions of CD8+ CD103+CD69+ T cells. This might suggest that Tregs substitute for exogenous TGF-β1 *in vitro.* However, these results should be handled with care, since the experiments were only performed twice. Whether and how our observations can be concluded into a regulatory impact of Tregs on Trm cell differentiation, needs to be further investigated.

4. Discussion

While the role of Trm cells in immune surveillance and regulation is already well established, detailed information about their development and formation is still lacking. So far, Trm cells have been identified in almost all epithelial tissues, such as lung, gut and skin, but also in the liver and in the brain [32,34,41,86,87]. Trm cells show a phenotype that distinguishes them from other CD8+ memory subtypes, featuring self-renewal and long-term residency. A large population of all CD8+ Trm cells display the co-expression of surface markers CD103+CD69+, both linked to tissue retention [32]. As they are antigen experienced, they respond quickly and specific upon re-infection, which makes them promising targets for therapeutic approaches [21,30,88]. However, in order to effectively use Trm cells for future therapies, e.g. for vaccination, better understanding regarding their differentiation, regulation patterns and maintenance, is needed. Effector CD8+ T cells, previously primed in SLOs, circulate the body, are directed to the site of infection and targeted to eliminate its source. Regarding Trm cell development, it is very likely that an effector precursor cell, possibly KLRG1-, receives specific tissue-derived signals to eventually differentiate into a Trm cell [34,41]. Some extrinsic effector molecules have been shown to be beneficial for their development, such as local inflammation signals and cytokines like TGF-β [43,46,89]. However, many other possible differentiation-factors are not known and there are many open questions regarding the maintenance of Trm cells.

Hence, we aimed to study Trm cell formation by analysing expression of the cell surface markers CD103 and CD69 *in vitro*. By testing the impact of cytokines, including IL-15 and TGF-β, and the role of secondary TCR stimulation, we were attempted to define a system in which T cells with a Trm cell phenotype develop.

4.1. Inducing CD69 and CD103 expression *in vitro* on CD8+ T cells

One of the questions we were aiming to answer regarding Trm cell development is which specific factors are responsible for the upregulation of Trm cell surface markers CD69 and CD103 *in vitro*. The cytokine IL-15 has been shown to have a supporting effect on the formation and long-term maintenance of memory T cells and was implied to have a homeostatic effects on Trm cells [89–91]. Furthermore, it was recently shown that mice deficient in IL-15 production had diminished capacity in forming Trm cells *in vivo* and *in vitro* [65]. Therefore, we wanted to investigate the effect of IL-15 on naive and effector CD8+ T cell survival *in vitro*. In cultures supplemented with IL-15, we observed similar levels of cell viability compared to IL-2. Since IL-2 is known to improve cell proliferation on T cells *in vitro*, the substitution with IL-15 suggested a similar role in T cell survival (**Supplementary Figure 1 B**) [17,21]. Additionally, we observed that IL-15 stimulation did not alter CD69 expression of CD8+ T cells *in vitro* (**Figure 3 A**). Although this does not imply that IL-15 is not critical for Trm development, our observation indicates that it alone does not induce CD69 expression and additional factors are required. For example, it is an on-going debate whether local antigen presentation is needed for Trm cell formation [64,66].

Previously, it has been shown that *ex vivo* obtained effector CD8+ T cells exhibited CD69 expression when cultivated with a cytokine combination of IL-33 and TGFβ, suggesting an antigen-independent regulation of Trm cells in skin [42] . For other tissues, including the gut and the female reproductive organ, it was suspected that local antigen recognition is not necessarily required for Trm cell differentiation [42,65,54]. Conversely, it has been shown that secondary antigen stimulation promotes effector CD8+ T cells to express CD69 in the skin, suggesting an enhancement of Trm formation and maintenance [63]. Moreover, Khan et. al have shown that activated CD8+ T cells rapidly re-express CD69, induced by TCR mediated signalling, but only in the presence of local antigen [64]. In agreement with these findings, we saw that CD69 levels on effector CD8+ T cells increased upon *in vitro* activation. In fact, when activated CD8+ T cells received a second TCR/CD28 stimulation *in vitro*, CD69 levels were significantly upregulated until 3 days of cultivation, compared to cells without secondary stimulation (**Figure 3 A**). On a molecular level, it is known that during T cell priming CD69 antagonizes S1Pr1, a receptor needed to follow migration signals, and thereby prevents egress from lymph nodes [18,38]. By re-encountering antigen in non-lymphoid tissues and inducing CD69 expression, a similar retention mechanism could be suggested [34]. Though it has been shown that other factors, such as TGF-β, IL-33 or TNF indirectly interfere with S1Pr1 expression as well, suggesting alternatives routes towards tissue maintenance [39].

To summarize, it appears that CD69 expression on effector CD8+ T cells can be stimulated by antigen. However, whether it is necessary for Trm development, is still debatable. Depending on the tissue and the presence of antigen and specific cytokines, different mechanisms might be responsible for Trm cell differentiation and their maintenance. In the end, we could observe *in vitro* upregulation of Trm cell surface marker expression in both TCR-dependent and independent ways.

One example by which tissue retention is governed in an antigen-independent manner, is by the expression of CD103 [41,64]. The integrin CD103 is universally expressed on tissue-residing lymphocytes, such as IELs. Since its main ligand is E-Cadherin, which is expressed on epithelial cells, the constitutive expression of CD103 is thought to ensure tissue retention [32,34]. One factor, that has been shown to induce CD103 expression on lymphocytes, is the transforming growth factor TGF-β1, which is present in many epithelial tissues [45]. Previously, Mackay et. al showed that CD8+ T cells of mice lacking the TGF-β1 receptor gene had a significantly reduced ability to upregulate CD103 [37,41]. Additionally, TGFβ1 was able to induce CD103 expression *in vitro* on primed CD8+ T cells [42]. Therefore, we asked whether TGF-β1 stimulation was necessary to induce and maintain a CD8+CD103+ phenotype *in vitro*.

As expected, we could observe a higher proportion of CD103 expressing naive CD8+ T cells when cultured in the presence of TGF-β1. But we also saw that the majority of naive CD8+ T cells express CD103. Hence it cannot be said whether TGF-β1 directly induced CD103 expression or whether proliferation and survival of CD103+ T cells was supported (**Figure 8 A**)

Nevertheless, effector CD8+ T cells showed initially very low levels of CD103 expression when cultured. There, we could observe CD103 upregulation *in vitro* as well when exogenous TGF-β1 was provided. Therefore, our findings are in line with literature, suggesting a positive effect of TGF-β1 on CD103 expression on CD8+ T cells (**Figure 8**). Nonetheless, a stable level of CD103 expressing cells could not be maintained for longer than 3 days. After 3 days of cultivation, both naive and re-stimulated effector CD8+ T cells showed reduced proportions of CD103 expression *in vitro*. The reason for this observation is unclear. Since TGF-β1 was only given at a single concentration at the beginning of cultivation its effect might differ if supplemented at a higher starting concentration or later during culturing.

4.2. Differential CD103 expression on CD8 T cell subtypes and upon T cell activation

How and from which precursors Trm cells derive, is one of the major questions regarding Trm development. It has been previously shown that Trm cells are generated from certain (mainly KLRG-) precursor memory T cell populations [41,62]. According to the findings of L.Mackay and others, it is strongly believed that circulating memory precursor cells receive tissue-derived signals and differentiate into non-circulating Trm cells, by upregulating Trm surface markers for tissue retention, such as CD103 [45,92]. Supporting literature, we found that circulating T cells exhibited lower CD103 expression. In fact, we observed that the majority of CD103 expressing T cells were naive CD8+ T cells, while CD8+ memory T cells (Tcm and Tem) showed low levels CD103 expression (**Figure 5**). Additionally, upon T cell activation CD103 was downregulated on CD8+ T cells and had a low level of CD44 co-expression, which confirms the phenotypically differences to circulating effector T cells (**Supplementary Figure 3**). As literature suggests, when effector CD8+ T cells receive specific tissue-derived signals, such as TGF-β1, they can acquire a CD8+CD103 Trm phenotype, which we confirmed with the *in vitro* studies, described above (**Figure 8**) [20,41,46]. While dynamic regulation of CD103 expression on CD8+ T cells is observed, the exact mechanisms underlying the differentiation pathway of CD8+CD103+ Trm cells, are still not fully understood [20,62]. It remains unclear whether Trm cell development follows the classical effector-memory formation and when a Trm precursor cell is generated [31]. For example, very recent findings suggest that already naive CD8+ T cells are preconditioned by dendritic cells in lymph nodes for a CD103+ Trm cell phenotype [73]. Additionally, Trm cells that can derive from KLRG1+ effector precursor CD8+ T cells, have also been proposed [93]. In contrary to previous beliefs that Trm cells are classically formed from memory precursors, both findings imply developmental plasticity during Trm differentiation [41,61,73,93].

4.3. Expression of transcription factor Eomes on CD8+CD103+ T cells

As stated before, Trm cells are phenotypically different than circulating memory T cells, which is reflected in their transcriptional landscape. Besides showing higher expression levels of the

retention markers CD103+ and CD69+, they exhibit low expression levels of the transcription factors T-bet and Eomes [46,52]. T-bet and Eomes are both involved in T cell differentiation after priming. Both transcription factors are highly expressed in circulating effector and memory CD8+ T cell sub-populations [53,54]. Upon T cell activation, we observed only a mild upregulation of Eomes expression in CD8+ T cells. At later stages of T cell activation, the percentage of Eomes expressing cells remains at rather constant levels, suggesting similar expression levels among circulating CD8+ T cell subtypes (**Figure 9**).

Previous findings showed that repression of both T-bet and Eomes is necessary to form CD8+CD103+ Trm cells in skin, with an important contribution of TGF-β1 [46]. Shortly before, it was found that TGF-β1 induced signal transduction resulted in downstream Smad3 activation and consequently lead to CD103 expression [59]. Consequently, it was thought that T-bet interferes with the Smad3-dependent transcriptional activation of CD103 and that T-bet repression in Trm cells is ultimately mediated by TGF-β1 [54,58]. Taken these findings into account, it seems likely that the suppression of Eomes, a homologue of T-bet, could work in a similar way. We found differential Eomes expression levels in CD8+ T cells, regarding their CD103 expression. In fact, CD103 expressing naive CD8+ T showed significantly lower Eomes expression levels than CD8+CD103- T cells (**Figure 10 A**). Additionally, upon T cell activation Eomes expression remained low for CD103 expressing T cells (**Figure 10 B**). Moreover, CD8+ T cells that were *in vitro* activated and received exogenous TGF-β1 signal, exhibited higher levels of CD103 expression but their Eomes expression levels remained significantly lower than non-treated controls (**Figure 11**). Thus, our findings support previous studies, suggesting transcriptional downregulation of transcription factor Eomes in CD8+ T cells in order to express CD103, most likely in a TGF-β dependent manner. However, complete suppression of Eomes, as it is observed in Trm cells, could not have been achieved by TGF-β1 stimulation *in vitro* [46].

4.4. Effects of CD8+ T cell *in vitro* co-cultured with dendritic cells and Tregs

When Trm cell develop and seed in their respective tissue, they adapt to their given environment. Different niches of the body provide different factors that influence Trm cell maintenance, which ultimately results in their phenotypical heterogeneity [40]. The effect of TGF-β1 on CD103 expression links Trm cell regulation to their microenvironment. It has been demonstrated that dendritic cells enzymatically activate TGF-β1 from a latent into an active form [71]. By providing active TGF-β1 and inducing CD103 expression, dendritic cells are believed to facilitate Trm cell maintenance and precondition their development [70,73]. In different epithelial tissues, Trm cells were observed to form clusters with antigen presenting cells, including dendritic cells, suggesting important cross-interactions [94,95]. Ultimately, by providing co-stimulation and cognate antigen presentation, dendritic cells can re-stimulation Trm cells and are likely involved during differentiation [69,73]. After we determined that *in vitro* TCR/CD28 stimulation successfully leads to expression of CD69 on CD8+ T cells (**Figure 3**), we asked

whether dendritic cells could substitute for successful co-stimulation. By co-cultivating BMDCs with naive CD8+ T cells, we observed that BMDCs were providing co-stimulatory signals to activated naive CD8+ T cells, resulting in the expression of CD69 and CD103 at similar levels compared to *in vitro* controls (**Figure 12A**). Since dendritic cells are major antigen presenting cells, their supportive role during T cell activation *in vitro* might be expected. However, we observed interesting results regarding Trm cell differentiation, when Tregs were added to the co-culture. Besides their immunosuppressive function, which Tregs are known for, they are also involved in CD8 memory formation during T cell priming [74]. Moreover, Tregs have been shown to be TGF-β1 producers which was recently suggested to benefit Trm formation in the brain [84,96]. We were aiming to test this potential role on Trm cell differentiation *in vitro*. Therefore, CD25+Foxp3+ Tregs were added to the co-culture, without exogenous TGF-β1 supplementation. Interestingly, we still observed a much higher expression of CD103 and CD69 on CD8+ T cells, compared to the single cultured controls not receiving TGF-β1 (**Figure 14A**). In fact, expression levels were similar to previously cultured naive CD8+ receiving *in vitro* activation and exogenous TGF-β1 (**Supplementary 4**). This could suggest that Tregs are producing TGF-β *in vitro* which affects CD103 expression on CD8+ T cells. Though, it must be mentioned that this was a preliminary study, which would be necessary to be repeated in order to generate more data. Also, to confirm TGF-β production by Tregs and transcriptional changes in CD8+ T cells. Nevertheless, from the observations we have there is a trend that regulatory T cells support the expression of Trm cell markers *in vitro*. Additionally, it was previously shown that dendritic cells can induce Treg functions in a TGF-β dependent manner in the intestine [72]. When dendritic cells were exchanged by B cells and co-cultivated with CD8+ cells and Tregs, we did not assess comparable levels of Trm cell expression markers (**Supplementary Figure 5**). Therefore, a supporting role of regulatory T cells on CD8+ Trm cells, possibly in combination with dendritic cells, could be hypothesized. Future investigation, both *in vivo* and *ex vivo* to determine cell clustering of dendritic cells and Tregs to confirm regulatory effects on Trm cells, would provide answers regarding their cross-interaction.

Concluding remarks and outlook:

Our health relies on complex mechanisms the immune system has developed. With intensive research, many of these mechanisms are nowadays well understood and used to our benefit in order prevent diseases [1,5]. But there is yet a lot to discover. Without question, Trm cells play an important part of our adaptive immune system. With their unique phenotype and antigen specific functions Trm cells are a central role of both basic and adaptive research [88]. However, in order to use Trm cells for therapeutic approaches, their differentiation mechanisms and cellular responses need to be fully understood. Studying CD8+ T cells *in vitro* and elaborate extrinsic effects on their development in a reconstructive way could be useful. Even though, keeping a stable Trm cell phenotype proved to be difficult *in vitro*, with our observations we could support

literature regarding the beneficial effects of some extrinsic factors on Trm differentiation. Summarizing, TCR/CD28 induced signalling on CD69 expression in effector CD8 T cells could suggest the antigen-dependent regulation of Trm cell development [64]. However, an alternative route of differentiation is suspected, at least in some tissues [42,65]. Furthermore, it was shown that TGF-β1 could induce elevated levels of CD8+CD103+ cells *in vitro*, which confirms its potential in Trm cell differentiation and tissue retention [41].

Additionally, we hypothesize a potential role of Tregs, possibly in combination with dendritic cells, to regulate CD8+ Trm cell formation in tissues which could be interesting to further investigate. Varying cytokines, the availability of local antigen and other unknown signals are suggested to create Trm cell populations with different phenotypes and requirements [54]. Hence, we further emphasize other tissue-specific signals, some of them yet to be discovered, driving Trm cells into such a heterogenic population. Altogether, there could be different molecular mechanisms for Trm cell development and regulation, that future research will adress. In the end, how Trm cells are created and which cellular interactions and factors define their differentiation, should be considered when studying Trm cells for applied research in order to develop Trm based therapeutic approaches.

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6. Appendix

6.1. List of abbrevations

Ab	Antibody
α-	refers to “anti”, as in antigen specific antibody
ACK	Ammonium-Chloride-Potassium
APC	Antigen presenting cell
BM	Bone marrow
BMDCs	bone marrow – derived dendritic cells
CD	Cluster of differentiation
Cre	Cre recombinase
DC	dendritic cell
eYFP	enhanced yellow fluorescent protein
FOXP3	Forkhead box protein 3
IEL	intraepithelial lymphopcyte
IFN-γ	Interferon-gamma
IL	Interleukine
ILC	Innate lymphoid cell
LN	lymph node
mAb	monoclonal Antibody
MAIT	Mucosal associated invariant T cells
NKT	natural killer T cells
RT	room temperature
TCR	T cell receptor
Tcm	central memory T cell
Tem	effector memory T cell
TF	transcription factor
TGF-β	transforming growth factor beta
TNF-α	tumour necrose factor alpha
Treg	regulatory T cell
Trm	tissue resident memory T cell
PBS	phosphate-buffered saline

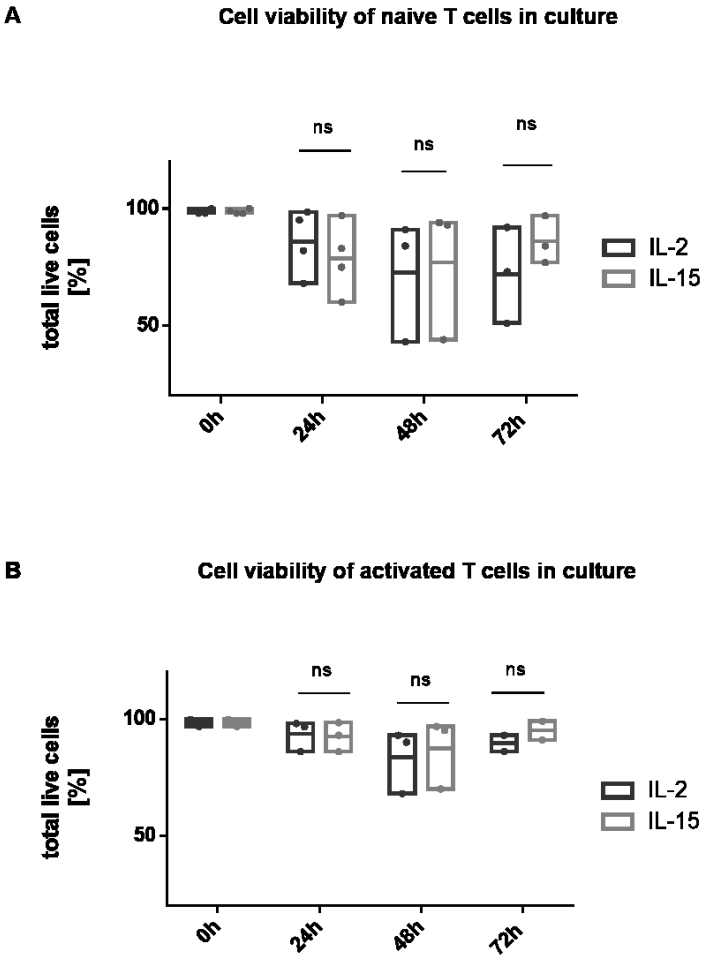
6.2. Key resources table

Supplementary Table 1: List of essential materials and devices that were used

reagent/resource	manufactuer	Id/ catalogue number	
CHEMICALS and BUFFERS			
PBS	as 1x PBS tabs, Alfagene		#LTI 18912-014
Trypan Blue	Sigma		#T8154-100ML
EDTA	Sigma		#03690-100ML
CELL CULTURE			
anti-CD28	BioxCell		#BE0015-1
anti-CD3	BioxCell		#145-2C11
Fetal Bovine SErum	Sigma		#F9665-500ML
IMDM	Sigma		#RNB47743
Penicillin/Streptomycin	Alfagene		#LTI 15140-122
COMMERCIAL KITS			
IC fixation+ Permeabilization Kit	eBioscience		#88882400
FOXP3 staining kit	eBioScience		#00552300
autoMacs Microbeads (anti-APC)	Militeny		# 130-090-855
ANTIBODIES			
Antibody	**Fluorochrome**	**Clone**	**Dilution**
CD8a	BV605	53-6.7 (BioLegend)	1/500
CD8a	APC	53-6.7 (BioLegend)	1/500
CD8a	SB600	53-6.7 (BioLegend)	1:500
CD4	V500	RM4-5 (BioLegend)	1/800
TCRβ	PerCP-Cy5.5	H57-597 (BioLegend)	1/300
CD103	PB	2E7 (BioLegend)	1/200
CD103	PE	2E7 (BioLegend)	1/200
CD69	AF647	H1.22F3 (BioLegend)	1/500

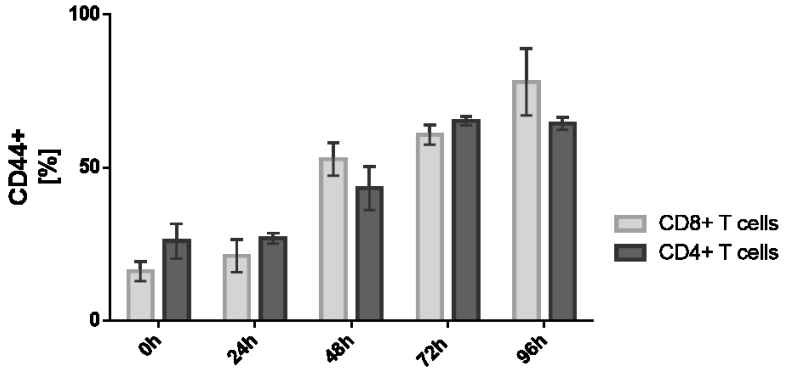
Eomes	AF488	DAN11MAG (eBioscience)	1/200
T-bet	PE-Cy7	4B10 (BioLegend)	1/200
T-bet	BV605	4B10 (BioLegend)	1/200
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit	APC-Cy7	Invitrogen by Thermo Fisher	1/1000
I-A/I-E (MHCII)	BV510	M5/114.15.2 (BioLegend)	1/3000
CD19	PE	6D5 (BioLegend)	1/500
CD25 (for cell sorting)	APC	PC61 (BioLegend)	1/500
FOXP3	PE	MF-14 (BeioLegend)	1/300
CD44	PE	IM7 (BeioLegend)	1/500
CD62L	FITC	MEL-14 (BioLegend)	1/500
DEVICES			
magnetic cell sorter	Milliteny	autoMACS	
Flowcytometer	BD Biosciences	FortessaX20	
SOFTWARE			
Flow cytometry analyses	Flo Jo		version V10
graphics and statistical analysis	Graph Pad		Prism 6
ANIMALS			
mus musculus	Charles River (kept and bred at the iMM Animal Facility)	C57BL6/J	
mus musculus	Jackson laboratories (kept and bred at the iMM Animal Facility	B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J	006148

6.3. Supplemental Figures



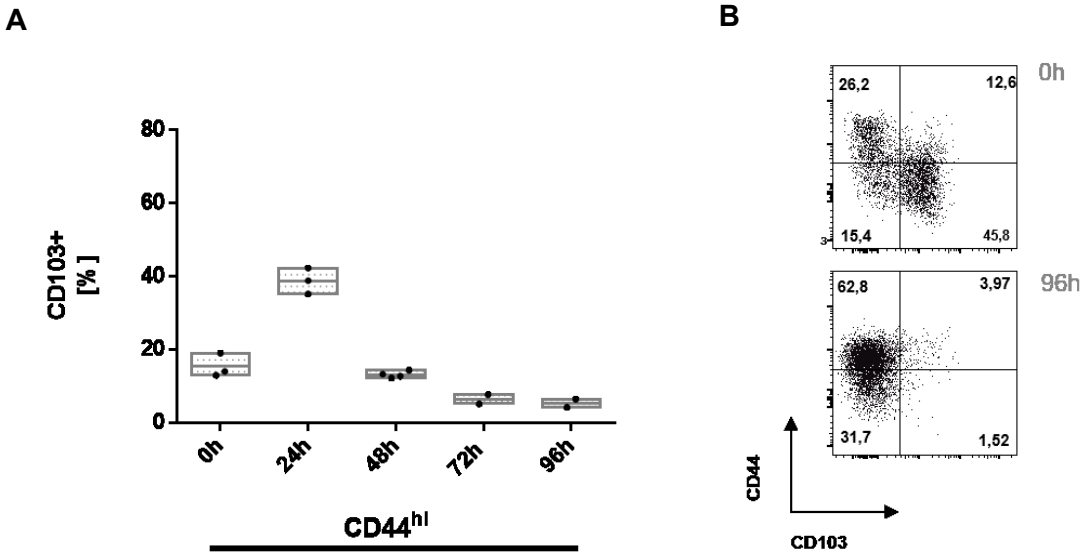
Supplementary Figure 1: Effects of IL-2 and IL-15 on the cell viability of CD8+ T cells in vitro by IL-15

A) CD8+ T cells were isolated from naive C57BL6/J mice or from **B)** mice 96h after i.p injecting with anti-CD3, autoMACS enriched and *in vitro* cultivated with either 10ng/µL IL-2 or 10ng/µL IL-15. At indicated time-points the level of viable cells was analysed by flow cytometry by measuring low uptake of a Live/Dead fluorescent dye. Experiments were performed individually (n ≥ 3)



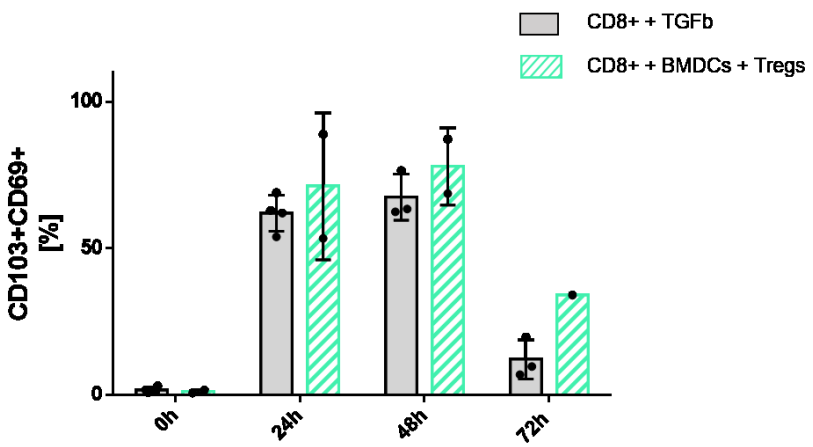
Supplementary Figure 3: Level of CD44+CD8+ and CD44+CD4+ T cells upon T cell activation

C57BL6/J mice were i.p injected with anti-CD3 and T cells were isolated from spleen and lymph nodes, at indicated time-points. Expression levels of CD44 in CD8 and CD4 T cells of lymphocytes pooled from SLOs was assessed by flow cytometry. Bars indicate calculated mean and standard of the individually performed experiments (n ≥ 3)



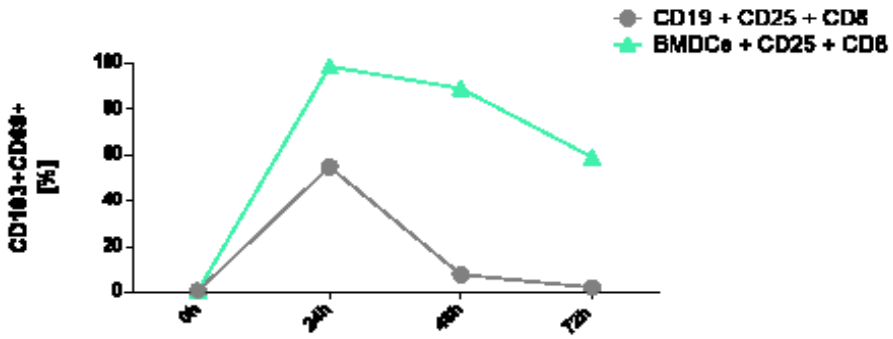
Supplementary Figure 2: Levels of CD103 expression in CD8+CD44^{hi} T cells in vivo

A) C57BL6/J mice were i.p. injected with anti-CD3 and proportions of CD103+ populations in CD8+CD44+ cells were identified at indicated time-points after injection by flow cytometry. Doublets were excluded and gated on live cells, TCRβ+ CD8+CD44^{hi} and CD69+ populations. Bars indicate standard deviation and calculated mean of individual experiments (n ≥ 2).
B) Representative flow cytometry plots presenting of CD8+ T cells plotting CD44 and CD103 expression levels at steady state (0h) and 4 days after activation (96h).



***Supplementary 4* Expression analysis of Trm surface markers on CD8+ T cells receiving exogenous TGF-β or when co-cultured with Tregs**

CD8+ T cells are analysed for their expression levels of CD69+ and CD103+ when co-cultured with BMDCs and Tregs (green) or single cultured receiving TGF-β1 (grey). For the co-culture, CD8+ and CD25+ T cells were isolated from C57BL6/J mice, *in vitro* activated (anti-CD3)and cultivated with BMDCs at the same concentration. As control, same isolated CD8+ T cells were in vitro activated and co-stimulated by (anti-CD3/anti-CD28) and received TGFβ1 at a concentration of 0.5 ng/µl. experiments. At indicated time-points of cultivation levels of simultaneously expressed CD103 and CD69 on CD8+ T cells were assessed by flow cytometry. Experiments were performed individually (n ≥ 2).



Supplementary Figure 5: Proportions of CD103+CD69+ CD8+ T cells in different co-cultures

CD8+ and CD25+ T cells and CD19+ C cells were isolated and autoMACS enriched from naive C57BL6/J mice. CD8+ T cells were actived *in vitro*m co-cultured with Tregs and either BMDCs (green) or CD19+ B cells (grey) at same cell concentrations. Expression of CD103 and CD69 was assessed by flow cytometry at indicated time points. For analysis, it was gated on CD4-CD8+ subpopulations co-expressing Trm marker CD103 and CD69. Tregs were confirmed by the expression of Foxp3+ CD4+ T cells, B cells by the expression of CD19 and BMDCs by the expression of MHCII.