Universidade do Minho
Escola de Medicina

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Characterization of CD8$^+$ tissue-resident memory T cells in the salivary gland and the impact of MCMV infection on T cell recruitment and retention

dezembro de 2019

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Professor Doutor Christopher M. Snyder
e da
Professora Doutora Margarida Correia-Neves

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Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

Caracterização das células T CD8+ residentes nas glândulas salivares e o impacto da infeção por MCMV no seu recrutamento e retenção.

Células T CD8+ residentes (T_{RM}) são mediadores cada vez mais importantes na resposta imunitária em diversos tecidos. Estas células têm como função a patrulha de tecidos, tendo sido descritas como parte da primeira linha de defesa em órgãos como a pele, pulmões, trato digestivo e cérebro. Devido à persistência das T_{RM} nos tecidos e à sua resposta efetora, a promoção destas células em barreiras naturais e em locais onde ocorre replicação viral pode ter um impacto significativo na patogénese destas infeções. Assim, as T_{RM} são alvos interessantes de estudo quando consideramos infeções víricas latentes como a causada pelo Citomegalovirus (CMV). Contudo, para otimizar o papel destas células é crucial compreender os mecanismos envolvidos na sua diferenciação e residência. Para além de descrever as alterações que ocorrem após infeção por CMV, este trabalho focou-se no estudo dos mecanismos envolvidos na diferenciação de T_{RM} na glândula salivar, órgão crucial para a replicação e disseminação do CMV. Os resultados demonstram que embora a infeção por CMV promova o recrutamento de células T CD8+ para a glândula, a diferenciação de T_{RM} ocorre de forma similar mesmo na ausência de infeção ou antigénio, o que contrasta com o que está descrito na maioria dos órgãos. Estes dados sugerem que mesmo nas glândulas salivares de ratinhos naïve existem sinais que possibilitam a diferenciação de T_{RM}. Assim, o recrutamento de células T CD8+ para a glândula salivar torna-se um passo essencial na formação de T_{RM}. Segundo o nosso trabalho a integrina $\alpha 4\beta 1$ surge como um mediador no recrutamento de células T CD8+ para glândula salivar independentemente da infeção por CMV. Por outro lado, embora a infeção resulte no aumento de quimocinas reconhecidas pelos receptores CXCR3 e CCR5 na glândula salivar, nenhum destes receptores tem um papel determinante no recrutamento de células T CD8+. Curiosamente, a expressão do receptor CXCR3 promove a acumulação de células T CD8+ na ausência de infeção por CMV.

Este trabalho representa uma mais valia não só pela caracterização das alterações que ocorrem na glândula salivar após infeção por CMV, mas também pelo estudo do impacto que a infeção tem no recrutamento de células T CD8+ e na formação de T_{RM}.

Palavras-chave: Células T residentes; Citomegalovirus (CMV); Glândulas salivares

ABSTRACT

Characterization of CD8+ Tissue-resident memory T cells in the salivary gland and the impact of MCMV infection on T cell recruitment and retention.

Tissue-resident memory $CD8^+$ T cells (T_{RM}) are crucial members of the adaptive immune system in different organs. These cells function as patrollers and first-responders of the immune response in organs such as the skin, lungs, digestive system and the brain.

Due to the long-lasting persistence and the prompt effector ability of the T_{RM} cells within the residing tissues, promoting these cells in barrier sites and organs that permit viral replication can significantly impact the pathogenesis of infection and the resulting disease. Consequently, T_{RM} cells are interesting cells to study in the context of a life-long latent infection such as the one caused by Cytomegalovirus (CMV). However, to take advantage of the potential role of these cells it is vital to understand the mechanisms involved in T_{RM} cells differentiation and residency. Besides describing the changes in the salivary gland following CMV infection, this work focused on understanding the mechanisms involved in T_{RM} differentiation in the salivary gland, where CMV replicates and spreads through saliva.

The results presented here demonstrate that although CMV infection promotes $CD8^+$ T cell recruitment to the salivary gland, T_{RM}, in contrast to most of the other organs, are able to differentiate in the absence of local infection or cognate antigen. This result suggests that the cues involved in T_{RM} differentiation exist in the salivary gland at a steady state which indicates that $CD8^+$ T cells entry in the salivary gland is a crucial step in T_{RM} differentiation in the salivary gland. According to our results, $CD8^+$ T cell migration to the gland is mediated by $\alpha 4\beta 1$ integrin both in infected and uninfected mice. Interestingly, while CMV infection increases the expression of chemokines recognized by the CXCR3 and CCR5 receptors, neither receptor was needed for T cell recruitment to the salivary gland during CMV infection. Surprisingly however, CXCR3 expression promoted the accumulation of $CD8^+$ T cells in the uninfected salivary glands.

The novelty of this work relies not only on the characterization of the changes caused by CMV in the salivary gland, but also on the impact of CMV infection in $CD8^+$ T cell recruitment and T_{RM} differentiation. Moreover, unveiling some of the mechanisms involved in $CD8^+$ T cell recruitment to the salivary glands may contribute to the development of preventive and therapeutic approaches to salivary gland-related diseases such as CMV.

Keywords: Tissue-resident memory T cells; Cytomegalovirus (CMV); Salivary glands.

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ABBREVIATIONS

ADCC: Antibody-dependent cellular cytotoxicity

ADCP: Antibody dependent cellular phagocytosis

aKIR: Activating killer immunoglobulin-like receptors

APC: Antigen-presenting cells

Bcl: B-cell lymphoma

Blimp-1: B lymphocyte-induced maturation protein-1

B2m: Beta 2 microglobulin

B6: C57BL/6 mice

CFSE: Carboxyfluorescein succinimidyl ester

CMV: Cytomegalovirus

Ctss: Cathepsin S

CTLA-4: Cytotoxic T-lymphocyte–associated antigen 4

DC: Dendritic cells

Eomes: Eomesodermin

F.p.: Footpad

gB; gH; gL: Glycoprotein B; H; L

Gbp: Guanylate-binding proteins

GI: Gastrointestinal

GSEA: Gene Set Enrichment Analyses

HCMV: Human Cytomegalovirus

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen

Hobit: Homolog of Blimp-1 in T cells

HSCT: Hematopoietic stem cell transplantation

HSV: Herpes simplex virus

I.n.: Intranasal

I.p.: Intraperitoneal

I.V.: Intravenous

I.V.+: Vasculature-localized

I.V.-: Parenchyma-localized

ICAM: Intercellular adhesion molecule

IE: Immediate early genes

IFITM: Interferon-induced transmembrane

IFN; IFN-α: Interferon; Interferon alpha

IFN-β; IFN-γ: Interferon beta; Interferon gamma

IFN-I; IFN-II: Type I; Type II interferon

Igtp: Interferon gamma-induced GTPase

Iigp: Interferon-inducible GTPase

iKIR: Inhibitory killer immunoglobulin-like receptors

Irf: Interferon regulatory factor

Irg: Immunity-related GTPases

Irgm: Immunity-related GTPase family M

KDN: Kidneys

KLF2: Krüppel-like Factor 2

KLRG1: Killer cell lectin-like receptor subfamily G member 1

KO: Knockout

Laptm5: Lysosomal protein transmembrane 5

LCMV: Lymphocytic choriomeningitis virus

LFA-1: Lymphocyte function associated-antigen 1

LG: Lungs

MCMV: Murine Cytomegalovirus

MHC: Major histocompatibility complex

NK: Natural killer cells

Oas(l): 2'-5'oligoadenylate synthetase (like)

Ova: Ovalbumin

PAMPs: Pathogen-associated molecular patterns

PD1: Programmed cell death protein 1

Psmb: Proteasome subunit beta

PTx: Pertussis Toxin

P2RX7: P2X purinoceptor 7

RNase L: Ribonuclease L

RPKM: Reads per kilobase of transcript per million mapped reads

Runx3: Runt-related transcription factor 3

SEM: Standard error of the mean

SG: Salivary glands

SIINFEKL: Ovalbumin 257-264 peptide

Slfn2: Schlafen 2

SPL: Spleen

STAT: Signal transducer and activator of transcription

STING: Stimulator of interferon genes

S1P: Sphingosine-1-phosphate

T_{CM}: Central memory T cell

TCR: T cell receptor

T_{EM}: Effector memory T cell

TGF-β: Transforming growth factor beta

TK: Thymidine kinase

TLRs: Toll-like receptors

TNF-α: Tumor necrosis factor alpha

T_{RM}: Tissue-resident memory T cell

VACV: Vaccinia virus

VCAM: Vascular cell adhesion molecule

WT: Wild-type

Zbp1: Z-DNA binding protein 1

2-5A: 2'-5' linked oligoadenylate

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Characterization of $CD8^+$ tissue-resident memory T cells in the salivary gland and the impact of MCMV infection on T cell recruitment and retention

1 Introduction

1.1 Tissue-resident memory T cells

CD8+ T cells

Early thymocyte progenitors migrate from the bone marrow to the thymus where T cells mature.[1,2] After a complex differentiation process, multiple subsets of T cells such as cytotoxic, helper, regulatory, γδ and natural-killer (NK) T cells emerge and are characterized by the expression of the T cell receptor (TCR).[3–5] These subsets differ not only in function but also in the expression of surface molecules. Simplistically, cytotoxic T cells express the CD8 co-receptor and can directly kill other cells and modulate other branches of the immune response.[6,7] Whereas helper T cells express the CD4 co-receptor and orchestrate the adaptive immune responses mainly by cytokines and chemokines production.[8] Both subsets are an essential part of the adaptive immune system and are commonly described as CD8+ T or CD4+ T cells according to their expression of co-receptors. As the TCR, both CD8 and CD4 glycoproteins also interact with the major histocompatibility complex (MHC)-I or MHC-II respectively, thus promoting T cell activation, differentiation and function.[9–11]

CD8+ T cells are particularly relevant in response to intracellular pathogens and since they are the main focus of this work, these cells will be further characterized. During the differentiation of T cells in the thymus, TCR recombination occurs providing a unique specificity to CD8+ T cells that can comprise either α/β or γ/δ chains.[12,13] As for the CD8+ T cell in this work, most of CD8+ T cells have TCRs formed by α/β chains.[14] T cells leave the thymus in their naïve form to circulate through the blood, lymph and secondary lymphoid organs until antigen recognition and T cell activation.[7,15] T cell activation usually happens in the secondary lymphoid organs and is a complex process that will only be succinctly described. Activation of T cells depends on their specificity and ability to recognize an antigen bound to a MHC, which leads to TCR aggregation and conformational changes that promote intracellular signals and T cell activation.[16] Besides TCR-MHC interaction (signal 1), costimulatory signals such as CD28/CD80; CD27/CD70; 4-1BB/4-1BBL and OX40/OX40L (signal 2) are necessary for T cell activation and play a role in peripheral tolerance.[17–20] Finally, pro-inflammatory cytokines such as type I Interferon (IFN-I) and IL-12 (signal 3), among several others, are also engaged in this process that ultimately leads to a robust interaction between cells, signal transduction and consequently CD8+ T cell activation with several transcriptional and functional changes.[7,20]

As mentioned before, despite their multiple roles, activated CD8+ T cells mostly patrol and kill target cells. This can be achieved in different ways. CD8+ T cells can produce and release cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). TNF-α signal, among others, leads

to a caspase mediated apoptosis, while IFN-γ, among other things, promotes MHC-I expression, both of which promote destruction of the target cell.[21–23] Additionally, direct killing also occurs either by the Fas-Fas ligand interaction between $CD8^+$ T cell and the target cells, or by the action of the highly cytotoxic granules with perforin and granzyme.[24–26]

The fate of activated $CD8^+$ T cells is very heterogenic and depends on multiple factors.[27,28] Although most of the $CD8^+$ T cells undergo apoptosis following most of the acute viral infections, a smaller fraction of cells survive and constitute the memory pool.[29,30]

All the studies done with $CD8^+$ T cells allowed us to use several markers to better define $CD8^+$ T cells and its subsets. Differences exist between human and rodent $CD8^+$ T cells and their markers. Therefore, it is important to note that all the experiments performed and the $CD8^+$ T cell markers used in this introduction refer to the mouse model, if not otherwise specified.

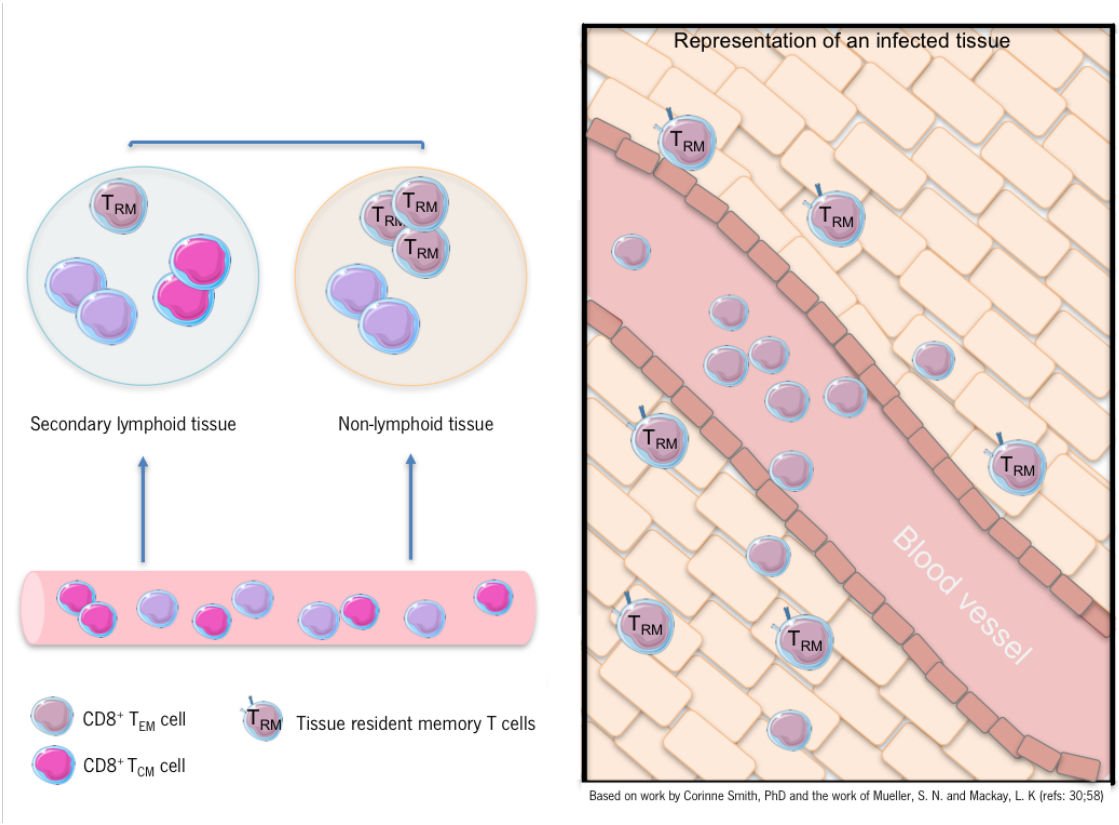
Classically, $CD8^+$ T cells can be simply divided into naïve, short lived effector and memory T cells.[31,32] After a viral infection and $CD8^+$ T cell activation, this population is dominated early on by short lived effector cells that are characterized by the expression of the killer cell lectin-like receptor subfamily G member 1 (KLRG1) and their brief lifespan and robust cytotoxic activity[32]. In contrast, memory T cells survive longer due to homeostatic proliferation, mostly through IL-15 and IL-7 signals, that lead to a higher expression of anti-apoptotic molecules, such as B-cell lymphoma (Bcl) 2.[30,33] For most viral infections, the short-lived effector population contracts after the first week, resulting in a dominant memory T cell pool that is usually divided in central memory T cells (T_{CM}) and effector memory T cells (T_{EM}).[34,35] T_{CM} ($CD62L^+$, $CCR7^+$ and IL- 7Rα/$CD127^+$) are highly proliferative and can be found in the blood and in the secondary lymphoid organs such as the spleen and lymph nodes.[30,31,36,37] T_{EM} ($CD62L^-$, $CCR7^-$, IL- 7Rα/$CD127^+$) have higher cytotoxic activity and were thought to be the only and main source of surveillance of non-lymphoid organs.[31,37,38] However, the characterization of $CD8^+$ T cells is still a complex topic and the fate of these cells is influenced by a variety of factors such as: cell division; priming conditions; antigen presenting cell; co-stimulation and the cytokine milieu. Thus, from activation to differentiation and survival of $CD8^+$ T cells, the extracellular inputs that modulate $CD8^+$ T cells are extremely diverse, which makes the study of these cells intricate as captivating.

Tissue-resident memory T cells, a subset of $CD8^+$ T cells

During the last decade, attention was brought to a distinct population of memory $CD8^+$ T cells. These cells did not completely fit the central/effector memory paradigm, since they remained for extended periods of time in the non-lymphoid organs without recirculating. Therefore, they were not in

equilibrium with the rest of the memory subsets.[39,40] These CD8+ T cells were named Tissue-resident memory T cells (T_{RM}).[39,41]

T_{RM} are, therefore, long-lived memory cells that reside in the non-lymphoid organs and act as first-responders in case of re-exposure to pathogens within those organs (illustration 1).[42–45] T_{RM} were primarily described in organs such as the skin, digestive tract, lungs, and reproductive tract, which elicited the idea that these cells would have a key role in protecting barrier tissues.[44,46–50] Interestingly, T_{RM} have also been shown to be present in multiple other organs such as kidneys, liver, heart, brain, and salivary gland.[41,44,49,51–61]



| Illustration 1 – Memory CD8+ T cells' subsets and tissue distribution.

Memory CD8+ T cells are divided in multiple subsets. Central memory T cells (T_{CM}) have higher proliferative capacity and preferentially home to secondary lymphoid organs. Effector memory T cells (T_{EM}) present high cytotoxic activity and are frequently found in circulation and in non-lymphoid organs. The tissue-resident memory T cells (T_{RM}) is the most recently described subset that mostly remains in the non-lymphoid organs and present limited recirculation [illustration adapted from the works of Smith *et al.* (2015) and Mueller *et al.* (2016)[59,62]]. |

The localization and the effector ability of T_{RM} were early indicators that these cells could be crucial for a prompt immune response in sites of pathogen entry and infection. It has been shown that in most tissues T_{RM} have a protective response to a wide variety of infections.[45] T_{RM} confer protection from viral, bacterial and parasitic infections, which highlights the essential role of T_{RM} in tissue immunity.[39,48,63–66] Besides the local T_{RM} differentiation, Kadoki *et al.* (2017), suggested that IFN-I production after infection can promote T_{RM} seeding to multiple organs allowing for local and distant protection.[67] The protective role of $CD8^+$ T cells relies on their effector function characterized by the cytotoxic elimination of cells as well as cytokine production.[7,68] These functions are shared by the T_{RM} and appear to be enhanced in this subset of $CD8^+$ T cells. T_{RM} also have a faster recall response that can be explained by the robust production of granzyme b, TNF-α, and IFN-γ.[65,68,69] After antigen recognition in the tissues, T_{RM} can also mediate a faster recruitment of multiple unstimulated memory T cells through IFN-γ signaling.[50] Therefore, it is thought that re-exposure of antigen in the tissue results in an inflammatory milieu and T_{RM} activation that, in turn, leads to a prompt cytotoxic response limiting pathogen replication and spread.[50] This initiate an alarm signal that further enhances the local immune response. Due to their immune roles and long persistence in the tissues, it becomes interesting to study T_{RM} in organs that are susceptible to multiple and frequent infections such as the lungs, where these cells might be relevant upon re-stimulation.[63,66] Similarly, T_{RM} are fascinating immune mediators in latent viral infections with reactivation potential, such as herpesvirus infections, that can benefit from an early *in situ* immune response.[43,70,71]

The fast response within the tissues and the ability to early control and limit infection has driven new efforts to promote these cells upon vaccination.[72–74] Zens *et al.* (2016), demonstrated that vaccine-induced T_{RM} were able to protect and reduce morbidity after an influenza challenge.[66] Indeed, understanding the mechanism involved in T_{RM} differentiation in different tissues is necessary to optimize their tissue homing and residence, which is crucial to consider in a vaccine design.

Remarkably, cells that share T_{RM} phenotype ($CD69^+$ $CD103^+$ $CD49a^+$) have also been described in tumors and due to their long life span, localization, and cytotoxic activity, T_{RM} have become an exciting topic in antitumor immunity.[75–77] In fact, T_{RM} have been associated with increased survival in breast; ovarian and lung cancer.[74,78–81]

On the other hand, the prompt response of these cells can lead to exacerbated immune responses. Therefore, it is not surprising that T_{RM} have also been linked to autoimmune diseases such as psoriasis and vitiligo.[82–84]

T_{RM} phenotype

Maintenance in the tissues is the hallmark of T_{RM}. Initially, to effectively validate T_{RM} residency, parabiosis techniques were used.[48,85] However, parabiosis experiments are laborious and challenging.[86] As such, with further phenotypic characterization of these cells, the expression of surface molecules as T_{RM} markers in some tissues (e.g. $CD69^+$, $CD103^+$, $CD49a^+$) became a convenient way to define likely T_{RM} populations without the need for parabiosis.[45,62,87]

Most T_{RM} express CD69, a C lectin type which, although not exclusive of T_{RM}, is an important marker.[44,62,88,89] Since it can be found in other immune cells such as NK, dendritic cells (DC) and effector $CD8^+$ T cells, the use of this marker to analyze T_{RM} requires a careful exclusion of such populations.[90,91] T cell activation and downregulation of Krüppel-like factor 2 (KLF2) are inducers of CD69 expression.[54,90] Upregulation of CD69 expression promotes Sphingosine-1-phosphate (S1P) receptor internalization, resulting in diminished signaling and, thereby, decreased tissue egress.[54,92] Actually, the lack of CD69 or the maintenance of the S1P signal results in a diminished T_{RM} population in most organs studied such as skin, lungs and the salivary gland.[54,93,94]

Another common T_{RM} marker (not exclusive to T_{RM}) is the αE chain of the αEβ7 integrin also known as CD103. Although less universally associated with T_{RM} than the CD69 marker, CD103 is expressed in T_{RM} in the lungs, skin, brain, gut and the salivary glands.[46,48,49,53,59–61,63,93,95–97] Most studies have shown that transforming growth factor beta (TGF-β) signaling promotes CD103 expression on $CD8^+$ T cells, however, other stimuli, such as IL-15 in the skin or CCR9 in the gut, have produced the same effect.[59,93,98–100] The αEβ7 integrin expressed by T cells can bind to E-cadherin expressed on epithelial cells.[101] This allows tethering to occur, which has been described as the main role of CD103 on T_{RM}.[101] Indeed, the manipulation of CD103 expression has a significant impact on T_{RM} numbers in the skin, brain, gut and lungs.[49,53,94,102,103]

Nonetheless, Bergsbaken *et al.* (2015) have shown that this may not be true for all gut compartments, since $CD103^-$ T_{RM} can be formed in the lamina propria.[104] As in the gut, $CD103^-$ T_{RM} have also been described in the skin.[93,105] Besides the gut, E-cadherin is also widely expressed in the epithelial cells of the salivary glands.[61106] However, even though T_{RM} in the salivary glands tend to be $CD103^+$, CD103 knockout (KO) did not significantly impact long-term T_{RM} population in this organ.[60]

Combined, these data suggest that CD103 is not universally crucial for T_{RM} differentiation even in organs enriched in E-cadherin. Some organs such as the brain have poor E-cadherin expression whilst CD103 is still present on T_{RM}, which suggests a different role for CD103 besides tethering.[53,107] Several alternative explanations are possible. For instance, alternative αEβ7 ligands may exist but remain unidentified in those organs. Alternatively, CD103 expression might tighten the adhesion with antigen-

presenting cells (APC), or simply that CD103 expression may be a bystander effect of the T_{RM} differentiation pathway. Therefore, it is important to note that although expressed by T_{RM} in most organs, CD103 is not a universal T_{RM} marker.

Another T_{RM} core marker is the CD49a (VLA-1α / α1 chain of integrin $\alpha1\beta1$). Its key function is binding to the collagen IV present in the basal membrane of the mucosal epithelium.[45] This interaction allows for cells to adhere but also facilitates migration along the collagen within the tissue.[108] CD49a has also been shown to impact $CD8^+$ T cell survival and differentiation within the tissue, being important for T cell localization in organs such as the lungs and intestine.[39,68,109–111]

CD44 has a similar effect since it helps to stabilize the T cells differentiation and has affinity for extracellular matrix components and selectins. Studies suggest that CD44 can associate with integrins (CD49d/VLA-4; $\alpha4\beta1$) on the surface of T cells promoting cell survival and T cell homing to the organs.[112,113] Although expressed by T_{RM}, CD44 is also detected on effector and memory T cells since its expression usually indicates T cell activation.[112,114]

Besides the previous markers, T_{RM} in multiple organs such as the brain and lung, also express programmed cell death protein 1 (PD1), a member of the CD28/CTLA-4 (Cytotoxic T-lymphocyte–associated antigen 4) family of inhibitory receptors.[45,79,115] PD1 has been classically described in exhausted $CD8^+$ T cells and its expression is induced by TGF-β signaling.[75,116] Most of the T_{RM} (especially the $CD103^+$) express this exhaustion marker, surprisingly however they seem to maintain some of their effector abilities.[117,118] Although PD1 expression limits T cell activation in some tumor studies, this effect is not universal and the role of PD1 seems to vary according to the organ/conditions.[119,120] Work by Campbell *et al.* (2008) suggests that Murine Cytomegalovirus (MCMV)-specific $CD8^+$ T cells in the salivary gland express PD1 that does not contribute to exhaustion or viral persistence in this organ.[121] Similarly, our unpublished data showed that T_{RM} in the salivary gland retain function even though expressing PD1. It is conceivable that the TGF-β signal in the organs promotes both CD103 and PD1 expression on T cells. More work is required to distinguish the role of PD1 in T_{RM}, however, an interesting hypothesis is that PD1 expression modulates the effector function of T_{RM}, possibly as a safety mechanism to avoid an exaggerated immune response.[75]

In line with mouse models, human T_{RM} also seem to express CD103, CD69, CD49a, PD1, downregulate CD62L, S1P receptor 1, KLF2 and have a distinct transcriptional profile from the T_{EM}.[68,87,93,122] However, making extrapolations from animal models should be done with caution since disparities in T_{RM} properties have been described. As an example, the homolog of B lymphocyte-induced maturation protein -1 (Blimp-1) in T cells (Hobit) is upregulated in T_{RM} in mouse studies, which does not seem to happen in

human T_{RM}.[87,123,124] CD101, that can be expressed by different lymphoid subsets and limits T cell proliferation and function, has been suggested as an additional marker for human $CD8^+$ T_{RM}, while it is associated with regulatory T cells in mice.[87]

Altogether, a common core has been described for most T_{RM} even though the markers used should be evaluated according to the subject and tissue of interest. As done previously by multiple groups, in this work T_{RM} were characterized using CD69 and CD103 as markers.[59–61,63,125,126]

T_{RM} differentiation

Although evidence suggests that there are cells committed to become T_{RM}, the precursors have not been fully identified. It is known that T_{RM} precursors cells lack KLRG1. Most studies indicate that T_{RM} arise from $KLRG1^-$ $CD127^+$ common precursors to memory cells, but it is still debatable if T_{RM} precursors can be $KLRG1^+$ effector cells that subsequently lost the KLRG1 expression.[93,96,127–130]

The T_{RM} differentiation may rely on specific transcriptional cues. In fact, differentiation of different T cell lineages depends on crucial transcription factors. Even at early differentiation stages the presence of transcription factors, such GATA-3 and Notch1, are crucial for the T cell differentiation.[131–134] Similarly, runt-related transcription factor 3 (Runx3) promotes the cytotoxic lineage, whereas, Id3 transcription factor T-cell factor 1, eomesodermin (Eomes) and Bcl6 are linked to T_{CM}, while T-bet, Blimp1 and Id2 are more related to T_{EM} differentiation.[127,135–139] Likewise, some transcription factors have been linked to mouse T_{RM} differentiation. Interestingly, as for T_{EM}, T_{RM} require Blimp1. Indeed, both Blimp1 and Hobit are crucial for T_{RM} maintenance in the skin, gut and liver, due to suppression of egress genes, at least in mice.[123] Moreover, Milner *et al*. (2017), demonstrated that Runx3 promotes not only T_{RM} differentiation, but also survival.[140] Additionally, T-box transcription factors are also important for T cell differentiation being T-bet associated with effector cells, while Eomes is linked to memory cells.[127,141] Interestingly, T_{RM} tend to be negative/low for these T-box transcription factors.[142–144] The low expression of T-bet/Eomes allows for upregulation of TGF-β signaling and CD103 expression.[145]

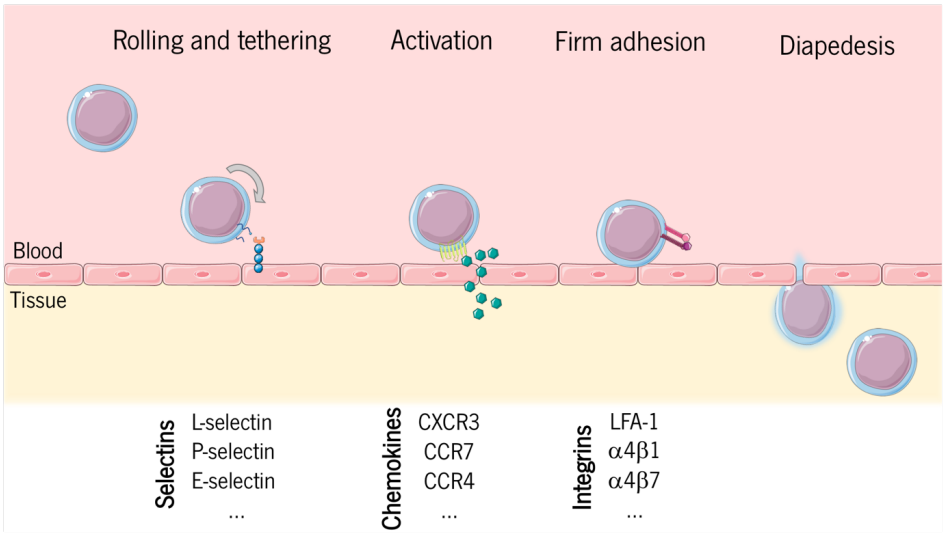
Although a single master transcription factor has not yet been identified for the T_{RM} lineage, T_{RM} tend to express transcription factors that partially overlap with both T_{EM} and T_{CM}, which is in line with their characteristics and might explain their effector functions and survival abilities.

Migration of $CD8^+$ T cells to the tissue

Although the T_{RM} precursors have not yet been clearly identified, it is thought that most of T_{RM} differentiate from $KLG1^-$ $CD8^+$ T cells within the residing tissue. Therefore, migration to and within the

organs are essential steps in T_{RM} differentiation. Migration of $CD8^+$ T cells is dependent on the homing receptors expressed on their surface and respective ligands found in the vasculature and tissues.

Although $CD8^+$ T cell migration from the blood to the extravascular compartment is a complex process, it can be divided in four essential steps: 1) rolling/tethering, 2) activation, 3) adhesion and 4) diapedesis, in which selectins, chemokines and integrins are the main mediators (Illustration 2).[146–148]



| Illustration 2 – Transendothelial migration.

Representation of the steps involved in leukocyte extravasation into tissues: 1) Rolling/tethering, 2) activation, 3) adhesion and 4) diapedesis. The transendothelial migration process is regulated by different adhesion molecules, both in the leukocytes and endothelial cells. These mediators can change depending on the tissue and the inflammatory condition. Although not limited to each step, for simplification, examples of selectins (represented in blue in the illustration), chemokines (represented in green) and integrins (represented in pink) are shown. |

Rolling/tethering of $CD8^+$ T cell

The migration process starts with the rolling and tethering of the $CD8^+$ T cells to the endothelial wall. This step is dependent on selectins that can be either expressed by the T cells, as the L-selectin (CD62L), or by the endothelial surface of the vasculature generally induced by inflammatory cytokines.[149,150] P and E selectins are examples of this last group and can be recognized by the P selectin glycoprotein-1, E-selectin ligand-1, CD44 and CD43 on T cells.[151–154] The skin is representative of this mechanism where inflammation induces the expression of E and P selectins in the post capillary venules that then bind to

P selectin glycoprotein-1, CD43 and CD44 facilitating the $CD8^+$ T cell recruitment.[152,155–157] In fact, mice deficient in E, P and L selectin-ligands show impaired recruitment of T cells to the skin.[158]

Activation

The resulting rolling motion created by the action of selectins allows for chemokines to bind the seven-transmembrane cell surface G-protein-coupled chemokine receptors expressed by $CD8^+$ T cells allowing for intracellular signaling.[159,160]

Chemokines were classically divided based on their function: pro-Inflammatory, produced as consequence of infection, inflammation, or by tumor cells (e.g. CCL2-CCL5, CCL11, CCL13, CXCL1-CXCL8, CX3CL1), or homeostatic chemokines that regulate cell migration during the development or maintenance of the tissue (e.g. CCL18, CCL19, CCL21, CXCL12, CXCL13). However, due to the mixed function of some chemokines (such as CXCL9-CXCL11, CXCL16, CCL1, CCL17, CCL22, CCL25), chemokines are now more commonly organized by structure, according to their first cysteine residue (C, CC, CXC, CX3C).[161–163] Chemokines can be produced by the endothelial cells of the vasculature or within the adjacent tissue.[161,164] Infection and inflammatory states lead to an increased and specific production of chemokines by immune cells, such as macrophages and DC in the tissue, which leads to T cell recruitment.[163,165–168] Thus, cells within the tissue can also modulate the migration pathways of new immune cells.

The interaction of the chemokine receptor with its cognate chemokine ligand promotes transmigration while inducing several signaling pathways that modulate polarization of the cell, actin reorganization, and gene transcription.[169–171] Moreover, chemokines are not only important for cellular recruitment but also for localization of the cells once in the organ.

The study of homing molecules is essential in understanding T_{RM} differentiation and has been gathering increasing attention. The chemokines involved in T cell homing are widely tissue-specific. Recently, it has been described that CXCL17-CXCR8 interaction mediates migration of T_{EM} and T_{RM} to the vaginal mucosa after herpes infection.[172] Similarly, CCL27 expressed by keratenocytes and CCL17 by endothelial cells in the skin, mediate T cell recruitment through CCR10 and CCR4 expression on T cells.[173,174] Moreover, CXCR3, CCR5, CCR3, CCR4 and CCR8 have also been implicated in T cell migration to the skin, which demonstrates the promiscuous and complex nature of the chemokine/receptor expression within the different organs and T cells.[175–179] This complexity can even occur within the same organ which is further exemplified in the gut where most $CD8^+$ T cells of the small intestine express CCR9, which correlates with the constitutive expression of its ligand (CCL25) by the epithelial cells in this portion

of the gut.[180,181] Surprisingly however, the CCR9-CCL25 interaction does not seem essential for $CD8^+$ T cell migration to all of the sections of this portion of the gut.[181] Therefore, the complexity of chemokine-ligand(s) that promote migration should be carefully studied by organs or even more particular tissues.

As suggested above chemokines not only promote transmigration but also localization of T cells within the organs. As examples, the chemokine receptor CCR5 is important for both $CD8^+$ T cell entry and placement in the lungs after viral infection and T cell proximity to tumor cells, whereas CXCR3 mediates T cell localization near infected cells and their effector functions in the skin.[182–184]

Since multiple chemokine-ligand pairs can exist and have similar results, finding the chemokine(s) involved in $CD8^+$ T cell migration and T_{RM} differentiation to different tissues is relevant but challenging.

Adhesion phase of $CD8^+$T cells transendothelial migration

The chemokine-receptor binding, besides chemotaxis, also allows for integrin activation, which is key for the adhesion phase of transendothelial migration.[150,185,186] Integrins are formed by an alpha and a beta chain. Vertebrates express 18α and 8β subunits that can generate a minimum of 24 different integrin heterodimers.[187,188] This panoply of heterodimers mediates firm adhesion of T cells to endothelial cells. The induction of $\alpha4\beta1$, $\alpha4\beta7$ and $\alpha L\beta2$ upon activation followed by binding to molecules such as Intercellular adhesion molecule (ICAM) 1 and 2 and the vascular cell adhesion molecule (VCAM)-1 have all been linked to T cells arrest and migration.[146,187,188]

Understanding these mechanisms allow us to modulate these components towards a clinical benefit. In fact, natalizumab, an anti-$\alpha4$ integrin monoclonal antibody, has been approved by the Food and Drug Administration for the treatment of Crohn's disease.[189,190]

Variables that impact $CD8^+$ T cell migration to the tissues

All the previously described mechanisms contribute and are synergistically important for the transendothelial migration of the $CD8^+$ T cells. However, a variety of intrinsic and extrinsic variables impact the expression of these homing receptors on T cells.

$CD8^+$ T cell subset:

The differentiation state of the T cells is important to consider when studying their migratory properties. While the Lymphocyte function associated-antigen 1 (LFA-1) is expressed in all subsets of $CD8^+$ T cells, naïve cells tend to express L-selectins and CCR7, whereas memory and effector cells express a wider pattern of homing molecules that can include P-selectin glycoprotein ligand-1, CD44, CXCR3,

CCR3-CCR6 VLA-4 and $\alpha4\beta1$.[191–193] The expression of these homing receptors may remain and be expressed by memory T cells as for the LFA-1 and VLA-4, or be transiently expressed as for the $\alpha4\beta1$.[191,194] These differences partially explain the localization and migratory patterns of the different $CD8^+$ T cell subsets.

$CD8^+$ T cell priming:

Additionally, the conditions and site of $CD8^+$ T cell activation are crucial for the differences in integrin expression and might also determine the $CD8^+$ T cell tropism. In the gut, Mora *et al.* (2003), showed that $CD8^+$ T cell activation with DC from Peyer´s patches induce the expression of $\alpha4\beta7$ integrin as well as CCR9 in opposition to DC from peripheral lymph nodes.[195] Moreover, the integrin binding potential can be promoted by TCR signaling, which further leads to the expression of glycotransferases, thus modulating the selectins being expressed on the T cell surface.[194,196,197] Similarly, the route of immunization can impact the type and amount of homing molecules expressed on T cell.[198,199] Therefore, differences in the priming conditions can significantly determine the migratory ability of the $CD8^+$ T cells.

Tissue environment:

The homing molecules involved in T cell transendothelial migration in each tissue can also change under different conditions. For example, the $\alpha4\beta7$ integrin is important for T cell homing to the gut under homeostatic conditions, however, this dependence was not severely seen after rotavirus infection, which may be related with the viral induction of chemokines in intestinal epithelial cells.[200,201] Besides directly inducing homing molecules in epithelial cells, infection and the resulting increase in immune cells to the tissue also influence the distribution of chemokines and integrins/ligands expressed in that tissue. This is demonstrated after influenza virus infection, after which human DC express multiple chemokines, which leads to further recruitment of other immune cells.[165] Similarly, neutrophils in inflamed tissues lead to 2-(ω-carboxyethyl) pyrrole accumulation that in turn serves as an integrin ligand facilitating migration of macrophages to the tissue.[202]

Nakanishi *et al.* (2009) demonstrated that after Herpes simplex virus (HSV)-2 infection, the production of IFN-γ by $CD4^+$ T cells within the female reproductive tract induces CXCL9 and CXCL10 expression, which promotes in $CD8^+$ T cell recruitment.[203] This once again suggests that the mechanisms of T cell migration can vary depending on the inflammatory and infectious status of the tissues.

As described before, multiple molecules are involved in $CD8^+$ T cell migration and homing. Moreover, several determinants impact the expression of these molecules from the type of cell to the environment of the tissue. Therefore, due to the complexity and promiscuous nature of these mechanisms, it is a daunting task to understand the players involved in $CD8^+$ T cells migration to the

different organs. Nonetheless, it is crucial to characterize these mechanisms and players to further understand the role of $CD8^+$ T cells and T_{RM} within the organs. Deciphering the mechanisms involved in $CD8^+$ T cell migration to different organs may reveal clues important for T_{RM} manipulation and thus promote local immunity.[204]

In situ signals involved in T_{RM} differentiation

Cytokines and chemokines

After reaching the organs, the signals received *in situ* are another essential part in T_{RM} differentiation. So far, TGF-β; IL-33 and TNF-α were capable of inducing T_{RM} phenotype ($CD69^+$ and $CD103^+$) in recently activated and memory $CD8^+$ T cells.[49,59,117,205] These cytokines were also able to induce a downregulation of KLF2, which contributes to tissue residency as previously described.[54] A recent report using MCMV demonstrated the *in vivo* importance of IL-33 in developing T_{RM} in the salivary gland and that even exogenous IL-33 promotes this differentiation.[206] As suggested before, the presence of chemokines within the tissue can also contribute to T_{RM} diferentiation. In fact, CCR9-CCL25 interaction has been shown to induce the expression of CD103 in $CD8^+$ T cells.[100] With further T_{RM} study in different organs, other local signals involved in T_{RM} differentiation are likely to be revealed.

Survival cues for T_{RM} in the tissue

Most T_{RM} form shortly after an infection and persist for long periods. Although they can be replenished from circulatory cells, this seems to be a secondary mechanism.[44,53,89,207] Even after infection, where high number of virus-specific $CD8^+$ T cells can be found in circulation long after the initial infection, as for Cytomegalovirus (CMV), T_{RM} are mostly formed at acute time-points.[59] Therefore, survival cues within the tissue are another crucial aspect when considering these long-lived resident T cells.

As briefly discussed before, the low levels of T-bet in both murine and human T_{RM} is essential for the TGF-β and Hobit signals to occur.[145] However, the remaining limited T-bet signal seems to be key for IL-15R expression in T cells.[145] This expression mediates not only homeostatic division in memory $CD8^+$ T cells, but also T_{RM} homeostatic proliferation and survival in some organs such as the skin and the salivary gland.[145,208] However, as most of the determinants of T_{RM} differentiation, the survival cues seem to be tissue-specific. For example, in opposition to the previous data, T_{RM} in the brain and the female reproductive tract do not seem to be dependent on IL-15 for survival.[143,209] Both T_{RM} differentiation and survival cues seem to be dependent on the characteristics of the tissue and organ studied. Consequently, it is logical

to consider conditions that alter the tissue environment, such as the presence of antigen and inflammation, as a stimulus for T_{RM} differentiation.

The role of antigen and local inflammation in T_{RM} differentiation

The inflammatory state and the recognition of local antigen within the tissues could potentiate T_{RM} differentiation, not only by increasing tissue-homing molecules, but also cytokines involved in T_{RM} differentiation. In fact, for most organs, the presence of infection/inflammation or antigen promote either T_{RM} differentiation or residency.[43,48,58,210] In the skin, one of the most well-studied organs in respect to T_{RM}, antigen is not absolutely required for T_{RM} differentiation. However, as for most organs, the presence of antigen promotes T_{RM} differentiation, CD69 expression, residency and modulates the $CD8^+$ T cells' specificity repertoire.[43,105,211,212] In a similar fashion, antigen is important for long-term residency of T_{RM} in the lung parenchyma and brain.[53,94,210,213–215]

After an acute viral infection, T_{RM} can be replenished in the lungs by T_{EM} in an antigen-independent manner, through IL-33 and TNF-α dependent signaling.[205,207] However, without infection the T_{RM} population tends to diminish with time.[89,105] A different T_{RM} niche has also been described in the lungs and it is believed to be promoted by tissue damage and the resulting local production of TGF-β and IL-15.[89,216] Therefore, local inflammatory milieu might overcome the necessity of local antigen in T_{RM} differentiation.[217,218] Although it is important to note that most systemic infection models lack significant T_{RM} numbers in the lung parenchyma, contrarily to what is seen after a direct lung infection.[63,66] This is possibly due to the lack of local antigen or absence of tissue inflammation or injury in a systemic infection without a lung tropism. Moreover, a prime and pull model (comprised of a first systemic immunization: prime, followed by a recruitment strategy as a local inflammatory signal: pull) induced T_{RM} differentiation in the female reproductive tract without local antigen.[51] As in this case, it starts to become apparent that an inflammatory milieu in the tissue may be sufficient to attract $CD8^+$ T cells and form T_{RM}. Nonetheless, the presence of antigen tends to promote and boost the T_{RM} numbers. In fact, so far, very few organs could harbor T_{RM} differentiation and their long-term residency in the absence of local infection or inflammation. The main exception is the gut, where persistent antigen seems to inhibit T_{RM} differentiation as demonstrated by the delayed induction of the T_{RM} marker CD103.[49,144] Recent data, including our own, suggest that T_{RM} form in the salivary gland of naïve mice.[60,61,219] Moreover, Woyciechowski *et al* (2017) showed that Poly(I:C) promotes $CD8^+$ T cell recruitment to the salivary gland in a $\alpha4\beta1$ dependent way, suggesting that inflammation allows $CD8^+$ T_{RM} differentiation in this organ.[125] Therefore, it was expected

that infection of the salivary gland by a virus such as CMV, would promote $CD8^+$ T cell recruitment and T_{RM} differentiation.

T_{RM} are recently described and exciting players of the adaptive immune system. Not only is it tempting to think that the robust and ubiquitous presence of these cells might confer a biological advantage, but more and more evidence continues to emerge revealing their multiple roles. Although some common characteristics can be defined, it is clear that the differentiation of these cells is tissue-specific, which limits the possibilities for extrapolations between different organs. Therefore, the characterization of these cells, the determinants of their differentiation and residency must be done for each of the target tissues. For most organs, this remains a challenge and an essential goal to further understand T_{RM}. These cells form after a variety of viral infections and, considering their long-term residency, T_{RM} become even more interesting to study in the context of a life-long latent infection. For all that has been said, it is appealing to think that the presence of antigen-specific T_{RM} in the tissues that support viral replication and latency can confer protection, control of viral replication and even transmission. Therefore, studying $CD8^+$ T_{RM} in the context of a prevalent latent infection such as the one caused by CMV is attractive and useful to better understand the acquired immune response to these infections. Hence, the work developed during this thesis focused on unraveling the mechanisms involved in $CD8^+$ T_{RM} differentiation in the salivary glands and the impact that CMV infection has in this process.

1.2 Cytomegalovirus

Human cytomegalovirus (HCMV) is an enveloped double-stranded linear DNA virus that belongs to the β-hespesvirinae subfamily and stands as the largest human herpesvirus.[220,221]

CMV is a highly species-specific virus that has evolved with its hosts for millions of years.[222] Therefore, animal models are useful tools to understand the physiopathology of infection. Multiple animal models have been described from rats to guinea pigs, pigs and rhesus macaques.[223,224] The mouse model, making use of the MCMV, has been extensively used due to its similarity to HCMV.[225] Both viruses share genetic similarities, the resultant cascade of protein expression and pathogenesis.[223,225,226] However, it is also important to note that besides all the similarities to HCMV, the resulting pathology after MCMV infection, varies depending on the mouse age and genetic background, inoculum dose and route of infection.[227–230] Additionally, different laboratory strains of MCMV and the methodology used to isolate and passage MCMV might not fully reflect the wild-type (WT) viruses.[227,231,232] Although both laboratory adapted and WT viruses share a conserved genome size with a low mutation rate in the absence of a specific selective pressure, differences in the viral tropism, virulence and pattern of mouse resistance have been described.[232,233] Most studies use serial passaged laboratory WT strains of MCMV, such as Smith and K181.

We used the MCMV model to study the immune response to CMV and its impact on T_{RM}. For most of our studies, we used the MCMV-K181 virus, which is a more virulent virus that results in higher viral titer in the salivary gland when compared to the Smith strain.[234]

CMV burden and disease

HCMV is a ubiquitous infection with a serum prevalence that ranges between 30-90% depending on the geographical localization, age, social and economic factors.[235–238] Contributing to its extensive prevalence, the primary HCMV infection is usually asymptomatic and results in a life-long latent infection in immunocompetent hosts.[239] Rarely, a mononucleosis syndrome develops, resembling a primary Epstein-Barr virus infection, with fever, myalgia, hepatomegaly and lymphadenopathy.[240–242] However, HCMV is a serious threat for immunocompromised individuals. HCMV is still an important opportunistic infection to consider and accounts for 85% of the retinitis in Human immunodeficiency virus $(HIV)^+$ patients.[243,244] Other manifestations include encephalitis, esophagitis, gastritis, hepatitis, colitis, and pneumonitis.[245,246] Moreover, depending on the transplant, up to half of the solid-organ transplant recipients can show evidence of HCMV infection.[247,248] Infections are more commonly seen after lung and small intestine transplantation and it remains a major cause of comorbidity and mortality especially for serological negative recipients where HCMV-specific immune response is absent.[249,250]

CMV is also a major complication of hematopoietic stem cell transplantation (HSCT).[251–253] After allogenic HSCT, the rate of CMV recurrence in seropositive patients varies according to the study, but a median value of 37% has been reported.[254]

HCMV infection also modulates the immune system by altering human leukocyte antigen (HLA) expression, cytokine production, and adherence molecules, which also potentiates the increased risk of secondary bacterial and fungal infections in transplanted patients.[253,255–257] Another critical population is pregnant women, in which the vertical HCMV transmission can severely impact the newborn. In fact, HCMV is the leading cause of viral congenital infection (around 0.6% of live births), although these values vary according to the seroprevalence and the characteristics of the population.[258,259] Around 10-15% of the infected newborns are symptomatic at birth and similar frequencies of the asymptomatic babies develop neurological sequelae or hearing loss.[259–261] In fact, congenital HCMV infection is still the leading congenic cause of hearing loss.[259,262,263]

Even with asymptomatic infections, HCMV has also been linked to poor response to vaccines, immunosenescence and increased mortality in elderly.[264–267] Moreover, the role of HCMV in diseases such as heart disease and atherosclerosis is being studied.[268,269]

Besides the significant silent burden of infection, HCMV is still an important cause of morbidity and mortality, which is the reason that a HCMV-vaccine is considered a priority.

Routes of CMV infection

HCMV is most commonly transmitted by contact with body fluids such as breastmilk, sexual contact and saliva but also by placental transfer and solid-organ transplantation (Illustration 3).[240,241,270,271] Interestingly, CMV replicates in the epithelial acinar cells of the salivary gland for an extended period and MCMV transcripts can be found in the salivary gland up to 7 weeks after infection.[59] In fact, infected infants can shed HCMV in their saliva for over a year, hence promoting horizontal transmission.[271–273] The salivary glands are also sites of CMV latency all of which contribute to saliva being a major route of shedding and emphasizes the importance of the salivary gland in CMV infections.[274,275] Therefore, understanding the immune response in the target mucosal sites of viral replication and shedding such as the salivary gland is crucial for the development of new therapeutic and preventive strategies.

Equally to HCMV, MCMV transmission occurs naturally through the same routes apart from the transplacental vertical transmission in immunocompetent mice.[276,277] Regardless of the similarities, most of the MCMV literature uses intravenous (i.v.) or intraperitoneal (i.p.) routes, which poorly represent the natural route of transmission in this model. Both i.p. and i.v. routes result in a significant direct access

to the blood and dissemination of the virus and consequently a bypass of the mucosal surfaces, which can modulate the resulting immune response.[278] Early after an i.p. infection, the virus infects the subcapsular macrophages of the mediastinal lymph nodes, followed by hematogenous spread to the liver and marginal zone of spleen.[278] Infection of the bone marrow was also described at early time points, however, other organs as the salivary gland are infected at later time points partially due to a monocyte-associated viremia.[279–281]

New efforts have been made to use animal models that mimic more natural routes of transmission. Along those lines, the footpad inoculation (f.p.) mimics transmission through a mouse bite, wound or grooming.[282] Differences in MCMV-specific $CD8^+$ T cell accumulation following i.p. or f.p. infection have been seen.[283] For instance, after f.p. infection the patrolling monocytes are recruited to the site of infection and play a critical role in viral dissemination to other organs as the salivary gland.[282] The limited direct viremia following the f.p. infection, due to a bottleneck effect caused by infection of $CD169^+$ subcapsular sinus macrophages where MCMV viral replication is poorly supported, may also impact the resulting pathogenesis and the immune response.[284]

As stated before, saliva is a major transmission vehicle for CMV infection, oral and intranasal (i.n.) infections, have also been suggested to resemble inhaled or ingested virions.[229,285] However, CMV is an enveloped virus and does not resist the stomach acid.[286] Thus, gastrointestinal infection is less likely than oropharyngeal infection.[286,287] In fact, a respiratory tract infection is thought to be a predominant mode of transmission in captive mice, resulting in a direct infection of the nasal mucosa and the olfactory neurons.[285] Following an i.n. inoculation, the virus is confined to the nasal area for the first 2 days in young Balb/c mice, where olfactory neurons have been shown to be primary targets.[231,285] Oduro *et al.* (2016) showed that the lungs of Balb/c mice can also be infected early after infection and these differences might be partially dependent on the volume, titer, and method of administration.[286] Farrell *et al.* (2017), showed that $CD11^+$ DC are responsible for spreading the virus to the mesenteric lymph nodes and later to the salivary gland.[288]

As for the i.p. route, monocytes are crucial for viral spread to the salivary gland, where CMV remains detectable for up to a month.[231,281,285,286,289,290] Differences between the immune response following the i.n. infection and the "classical" i.p. infection were also reported. The $CD8^+$ T cell response after an i.n. infection was reduced in comparison to an i.p. infection, which may be related to differences in the viral titers that are lower after the i.n. infection in comparison to the i.p. infection.[286,291] However, a large number of certain CMV-specific T_{EM} (inflationary responses) were still detected and maintained after an i.n. inoculation in the blood and spleen.[286,291,292] An acute presence of antigens in barrier and mucosal

tissues are often avoided by the use of systemic infections. Notably however, the infection of these barrier tissues correlates better with natural viral infections. The first line of immune defense is within these barrier tissues, which impacts not only the local but also the following systemic immune response as exemplified by the role of T_{RM} in multiple infections. The route of infection is therefore a crucial determinant for the resulting immune response and protection, especially in barrier tissues.

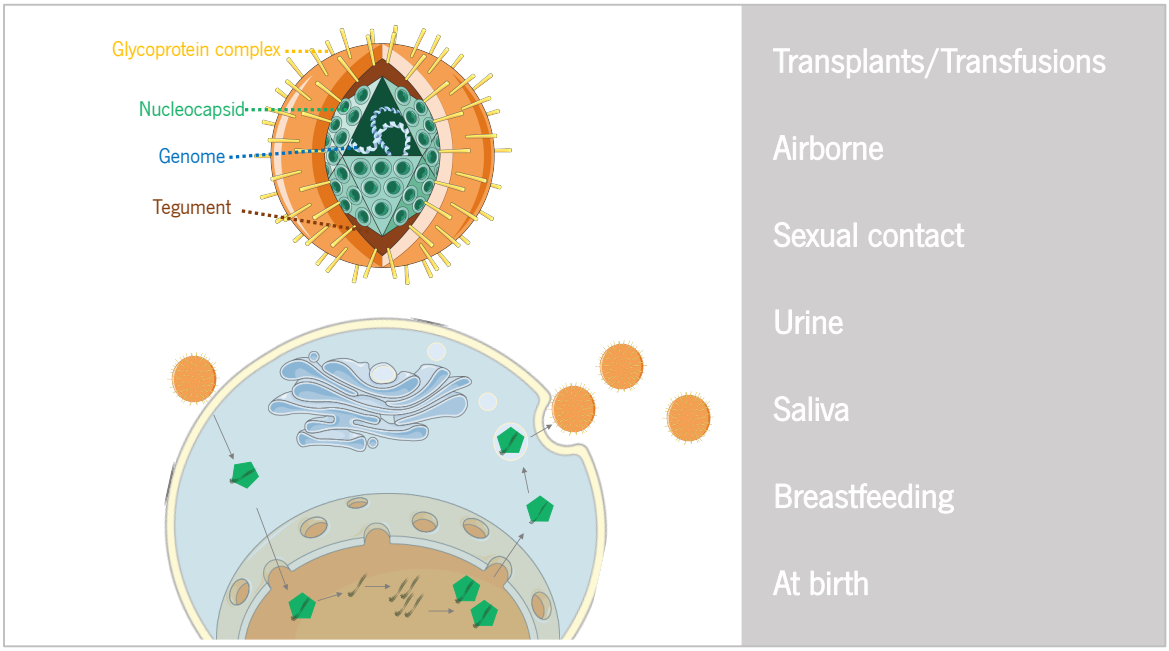


Illustration 3 – HCMV structure, life cycle and routes of infection.

HCMV is a double-stranded DNA virus with an icosahedral capsid surrounded by a proteinaceous tegument and an outer envelope enriched with glycoproteins. These glycoproteins allow membrane fusion and the release of both tegument proteins and nucleocapsids into the host cell. The nucleocapsid is translocated to the cell nucleus, where the genome is released initiating the cascade expression of viral genes. Newly synthetized DNA is encapsulated and transported to the cytoplasm, where it is trafficked to the viral assembly complex for further envelopment. Exocytosis of newly formed infectious virions promotes viral spread and transmission that can occur through multiple routes as the ones represented on the right. [illustration adapted from the work of Crough *et al.* (2009) and Beltran *et al.* (2015) [240,293]].

Immune response to CMV

CMV infects a broad variety of cells from fibroblast, monocytes, epithelial and endothelial cells to myocytes.[294,295] CMV enters the cells either by the endocytic pathway or by direct fusion using mostly the glycoproteins L and H (gL and gH) on its surface.[296] Once in the nucleus, viral replication occurs in a

programmed cascade: Immediate early (IE), early and late genes.[297–299] IE genes encode proteins in the first 2 hours after infection that are mostly related to the transcription of the subsequent genes.[300–302] During the first 24 hours, Early genes give rise to proteins that, in combination with the IE genes, are important for the induction of several promoters involved in DNA synthesis.[303,304] Late genes are generally responsible for structural proteins and are involved in the formation of virus particles.[304] This cascade of protein expression is relevant not only to understand CMV pathology, but also the resulting immune response.

CMV acute infection triggers a robust immune response. The innate immune system functions as the primary response to infection, being important for the acute CMV control especially in neonatal infections due to the reduced $CD4^+$ T cell response and immune system immaturity.[305,306] The recognition of the virus can be mediated by pathogen-associated molecular patterns (PAMPs), including glycoproteins from CMV surface, such as glycoprotein b (gB) and gH, and viral DNA. These viral PAMPs are recognized by Toll-like receptors (TLRs) and by stimulator of interferon genes (STING), which leads to cytokine production and activation of some of the main mediators of the innate immune response to CMV, like innate lymphoid cells 1 and NK.[307–309] Both group 1 innate lymphoid cells and NK, produce antiviral cytokines such as IFN-γ acutely after infection.[310,311] NK produce perforins and granzymes as well, allowing for their cytotoxic activity.[312] Although NK have direct effects on infected cells, they are thought to also mediate a bridge between innate and adaptive immune system.[313] The role of NK is clearly highlighted in the MCMV model, in which the susceptibility to the virus is determined. C57BL/6 mice (B6) express the Ly49H receptor that recognizes the MCMV m157 protein, which induces a NK response allowing this to be a MCMV-resistant strain.[314,315] The lack of Ly49H receptor is the explanation for the susceptibility seen in the Balb/c strain.[314] The activating and inhibitory killer immunoglobulin-like receptors (aKIR and iKIR) are expressed by NK in humans and are specific for MHC class I molecule.[316] Similarly to Ly49H molecules, killer immunoglobulin-like receptors are also important for destruction of infected cells and virus control.[317–319] Although the redundancy is presumed to exist, given the preponderant number of these receptors, rare genetic abnormities that lead to overexpression of these iKIR have been described and can result in recurrent episodes of HCMV disease.[320] Moreover, NK are able to confer protection to HCMV after transplantation, while the lack thereof increases the susceptibility to infection.[321,322]

The importance of the NK response can also be estimated by the immune evasion mechanisms that MCMV has developed to avoid NK recognition. These include downregulation of activating receptors in NK such as NKG2D.[323] Moreover, CMV encodes an IL-10 homolog, which results in a decreased pool of NK and multiple deficits in the adaptive immune response.[256,324,325] Although NK and group 1 innate lymphoid cells are essential for a rapid response against CMV, help arises from other immune cells. CMV

recognition prompts a robust humoral response usually targeted to structural tegument proteins and envelope glycoproteins.[326–328] Although antibodies are not crucial for acute viral infection control, MCMV-specific antibodies have been shown to prevent viral dissemination and virus reactivation.[329–331]

Although the acute immune response to MCMV is a combined effort from several branches of the immune system, T cells are essential for the control of the infection.[126] $CD4^+$ T cells are important mediators of acute immune response and accomplish antiviral activity partially due to IFN-γ and TNF-α production.[332,333] Cytolytic $CD4^+$ T cells specific for CMV are also evident in HCMV-infected people.[17,334,335] In fact, reduced $CD4^+$ T cells results in increased HCMV shedding in children possibly through the loss of these direct anti-viral functions of $CD4^+$ T cells.[336,337] However, $CD4^+$ T cells also promote the function of other immune cells, as the $CD8^+$ T cells, and it is still unclear whether and how $CD4^+$ T cells help may contribute to $CD8^+$ T cells function after CMV infection.[338–340]

CMV induces a robust $CD8^+$ T cell response with some CMV-specific T cells remaining active at an increased number even at later time points. This process is called memory inflation and reflects the accumulation of T cells with certain specificities.[341–343] In B6 mice, $CD8^+$ T cells specific for the $M38_{316}$-epitope are one of the best studied examples of T cells that undergo memory inflation.[344,345] In contrast, in their response to the M45-epitope, among others, T cells behave like classical anti-viral T cells and the population decreases after the first week of infection.[343,344] The differences between these responses are correlated with the generation of the epitopes. The M45 epitope is dependent on the immunoproteasome, which is less available after the acute infection.[346] In contrast, the $M38_{316}$-epitope can be processed by the constitutive proteasome and therefore presented during latency.[346] Memory inflation is also seen in humans where approximately 10% of the memory T cells are HCMV-specific and can dominate the $CD8^+$ T cell repertoire.[347] This increased pool of MCMV-specific $CD8^+$ T cells is a response to the sporadic reactivation events that occur in the endothelial cells of the vasculature.[343] Cytotoxic activity and IFN-γ production by $CD8^+$ T cells are essential to acute MCMV control.[348–350] Indeed, $CD8^+$ T cell transfers prevent pathology and confer protection to lethal MCMV infection in immune compromised mice.[351–354] Similarly in humans, HCMV-specific $CD8^+$ T cell transfer conferred protection against reactivation.[355–358] Viral control is therefore largely dependent on $CD8^+$ T cells in most organs. However, the salivary gland stands as the exception.[121,359,360]

As suggested before, the salivary glands are crucial for CMV infection. The acinar glandular epithelial cells of the salivary gland are infected 3-6 days after systemic MCMV infection which results in local viral replication, viral shedding and latency.[59,361]

After MCMV infection, the salivary glands are flooded with DC, γδ, $CD4^+$ and $CD8^+$ T cells.[361] This migration of immune cells to the salivary gland, especially lymphocytes, results in increase of cytokine production, being that the infected salivary glands are enriched for IL-10, IFN-γ, CCL3, CCL4 and CCL5 in comparison to the secondary lymphoid organs.[361] However, the immune response in the salivary gland is less efficient than the other infected organs, allowing for most of the acute viral replication to occur for a prolonged time.[121] The peculiar immune response within this organ may be the explanation. Besides the low rate recruitment of NK to the salivary gland after MCMV infection, the NK within the salivary gland show a delayed activation and are hyporeactive to the activating receptor link and cytokines´ stimuli in comparison to other NK population seen in spleen and liver, which in the end results in a humble IFN-γ response.[91] Additionally, in contrast to what happens in the other organs, $CD8^+$ T cells are unable to control viral replication in the salivary glands.[360,362] Viral infection of the salivary glands induces a downregulation of MHC-I on the epithelial cells thus preventing $CD8^+$ T cells recognition of the infected cells.[360] Indeed, $CD8^+$ T cells are able to control viral infection in the salivary gland in the absence of the immune evasion genes (m04/m06/m152).[360] Moreover, there is a lack of cross-presenting APC cells in the salivary gland, although antigen processing and presentation in MHC-II context still happens.[360,363] Additionally, $CD4^+$ T cell responses in the gland are also impaired in comparison to the periphery. In contrast to the blood and lymphoid organs, where the $CD4^+$ T cell response can be detected in the first week of infection and is followed by contraction, $CD4^+$ T cells reach the salivary glands with some delay and peak after the first week.[364] Moreover, increased levels of IL-10 in the gland also reduce IFN-γ production by T cells.[365] These mechanisms help explain the immune evasion that leads to the prolonged MCMV replication and latency in the salivary glands.[274]

Acute viral infection is followed by viral latency. In spite of the fact that, by definition, infectious viruses are not detectable in this latent phase, viral DNA has been detected in cells such as the hematopoietic cells and multiple organs such as the spleen, heart, kidneys, lungs and the salivary glands.[280,366–369] Nonetheless, the detection of low levels of DNA can be challenging and therefore the characterization of latent sites remains as an open question. Notably, due to sporadic viral reactivations, continued immune surveillance is required to keep the virus from causing disease.[370,371] During latency, viral transcription occurs with IE1 mRNA being one of the first mRNAs detected.[371] This does not equal a full reactivation with the production of new infectious viruses since even in the presence of transcripts there are multiple checkpoints that prevent a complete productive cycle.[372] An open chromatin structure of the major IE and the differential splicing that allow for IE3 mRNA and the subsequent protein are important for the early gene transcription, but not the only checkpoints.[373–375] Moreover, the occurrence of

gene desilencing results in viral transcription that allows presentation of antigenic peptides and re-exposure of antigen to the T cells, which led to the immune sensing hypothesis of latency control.[370,371,376–380]

Contrary to the acute infection, neutralizing antibodies are crucial at preventing viral reactivation both systemically as in the salivary glands at latent times.[329,381] Moreover, the use of B-cell depleted animal models allowed for high reactivation rates upon deficiency of NK, $CD4^+$ T cells and $CD8^+$ T cells.[370] It is likely that NK and T cells have a redundant role, since the isolated defect of one of these subsets is not enough to produce viral particles even in a B-cell depleted model.[370] Interestingly however, $CD8^+$ T cells seem to have a more predominant role in preventing MCMV reactivation and viral shedding than $CD4^+$ T cells, even in the salivary glands.[370,376]

These data suggest that CMV-specific T_{RM} could have an important role in controlling CMV reactivation in the salivary gland.

HCMV vaccine

Since the late 1970s, effort has been made to develop a HCMV vaccine.[382] A HCMV vaccine could have a tremendous impact, especially in solid organ and hematopoietic stem cell transplant recipients, preventing viremia and disease. Moreover, women of childbearing age can be another target since a primary infection or re-infection/reactivation can have a severe impact during pregnancy. Although the seropositive-status of the mother is not sufficient to confer protection to congenital infection, it seems that maternal antibody and T cell responses are beneficial in limiting transmission and disease.[383–385]

The immune response that needs to be induced by vaccination to confer protection is still debatable. Neutralizing antibodies are important in the case of solid organ transplants, while T cells have been more associated to protection in hematopoietic stem cell transplants.[386,387] In cases of reactivation, while neutralizing antibodies have been frequently associated with virus control, this is not universal.[388–393] In fact, neutralizing antibodies seem to have a limited role in cell-to-cell transmission.[390] Therefore, the immune response responsible for controlling the viral spread requires further analysis. Furthermore, some vaccination strategies are able to reduce viral shedding in saliva, independently of the neutralizing antibody titers, which also have been shown to be important in preventing mortality in mouse studies and contribute to HCMV protection.[394,395] This suggests that another branch of the immune system has impact in shedding. $CD4^+$ T cells have also been associated with protection and studied in vaccine scenarios.[383,396]

$CD8^+$ T cells may also play a critical role in CMV protection and pp65 and IE1 are commonly studied targets.[397] Although several vaccine candidates induced substantial $CD8^+$ T cell responses, the immune

dominance of the response does not seem to be essential for protection since subdominant responses have elicited protection in mice.[398,399] However, more studies are needed to see if the same applies in humans. It is interesting that some of these findings can change according to the vaccine strategies used, which may suggest that the immune responses, including the T_{RM}, vary according to the specificity, priming conditions and the presence of other immune cells in the gland.[400] All of which increase the complexity in developing a successful HCMV vaccine.

So far, most of the vaccine strategies used fall under one of these categories: replication-deficient virus/attenuated vaccines; recombinant vaccines; vectored vaccines; DNA vaccines; RNA vaccines; peptide vaccines.

Replication deficient/Attenuated vaccines

Several strains have been used to develop attenuated vaccines such as AD169, Town and Toledo able to confer protection in specific scenarios. However, some of these approaches failed to elicit enough neutralizing antibody titers.[401,402] One of the greatest advantages of this strategy is that it allows the induction of different branches of the immune system from neutralizing antibodies to cytotoxic T cells. However, concern exists about establishment of infection, latency and the development of conditions that may be associated with CMV, such as atherosclerosis.

The V160 vaccine developed by Merk is an interesting vaccine candidate that is now starting phase II trials. The V160 is a recombinant vaccine from an attenuated strain (AD169). However, differently from other attenuated vaccines that lack the pentameric complex, its expression was restored in the V160 vaccine which allowed for increased levels of neutralizing antibodies.[403,404] In fact, neutralizing antibodies and cellular mediated immune responses are comparable to natural CMV infection.[404]

Recombinant vaccines

Vaccines using the gB subunit are probably the most tested vaccine so far, and demonstrated some efficacy preventing primary HCMV infection in women as well as a reduction of both viremia and time of ganciclovir treatment in transplanted patients.[386,405,406]

Vaccines expressing gB result in a robust neutralizing antibody response, similarly to the UL128/UL130/UL131a proteins, that interact with the gH/gL heterodimer forming the HCMV pentameric complex needed to enter epithelial cells.[407–410]

Vectored vaccines

These vaccines rely on heterologous viral vectors, such as Canarypox, lymphocytic choriomeningitis virus (LCMV), Venezuelan equine encephalitis and Vaccinia virus (VACV) that deliver CMV immunogens, mostly gB, pp65 and/or IE1.[411–414]

DNA plasmid

DNA vaccines consist of plasmid preparations containing DNA sequences that induce an immune response against a pathogen.[387,415] An example is the ASP0113 vaccine candidate, which is a DNA-plasmid that targets both gB and pp65.[416] This approach reduced CMV viremia and reduced use of antiviral drugs following hematopoietic cell transplantation.[416]

RNA vaccines

These vaccine strategies use mRNA to elicit a robust humoral and T-cell immunity. Interestingly, these mRNA can be used to combine multiple target in a single vaccine. This was tested and surprisingly the combination of epitopes led to competition of the resulting T cell responses, which might be a challenge in designing a T cell-targeted HCMV vaccine.[417] However, this can probably be overcome with co-dominant epitopes combined in a single vaccine or a combination of multiple inoculations.

Table 1: HCMV vaccines in enrollment or active development according to the National Institute of Health - clinicaltrials.gov (accessed on 26/04/19).

Type	Details	Phase	Sponsor
mRNA vaccines	mRNA-1647 (gB) mRNA-1443 (pp65)	I	ModernaTX, Inc.
Replication deficient vaccines	V160	I/II	Merck Sharp & Dohme Corp
Vectored vaccines	Multi-antigen CMV-Modified Vaccinia Ankara Vaccine	I/ II	City of Hope Medical Center
	HB-101	II	Hookipa Biotech
Peptide vaccines	Multi-peptide CMV-Modified Vaccinia Ankara Vaccine	II	City of Hope Medical Center
	Tetanus-CMV fusion peptide vaccine	I	City of Hope Medical Center
	CMVpp65-A*0201 peptide vaccine	II	City of Hope Medical Center
DNA vaccines	ASP0113	II/III	Astellas Pharma Global Development, Inc
	BD03	I	SL VAXiGEN

Developing a HCMV vaccine has been a challenging goal. The species-specificity of HCMV represents a limitation to pre-clinical trials in vaccine development, which is one of the many difficulties that has been assumed for the lack/diminished efficacy in some clinical trials.[418,419] The failure in inducing enough neutralizing antibodies titers, their limited binding capacity and defining the protective antibody level justify some of the disappointing results.[394,420] Moreover, the inability to induce a neutralizing-independent antibody response (e.g. antibody-dependent cellular cytotoxicity/phagocytosis – ADCC/ADCP) can be another limiting factor.[395,421,422] The lack of T cell response is another possible justification, which is aggravated by the difficulty in covering the antigen diversity both within and between hosts.[423–426] These factors add to the complexity of designing a HCMV vaccine, which will remain intricate until the protective immune response and the immunogens that elicit a robust response are identified and characterized.

In sum, CMV causes a ubiquitous infection that remains as an important cause of morbidity and mortality. The vast CMV prevalence can be partially explained by the inability of the immune system to clear the infection, which leads to viral latency in multiple organs, including the salivary gland. After infection, a significant proportion of MCMV-specific T_{RM} develop and although $CD8^+$ T cells have a secondary role in the acute CMV control in the salivary gland, this does not seem to be true during latency. To maintain this state of latency, a continuous immune surveillance of the tissues is needed. Intrinsic characteristics of T_{RM}, such as their localization, cytotoxic ability and potential for immune cell recruitment, makes them the ideal candidates and the perfect tool to keep CMV from reactivating. In fact, sporadic viral transcription could activate T_{RM} in the lungs, another organ that harbors virus during latency. Moreover, Thom *et al*. (2015), have shown that $CD8^+$ T cells residing in the salivary gland help control an intra-glandular viral challenge.[60] Although the promising role of T_{RM} in the salivary gland, the requirements for T_{RM} differentiation and function in the salivary glands, a vital organ in CMV pathology, remains poorly characterized.

1.3 Aims

1. Study the impact of MCMV infection in T_{RM} differentiation in the salivary gland

    1.1. Determine the effect of different routes of MCMV infection in the resulting $CD8^+$ T cell migration and T_{RM} differentiation in the salivary gland;

    1.2. Identify the impact of acute MCMV infection in promoting T_{RM} differentiation.

2. Characterize the mechanisms involved in $CD8^+$ T cell migration to the salivary gland

    2.1. Unravel the chemokines expressed in the salivary gland with or without MCMV infection;

    2.2. Characterize the chemokine receptors expressed on MCMV-specific $CD8^+$ T cells;

    2.3. Determine the role of relevant adhesion molecules in $CD8^+$ T cells migration to the salivary gland.

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Experimental work 2

α4β1 and CXCR3 facilitate $CD8^+$ T cell accumulation in the salivary glands where T_{RM} maintenance is constitutively supported in the absence of local infection.

Most of the presented results were published in the Journal of Immunology:

Caldeira-Dantas, S., Furmanak, T., Smith C., Quinn, M., Teos, L. Y., Ertel, A., Kuruo, D., Tandon, M., Alevizos, I. & Snyder, C.M. The Chemokine Receptor CXCR3 Promotes $CD8^+$ T Cell Accumulation in Uninfected Salivary Glands but Is Not Necessary after Murine Cytomegalovirus Infection. J. Immunol. **200**, 1133-1145 (2018).

2.1 Abstract

Recent work indicates that salivary glands are able to constitutively recruit $CD8^+$ T cells and retain them as tissue-resident memory T cells (T_{RM},), independently of local infection, inflammation or antigen. To understand the mechanisms supporting T cell recruitment to the salivary gland, we compared T cell migration to the salivary gland in mice infected, or not, with Murine Cytomegalovirus (MCMV), a herpesvirus that infects the salivary gland and promotes the accumulation of T_{RM} in this organ. We found that acute MCMV infection rapidly increased T cell recruitment to the salivary gland, but that equal numbers of activated $CD8^+$ T cells eventually accumulated in both infected and uninfected glands. T cell recruitment to uninfected salivary glands depended on chemokines and the integrin $\alpha 4$. Several chemokines were expressed in the salivary glands of both infected and uninfected mice and many of these could promote the migration of MCMV-specific T cells *in vitro*. MCMV infection increased expression of chemokines that interact with the receptors CXCR3 and CCR5, but neither receptor was needed for T cell recruitment to the salivary gland during MCMV infection. Unexpectedly, however, the chemokine receptor CXCR3 was critical for T cell accumulation in uninfected salivary glands.

Together, our data suggest that CXCR3 and the integrin $\alpha 4$ mediate T cell recruitment to uninfected salivary glands, but that redundant mechanisms mediate T cell recruitment after MCMV infection.

2.2 Introduction

The salivary gland is an exocrine organ, composed mostly of acinar cells producing a water-based fluid that is mucous-, ion-, enzyme-rich, and released in the oral cavity via a network of epithelial ducts. Because of this, several viruses, including all human β- and γ-herpesviruses, that infect the salivary gland use saliva as a major route of transmission to new hosts.[1–3] How the immune system responds to pathogens in the salivary gland, and prevents or limits viral shedding, remains poorly defined. In addition, how pathogens alter the salivary gland environment by infection is unknown.

Cytomegalovirus (CMV) is a β-herpesvirus that infects most organs in the body but undergoes prolonged replication in the salivary gland. CMV infects the acinar and ductal epithelial cells in the salivary gland and is thus shed into saliva during replication.[4–6] Using the Murine Cytomegalovirus (MCMV) model, it was shown, over 40 years ago, that lymphocytes enter the salivary gland, resulting in the death of MCMV-infected epithelial cells.[7,8] MCMV also establishes latency in the salivary gland and readily reactivates in this organ during periods of immune suppression, particularly when $CD8^+$ T cells have been depleted.[9] It might be assumed that lymphocytes are drawn to the salivary gland in response to the CMV infection. Interestingly however, recent data from several laboratories, including our own, suggested that antigen-stimulated $CD8^+$ T cells could be recruited to the salivary gland constitutively after MCMV infection or even without specific infection of the gland.[10–12] MCMV-specific T cell populations were abundant in the salivary gland after MCMV infection where they adopted an intra-epithelial localization and expressed CD69 and CD103, markers of tissue-resident memory T cells (T_{RM}).[10,11] T_{RM} are memory T cell subsets that are retained in tissues, independently of the circulating pool of T cells, thus providing a rapid defense against reactivation of a latent infection or local reinfection of a previously encountered pathogen.[13,14] Surprisingly, even *in vitro* activated T cells could migrate to the salivary gland where they developed into protective T_{RM} in the complete absence of infection or specific inflammation, leading to the description of the salivary gland as a "sink" for $CD8^+$ T_{RM}.[10–12]

It is currently unknown whether local inflammation or antigen can enhance the recruitment or retention of $CD8^+$ T_{RM} in the salivary gland. However, this ability of salivary glands to attract and retain protective numbers of $CD8^+$ T_{RM}, without a local infection or inflammation, is quite unexpected. In most other sites in the body, tissue-localized inflammation and/or antigen are critical for the efficient recruitment of T cells or their retention as T_{RM}.[15–24] The best-studied example of the interplay between antigen, inflammation and T_{RM} differentiation is the skin, where inflammation alone is sufficient to enable T cell egress from the blood and differentiate into T_{RM} phenotype.[22] Interestingly, while infection at one skin site could lodge T cells at distant skin locations[23,25], the efficiency is very poor without local inflammation

and local antigen enhanced the maintenance of T_{RM} populations and shaped the specificity of the cells that were retained.[16,23,24] Thus, although antigen and inflammation within a particular skin site are not absolutely required for T_{RM} differentiation, they markedly enhance the number of protective T_{RM} that are established in the skin. Other tissues have been less well studied, but a similar theme is repeated. In the vaginal mucosa, $CD8^+$ T cell entry during Herpes simplex virus (HSV) infection was poor unless $CD4^+$ T cells promoted in the tissue local chemokines, in an interferon (IFN)-γ dependent manner.[17] In the lungs, the differentiation and maintenance of protective numbers of T_{RM} after multiple infections, including after MCMV, depended on both antigen and infection of the lungs.[26,27] The brain is even more restrictive, requiring infection or antigen for any detectable T_{RM} differentiation.[20] In fact, other than the salivary gland, only the small intestine has been described as permissive of T_{RM} differentiation and maintenance in an antigen- and infection-independent manner.[28] Thus, the salivary gland and the small intestine may be uniquely capable of both recruiting and retaining T cells with no specific infection. While many studies have addressed the mechanisms of T cell recruitment to the intestine very little is known about the mechanisms of T cell recruitment to the salivary gland.[28–31] A recent study demonstrated that systemic inflammation could induce expression of the vascular cell adhesion molecule (VCAM)-1 on vascular endothelial cells in the salivary gland, and that this boosted the recruitment of activated T cells via the integrin $\alpha4$, which pairs with the $\beta1$ integrin to form the ligand for VCAM-1.[32] At a first glance, this supports the notion that inflammation enhances T cell recruitment to the salivary gland. However, T_{RM} differentiation and maintenance was not studied. Moreover, the chemokines that recruit T cells to the salivary gland remain undefined.

We examined $CD8^+$ T cell recruitment to the salivary gland in the presence or absence of active MCMV infection. Our data confirm and extend recent observations that uninfected salivary glands are permissive to the recruitment and retention of activated $CD8^+$ T cells in a manner dependent on the integrin $\alpha4$. Moreover, active MCMV infection of the salivary glands increased the rapid recruitment of activated T cells. Remarkably however, inflammation induced by MCMV infection did not enhance the number of T_{RM} that were ultimately lodged in the salivary gland. Indeed, many chemokines, abundantly expressed in the salivary gland of both infected and uninfected mice, could attract MCMV-specific T cells *in vitro*, including CXCL9 and CXCL10 ligands for the receptor CXCR3.

CXCR3 is a chemokine receptor that has been described to be important for $CD8^+$ T cell migration to a variety of tissues, typically in the context of inflammation and infection.[33] Unexpectedly, we found that CXCR3 expression by T cells was critical for efficient T cell accumulation in the salivary gland in uninfected mice, but dispensable for their accumulation in this organ during MCMV infection. These data establish

a mechanism for the surprisingly efficient recruitment of activated T cells to salivary glands of mice with no local infection.

2.3 Materials and Methods

Mice and infections

All mice were purchased from the Jackson laboratory and bred in house. B6 C57BL/6 (B6), B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ (CD45.1), B6.PL-Thy1^{a}/CyJ (Thy1.1) and B6.129S7-Ifng^{tm1Ts}/J (IFN-γ KO) mice were used as recipients in the adoptive transfer experiments and to assess chemokine expression in the salivary gland. OT-Is on a B6 background [C57BL/6-Tg(TcraTcrb)1100Mjb/J] were bred to CD45.1, CXCR3 KO mice (B6.129P2-Cxcr3tm1Dgen/J) and CCR5 KO mice (B6.129P2-Ccr5tm1Kuz/J) to generate congenic CXCR3 KO or CCR5 KO OT-I mice.

MCMV-K181 virus (kindly provided by Ed Mocarski) was used in figures 1, 3, 4, 5A-7, figure 9F. MCMV-SL8-015 or MCMV-K181-trf-Ova (MCMV-Ova), have been previously described and are recombinant viruses that express ovalbumin (Ova). [34,35] These viruses were used in figure 8, figure 9A-D and to expand OT-I T cells after the first adoptive transfer on figure 5. OT-I T cells are CD8^{+} T cells specific for the Ova peptide SIINFEKL.

For the experiments in figure 2 MCMV-TK virus was used. MCMV-TK virus is a recombinant virus where the m157 gene was replaced by the thymidine kinase (TK) gene derived from HSV-1, allowing for the replication of this virus to be blocked with Acyclovir or Famcyclovir treatments, since these act as DNA chain terminator once phosphorylated by the TK.[36] Briefly 2mg/mL of Famcyclovir was mixed with the drinking water of the treated mice starting 3 days before MCMV-TK intraperitoneal (i.p.) infection. Famcyclovir was replaced every other day until sacrifice. The control groups, with replicative MCMV-TK infection, were given water with no Famcyclovir treatment. Mice were sacrificed at 7, 4 or 28 days after infection, organs were collected and lymphocyte isolation and FACS staining performed as described below.

Infections were performed using 2x10^{5} pfu and the i.p. route (100 µl per injection) in all experiments except figure 1, where some of the infections were performed via intranasal (i.n.) or footpad (f.p.) routes in a total volume of 20-25 µL per inoculation after anesthesia with Isoflurane. MCMV-K181, MCMV-Ova and MCMV-SL8-015 were produced as described in [35–38]. All protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Lymphocyte isolation and FACS Staining

For all the experiments, except for the experiment represented in figure 2 and sup. figure 2, intravenous (i.v.) antibody injections were performed, as described in [39,40] without perfusion, to distinguish

between vasculature-localized (i.v.+) and parenchyma-localized (i.v.-) $CD8^+$ T cells. In brief, mice were injected intravenously with 3 μg of an anti-CD8α antibody (clone: 53-6.7 conjugated to BV650 or BV421) 3 minutes before sacrifice. Blood was collected from the retro-orbital sinus or from the chest cavity, after cutting the pulmonary vein at sacrifice and the organs were collected in media containing an unlabeled CD8α antibody (clone: 53-6.7). Total $CD8^+$ T cells were identified by CD8β staining (± CD8α of the intravascular portion) during the phenotypic analyses as described below.

Lymphocytes from the blood, spleen, inguinal lymph nodes, sub-mandibular salivary glands (referred to as "salivary glands" throughout), kidneys and lungs were isolated as described previously [40] with minimal modifications. Briefly, the mucosal organs were minced using the gentleMACS Dissociator (Miltenyi Biotec) and incubated at 37°C for 1-1.5 hours in digestion media containing 1 mg/ml collagenase type IV, 5 mM CaCl2, 50 mg/ml DNase I, and 10% FBS in RPMI. Salivary glands were suspended in 40% Percoll and overlayed on top of a 75% Percoll layer, while the kidneys and lungs were suspended in 40% Percoll. Suspensions were centrifuged at 600 xg for 25-30 minutes and the lymphocytes were collected from the 75/40 interface (salivary glands) or pellets (kidney and lung).

Phenotypic analyses of T cells were performed using the following antibodies: CD8α (clone: 53-6.7); CD8β (YTS156.7.7); CD69 (clone: H1.2F3); CD103 (clone: 2E7); CD44 (clone: IM7); Killer cell lectin-like receptor subfamily G member 1 (KLRG1) (clone: 2F1); CXCR3 (clone: CXCR3-173); CXCR4 (clone: L276F12); CXCR6 (clone: SA051D1); CX3CR1 (clone: SA011F11); CD4 (clone:RM4-4). OT-Is were identified by their congenic markers with CD45.1 (clone: A20) and/or CD45.2 (clone: 104) and by the T cell receptor (TCR) chains Vα2 (clone: B20.1) and Vβ5 (clone: MR9-4). In figure 10E (exp. 2) donor cells were identified also by the Thy1.2 marker (clone: 30-H12). All antibodies were purchased from Biolegend or BD Bioscience. All MHC-tetramers, loaded with peptides from M38, were provided by the National Institutes of Health Tetramer Core Facility (http://tetramer.yerkes.emory.edu/) and used as described[41]. A tetramer loaded with the B8R peptide derived from Vaccinia virus was used as a negative control for the tetramer staining following a MCMV-infection. All samples were collected on a BD LSRFortessa or LSR II flow cytometer and analyzed using FlowJo software (TReeStar). The gating strategy is represented in the sup. figure 1.

In vitro T cell activation and expansion

OT-Is were activated *in vitro* based on the protocol described [12] with modifications. Briefly, splenocytes from OT-I mice were harvested and $4x10^6$ cells/mL were cultured with 1 μg/mL of the SIINFEKL peptide for 2 days. On the second day the cells were resuspended to $5x10^5$ cells/mL and

incubated with 0.03 U/mL of IL-2 that was renewed every 2 days, for a total of 4-5 days, until the adoptive transfer.

Adoptive transfers

All the adoptive transfers were performed via retro-orbital injections in a volume of 100 μL between congenic donor and recipient mice (differing in CD45.1/.2 or Thy1.1/1.2 expression).

In vitro activated T cells:

For the adoptive transfers in figures 3, 4, 5(C), 9E-F and 10, *in vitro* activated, $CD8^+$ $CD44^+$ OT-I T cells were transferred to congenic naïve recipients or recipients infected for 9 weeks (figure 3) or 11 days (figure 4 and 9) with MCMV-K181 (lacking Ova). For treatment with Pertussis Toxin (PTx), OT-Is were suspended at a concentration of $1.5x10^7$ cells/mL and treated, or not, with 50 ng/ml PTx (Sigma-Aldrich) for 1 hour at 37 °C prior to transfer. In figure 9F, wild-type (WT) and CCR5 KO OT-I T cells were mixed and co-transferred to B6 mice that have been previously infected with MCMV-K181 for 11 days. The mixture of donor cells was treated with anti-CXCR3 blocking antibody (clone: CXCR3-173) or isotype control antibody (Polyclonal Armenian Hamster IgG) at a concentration of 30 μg per $4x10^7$ cells for 15 minutes. Recipient mice were also treated with the anti-CXCR3 antibody or isotype control (250 μg/mouse) via i.p. injections on days -2; day 0 (the day of transfer) and day 2. Blocking antibodies and isotype controls were purchased from Bio X cell. In figure 10 the same approach was used but only WT OT-Is were transferred to naïve recipients.

In vivo activated T cells:

For *in vivo* activation (figures 5A-B, figure 8, figures 9A-D), naïve OT-I T cells were transferred into naïve congenic recipients, followed by infection with MCMV expressing the cognate SIINFEKL peptide (either MCMV-SL8-015 or K181-MCMV-Ova) 1-3 days after transfer. The number of cells transferred is indicated in each figure legend. In figures 5A-B, 5×10^4 OT-Is were transferred into congenic recipients to produce large numbers of OT-Is for a secondary transfer by day 5 post-infection. In this experiment, OT-Is were recovered from the spleen 5 days after infection, treated for 30 minutes with 60 μg/mL of either anti-α4 (clone PS/2), anti-α4β7 (clone DATK32) or the respective isotype controls and transferred to a new group of infection-matched or naïve recipients. The recipients were treated on the day of the transfer with 300 μg of each antibody or isotype control via i.p. injection. Organs were collected 2 days after the secondary transfer and the tissues were processed as described above.

CD4 antibody treatment

To achieve $CD4^+$ T cell depletion, 100ug of anti-CD4 monoclonal antibody from Bio X Cell® (clone: GK1.5) or the isotype control IgG2b (clone LTF-2) were administered i.p., every other day starting 7 days prior to MCMV infection. The $CD4^+$ T cell depletion was confirmed before infection and the antibody blockade was maintained by repeated administrations (1-2 days interval) untill sacrifice.

Cell proliferation assays

To assess proliferation of OT-I T cells in naïve or MCMV-infected mice (figures 4E-G), OT-Is were labeled with a cell tracer dye: CellTrace Violet or Carboxyfluorescein succinimidyl ester (CFSE) following manufacturer's instructions before the adoptive transfer. Briefly, cells were suspended at a concentration of 10^6 cells/mL and incubated with 5 μM of the Cell Trace Violet dye in for 20 minutes or suspended at a concentration of $1x10^7$ cells/mL and incubated with 1 μM CFSE for 10 minutes.

Transwell migration assays

For Transwell migration assays, $CD8^+$ T splenocytes were isolated from MCMV-K181 infected mice (7 days) using the EasyStep Biotin selection kit (StemCell Technologies) and biotinylated antibodies against erythrocytes (Ter119), CD19 (6D5), NK1.1(PK136), I-A/I-E (M5/114.15.2) and CD4 (GK1.4) following the manufacturer's protocol. Typically, $CD8^+$ T cells were 80-90% pure following this protocol. Purified cells were resuspended in RPMI media containing 2% BSA and 25 nM of Hepes buffer (migration media) at a concentration of $5x10^6$ cells/mL and incubated for 1 hour at 37 °C. Subsequently, $5x10^5$ cells, in a total volume of 100 μL were added to the upper chamber of a 6.5 mm Polycarbonate Transwell system (from Corning Inc.) with a pore diameter of 5.0 μm. Chemokines (all from Biolegend) were diluted in migration media and added to the lower chamber at titrating concentrations in a total volume of 600 μL (3-4 replicate wells per concentration). Control (media alone) samples without chemokines in the lower chamber of the Transwell plates, were included on every plate to account for plate-to-plate variations in T cell migration. All tests were run in duplicate, or triplicate on every plate.

Chemokine concentrations, shown in figure 7, represent the optimal migration over control (media alone) wells based on replicate titrations of each chemokine. Cells in the Transwell plates were incubated for 1.5 hours at 37°C. At the end of the incubation, 200 μL of cells from the bottom chamber were mixed with counting beads (CountBright Absolute Counting Beads, Invitrogen), while the remaining

volume was used for FACS analyses of tetramer-binding cells. Cells were collected by flow cytometry as above.

qRT-PCR

For assessment of chemokine receptors expressed by T cells in figure 8C-D, OT-I T cells were transferred to B6 mice that were then infected with MCMV-K181-Ova (n=3 mice). 7 days after infection, spleen and salivary glands were collected and $CD8^+$ T cells were enriched using the EasySep Biotin selection kit (Stemcell Technologies), as described above for the Transwell assays.

OT-Is were sorted based on the congenic markers CD45.1 and CD45.2. RNA was extracted from the sorted OT-Is (figures 8C-D) and whole salivary glands of naïve B6 or IFN-γ KO mice (figure 10F) using the RNeasy Mini Kit (QIAGEN), cDNA was recovered using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In both cases the β-actin and chemokine receptor transcripts expressed were detected on a StepOnePlus system (Applied Biosystems) using predesigned qPCR assays from Integrated DNA Technologies and 6-carboxyfluorescein for detection. The relative concentration of chemokine receptors on these cells was determined by comparing the chemokine receptor signal to the β-actin signal for the same sample on the same plate and the data is expressed as the $2^{\Delta_{CT}}$ value (e.g. $2^{-(CT\ value\ for\ chemokine\ A\ -\ CT\ value\ internal\ reference\ control\ A)}$).

RNA-Seq

For the RNA-Seq analysis salivary glands from mice that were infected, with $2x10^5$ pfu of MCMV-K181 i.p., for 14 days or naïve mice were collected and RNA was obtained with the miRCURY RNA Isolation kit Tissues (EXIQON).

RNA Clean-up and ribosomal RNA (rRNA) depletion for the RNA-Seq analysis

Prior to cDNA library preparation, RNA samples were purified using RNA Clean & Concentrator (Zymo research, R1015) and treated with DNase I to remove contaminating genomic DNA.

Validation of RNA quality and concentration was determined using the RNA 6000 Pico Kit (Agilent Technologies, catalog number 5067-1513) for the BioAnalyzer 2100 instrument (Agilent Technologies).

Four micrograms of total RNA per sample were used as input for the depletion of ribosomal RNA (RiboMinus kit; Ambion, A15020), yielding 6% recovery of input RNA on average.

RNA-Seq Library Preparation and Sequencing

Library preparation was performed using the Ion Total RNA Seq Kit v2 (Thermo Fisher Scientific) according to the manufacturer's protocol. The yield and size scatter of the cDNA libraries were evaluated using High Sensitivity DNA Chips (Agilent Technologies, catalog number 5067-4626) on the BioAnalyzer 2100 instrument. The barcoded cDNA libraries were diluted to a final concentration of 100 pM, and used for template preparation on the Ion Chef instrument using the Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific, catalog number A27198). Sequencing was performed on the Ion Proton sequencer system from Thermo Fisher Scientific using Ion Torrent PI v3 chips (Thermo Fisher Scientific, catalog number A26771). Sequenced data were preprocessed on the Ion Torrent server with Torrent Suite version 4.4.3.

RNA-seq data Analysis

IonTorrent single-end sequence reads were mapped to the mm10 genome using the IonTorrent Torrent Server tmap aligner, version 4.4.11. Gene abundances were estimated by counting strand-specific reads using the Subread package featureCounts tool, where reads with mapq of 0 or multiple mappings were filtered out.[42] Reads overlapping more than one gene feature were not counted because the transcript of origin could not be confidently determined, as recommended by the Subread/featureCounts manual. Resulting read counts were then used to analyze differential expression between infected and uninfected samples using the DESeq2 package for R/bioconductor, with default settings.[43] For additional data exploration and visualization, reads per kilobase of transcript per million mapped reads (RPKM) estimates were calculated from featureCounts results.

Gene Set Enrichment Analyses (GSEA) was also performed to identify up- and down-regulated gene groups among available pathway and gene ontology annotations[44]. Mouse gene symbols were converted to their human orthologs, obtained from MGI on March 3rd 2017, for compatibility with GSEA. GSEA preranked analysis was performed using the list of available human orthologs and the DESeq2 test statistic as the ranking metric, and GSEA enrichment score set to "classic" as described in the GSEA FAQ. Data have been submitted to the NCBI-GEO database (https://www.ncbi.nlm.nih.gov/geo/). The accession number is: GSE107338.

ELISAs for chemokine expression

Whole salivary glands of naïve B6 and IFN-γ KO mice were collected in PBS with 10 μL/mL of the Halt™ Protease Inhibitor Cocktail (Thermo Scientific) and homogenized for 2 cycles of 30 seconds in a mini bead beater. After centrifugation (14000 xg for 15 minutes) the supernatant was stored at -80°C.

The total protein concentration was determined using the Quick Start™ Bradford Protein Assay from Bio-Rad. Samples were normalized to 8 mg/mL of total protein. The concentration of CXCL9 in each sample was quantified in duplicate with the CXCL9 (MIG) ELISA kit (Thermo scientific) according to the manufacturer's specifications.

Statistical analysis

Differences in absolute numbers were determined after Log_{10} transformation and the fold change was determined based on the ratios of the geometric mean. The specific statistical test used for each experiment is indicated in the figure legend. Prism 6 for Mac OS X was used to determine the Log_{10} transformed values, geometric mean, standard error of the mean (SEM) and to perform the statistical analysis.

2.4 Results

2.4.1 Impact of MCMV infection in T_{RM} differentiation

Impact of MCMV infection in the gene expression of salivary glands

To understand the modifications in the salivary glands' gene expression after MCMV infection, salivary glands were extracted from naïve mice or mice that had been infected for 2 weeks with MCMV strain K181 and used for a RNAseq analysis. Table 2 shows the top 50 genes, which expression was increased in MCMV infected mice (complete gene list in sup. tables S1A and S1B). Perhaps not surprisingly, the dominant changes in overall gene expression could be traced back to immune responses, including increases in genes encoding MHC molecules such beta 2 microglobulin (B2m) and genes involved in the antigen processing machinery such as Tap1 and Protease subunit beta (Psmb)8 (Table 2)

Interestingly, several IFN-γ-induced guanylate-binding proteins (Gbp) such as Gbp 2, 3, 6, and 7 and interferon gamma inducible protein 47 and IFN-γ-induced GTPases (Iigp) as Iigp 1, immunity-related GTPase family M (Irgm)1, 2 and the interferon-inducible GTPase (Igtp) were among the most expressed genes after infection, suggesting a dominant IFN-γ-induced change in gene expression. Gene Set Enrichment Analyses (GSEA) using the Gene Ontology Biological Process database revealed that the top 20 enriched gene sets (over-represented set of genes that are statistically different in two conditions) were all related to innate and adaptive immune responses, cytokine signaling, IFN-I, IFN-γ and leukocyte activation (Table S1C). In contrast, very few genes were significantly downregulated after MCMV infection and many of these were small nucleolar RNAs (SNORDs, Table S1A).

Table 2. Top 50 genes with differential expression between salivary glands of MCMV infected and uninfected mice.

Salivary glands from mice that were infected, with MCMV-K181 for 14 days or naïve mice were collected and RNA was obtained. The read counts were then used to analyze differential expression between infected and uninfected samples using the DESeq2 package for R/bioconductor, with default settings. RPKM estimates from infected and uninfected were calculated from featureCounts results and shown in the left panel. log2, mean centered RPKM is shown in the right panel. The top 50 differently expressed genes between naïve and MCMV infected salivary glands are presented. The log2 fold change between infected and uninfected based on the average of the 3 sample's log-2 mean-centered RPKMs in each group is ranked. The colors display the pattern of the gene expression across the samples (red indicating

higher values and blue lower values). This table is a detail of table S1A for all the genes that increased or decreased significantly with infection (FDR<0.05) available in: https://www.jimmunol.org/content/suppl/2017/12/29/jimmunol.1701272.DCSupplemental

Top 50 with differential expression

		Sample Reads Per Kilobase Per Million Reads (RPKM)						Log2, mean-centered RPKM					
		uninfected			infected			uninfected			infected		
Gene	log2 Fold Change	DK1-R	DK1-L	DK1-N	DK5-R	DK5-L	DK5-N	DK1-R	DK1-L	DK1-N	DK5-R	DK5-L	DK5-N
Igtp	1.613575115	3.16320126	2.78539685	4.80376721	18.2338811	13.2650395	11.2167383	-1.1401351	-1.3236375	-0.5373542	1.38702933	0.92803662	0.68606088
Iigp1	1.59498252	2.47791047	1.74416562	2.61568086	9.07148568	8.92183955	6.80213591	-0.7914619	-1.2980489	-0.7133994	1.08075293	1.05675528	0.66540191
Gbp2	1.529682012	3.63708965	4.13146399	4.81899565	17.9455924	17.1637206	9.57072235	-1.0878232	-0.9039546	-0.6818752	1.21494998	1.15068276	0.30802016
Irgm2	1.270279995	3.43034273	4.008863	4.80959197	10.3655683	10.626312	10.0754461	-0.9155687	-0.6907283	-0.4280069	0.67980588	0.71564765	0.63885038
B2m	1.251512625	69.3788274	74.2119971	90.4467687	178.976127	172.486171	188.129544	-0.7666671	-0.6695101	-0.3840936	0.60053269	0.54724622	0.67249195
Psmb8	1.235543706	13.0200176	10.4434541	11.1705362	22.3136114	34.8272306	28.2914601	-0.4628578	-0.7809902	-0.6838908	0.31433482	0.95662655	0.65677743
CxcL9	1.212842206	0.15368425	0.64657003	0.09696446	2.03005759	2.51576693	1.938944	-2.1370104	-0.0641731	-2.8014517	1.58646902	1.89594664	1.5202195
Psmb9	1.163060538	8.67794849	6.75810079	7.16436263	16.3551348	21.393334	21.1648006	-0.477507	-0.8382432	-0.7540227	0.43681068	0.82422836	0.80873392
CCL5	1.131996304	2.52233195	3.89098783	1.59142243	8.05733688	14.8385388	14.2444831	-1.1150957	-0.4897174	-1.779537	0.56044924	1.44142329	1.38247753
Ifi47	1.111818966	1.77427821	2.51931051	1.60921031	4.94013703	5.78619364	6.23582884	-0.8934946	-0.3876979	-1.034374	0.58382421	0.81188776	0.91985448
Ctss	1.103317736	22.5414871	13.6728168	20.4012164	31.6292104	48.7835938	39.8780901	-0.2600503	-0.9813226	-0.4039779	0.2286245	0.853763	0.56296327
H2-K1	1.091839072	318.344123	169.837783	239.76888	406.389666	567.934384	485.123789	-0.0799679	-0.9863975	-0.4889106	0.27230873	0.75516928	0.52779794
Oasl2	1.081462425	6.97582429	3.71344798	4.85038762	11.9099733	11.8343354	9.80168342	-0.0985126	-1.0081169	-0.6227762	0.67322199	0.6640305	0.39215326
Apof	1.066056299	6.32281966	3.211577	5.98391324	8.44784861	16.330081	11.5904647	-0.2674069	-1.2446931	-0.3468857	0.15060902	1.10148509	0.60689155
H2-Ab1	1.051072685	62.3711587	64.3938769	58.555808	88.3972417	165.497377	129.501306	-0.4866622	-0.4406177	-0.577729	0.01646012	0.92119522	0.56735351
Il2rb	1.041490132	0.71759221	2.71710504	1.14929505	4.37486446	4.8709595	5.61114283	-1.823063	0.09777123	-1.1435499	0.78493921	0.93990688	1.14399553
Tap1	1.040515845	11.9054068	10.8240207	9.11885051	19.5989275	24.1158513	20.1301823	-0.3288654	-0.4662458	-0.7135585	0.39029238	0.68949941	0.42887791
Cxcr3	1.022376938	0.64743511	0.11673623	0.0000001	2.08413704	2.83883737	2.40048256	3.23135585	0.75987263	-19.394948	4.91799849	5.36384855	5.1218728
Zbp1	0.98894977	0.68833295	0.4343862	0.97715693	2.40680632	2.58807559	2.45641425	-0.8988956	-1.5630239	-0.3934119	0.90704596	1.01180567	0.93647979
Lgals3bp	0.985217966	11.188776	12.3024264	18.6445267	24.5881564	28.2846998	29.8407774	-0.7937841	-0.6568934	-0.0570841	0.34212726	0.54418556	0.62144882
H2-Eb1	0.984557663	47.3469967	37.1380684	32.5731465	59.237142	98.3188971	69.0398826	-0.1719363	-0.5223105	-0.7115262	0.15129278	0.88225947	0.37222075
Irgm1	0.979183699	13.298024	9.78361785	19.3521827	30.7387221	25.9457881	25.7260518	-0.5372774	-0.9800493	0.004007	0.6715679	0.42701107	0.41474077
H2-D1	0.975966992	34.2040417	38.0850606	46.5683571	64.8774176	91.4985003	68.2293842	-0.6565998	-0.5015414	-0.2114166	0.26694978	0.76298148	0.33962658
Stat1	0.973509012	10.7528763	8.34637891	10.9390979	18.6749677	16.8218208	19.740955	-0.3296224	-0.6951227	-0.3048513	0.46676073	0.31598885	0.54684677
Irf7	0.971301689	11.367454	6.87054568	18.2585547	19.7180603	30.0376558	24.7588234	-0.546126	-1.2725386	0.13753741	0.24848248	0.85573707	0.5769076
CD52	0.963556189	4.73376015	4.85441695	3.08001935	7.81619258	13.8483623	21.1163722	-0.6409507	-0.6046392	-1.2609979	0.08252874	0.9077062	1.51635282
Rnu11	0.943832884	786.804082	1302.62609	1789.20087	2490.40669	4564.51732	3738.69695	-1.3932893	-0.6659426	-0.2080503	0.2690157	1.14309665	0.85516988
Gbp3	0.942197725	2.35344094	2.46477631	2.27468455	5.05800048	5.57128998	4.33196102	-0.5408106	-0.4741256	-0.5899158	0.56298489	0.70242913	0.33943798
Nlrc5	0.934232705	1.47853324	2.08247686	1.25732683	3.52978237	3.53510873	2.98924023	-0.6314899	-0.1373561	-0.8652969	0.62392265	0.626098	0.38412226
Mir6240	0.928596804	57.2375326	131.640553	80.6524725	188.7695	309.392632	143.050979	-1.1956369	0.00593367	-0.7008797	0.52595536	1.23876852	0.12585905
Cited2	0.926032554	4.91271864	4.67425146	1.93128689	5.63780222	10.4391072	12.0147566	-0.1929141	-0.2647004	-1.5398732	0.00569716	0.89449071	1.09729981
Ly6a	0.905897585	28.8129855	25.6811551	39.6378538	50.6618585	67.1249754	51.8773949	-0.5294748	-0.6954839	-0.0693151	0.28470604	0.69065569	0.31891207
Laptm5	0.905071857	7.50914419	9.39926244	5.7557993	12.7303126	13.1430594	19.8648775	-0.4854824	-0.1615834	-0.8691146	0.27606502	0.32209832	0.91801707
Cxcr6	0.902946214	0.0000001	0.29766814	0.0000001	1.64929168	1.38743865	1.53026008	-15.512302	5.99297157	-15.512302	8.46304144	8.21361885	8.35497174
Gzma	0.899196608	1.01697666	0.42785557	0.32082206	2.50232321	5.33243914	3.66588501	-0.4376389	-1.6867297	-2.1020802	0.86134267	1.95287012	1.41223605
Slc25a29	0.898340674	6.81954309	5.54687295	4.87636465	8.53710922	14.1286724	11.0620507	-0.215159	-0.5131594	-0.699028	0.10891358	0.83571995	0.48271289
Prf1	0.875333012	0.36226286	0.45722637	0.54855262	1.40742055	2.05701686	2.11546909	-1.3239538	-0.9880821	-0.7253607	0.63399082	1.18149095	1.22191494
Hist1h2ai	0.865109761	2.65745687	2.93482179	4.40127765	6.4527719	13.2544556	13.650512	-1.1383619	-0.9951353	-0.4104858	0.14151082	1.1799973	1.22247495
Ifitm3	0.857495228	19.0615116	15.6523829	13.9102109	31.0497824	27.4907228	32.5341637	-0.2107829	-0.4950631	-0.6653011	0.49313773	0.31749941	0.56051004
H2afx	0.843058774	5.03723981	2.90242861	2.90179895	6.12628729	8.90682614	7.81569921	-0.0245567	-0.8199296	-0.8202426	0.25782286	0.79772127	0.60918478
Gbp6	0.84189252	0.48272618	0.87038274	1.37055541	2.57201867	2.27537814	1.64064492	-1.4618947	-0.6114498	0.043589	0.95172945	0.77493467	0.30309138
C1qb	0.839781199	35.1725177	17.5094452	21.7494398	42.0288113	53.3327191	35.4596708	0.13842546	-0.8678898	-0.5550449	0.3953555	0.73899773	0.15015599
Slfn2	0.832163958	5.90262055	5.86964357	7.36367607	10.4237085	11.8721493	12.4761258	-0.5365542	-0.5446369	-0.2174836	0.28388695	0.47159944	0.54318832
Nkg7	0.82699672	0.0000001	0.92412593	0.34647204	1.99123175	5.89922976	3.76102667	-20.014962	3.12469639	1.70934587	4.23219617	5.79906163	5.14966157
Lgals1	0.825345197	5.95270339	5.40007209	10.2109642	13.7314986	17.128835	12.6734227	-0.7431346	-0.8837009	0.03536761	0.4627376	0.78167555	0.34705472
Selplg	0.825301868	2.05738596	2.30818549	1.13581359	3.06402588	4.51682029	4.44974245	-0.3551468	-0.1892001	-1.2122332	0.21946923	0.77934826	0.75776258
H2-Q4	0.823928738	15.8528492	11.5232266	11.6254607	17.7352258	26.4304388	22.7980019	-0.0859745	-0.546172	-0.5334288	0.07590099	0.65148367	0.43819068
Stat2	0.812234407	7.24782957	4.70212685	8.81457518	12.6318076	10.4992404	11.6835276	-0.2805109	-0.9047465	0.00183107	0.52092923	0.25415308	0.40834406
H2-DMa	0.809529553	3.32101968	3.96501858	2.54838754	4.74251492	7.50720954	8.2504523	-0.4795305	-0.2238292	-0.8615721	0.0344955	0.69711994	0.8333164
Rps11	0.795539183	8.15777458	16.2243969	17.3126669	17.2874261	44.1973262	26.7329488	-1.2185094	-0.2265921	-0.1329289	-0.1350338	1.21920217	0.49386206

The route of MCMV infection impacts the CD8+ T cell migration to the salivary gland but not the rate of T_{RM} differentiation

We have previously shown that MCMV infection by the i.p. route leads to robust differentiation of T_{RM} in the salivary gland after infection.[11] However, factors outside of the tissue such as the site of T cell priming may influence $CD8^+$ T cell trafficking and T_{RM} differentiation in some tissues.[45–48] To determine whether the route of MCMV infection influenced the migration of $CD8^+$ T cells and differentiation of MCMV-specific T_{RM}, we compared T_{RM} numbers 14 days after infection via the i.p., i.n. and f.p. routes. Here, and throughout, cells that were still within the circulation were distinguished from those that had reached the parenchyma by intravascular staining with anti-CD8 antibodies, as described in the materials and methods. As shown in figures 1A and 1B, MCMV-specific $CD8^+$ T cells reached the parenchyma of the salivary gland after all 3 routes of infection (i.p., i.n. and f.p.) and developed the T_{RM} phenotype. Interestingly, comparing the different routes of infection, the f.p. route was associated with lower overall numbers of MCMV-specific T cells in the salivary gland. Notably, this effect was not due to an overall difference between circulating populations, as seen by the similar number of MCMV-specific T cells in the spleen at the same time point after all 3 infections. Importantly however, the frequency of T cells that developed the T_{RM} phenotype in the salivary gland was identical after all 3 routes of infection (figure 1C), implying that once T cells arrived in the gland, they were equally capable of differentiating into T_{RM}. In contrast, differentiation of MCMV-specific T_{RM} mostly occurred in the lung after i.n. infection (figures 1B and 1C), which confirms the results of a recent study.[19] These data suggest that the route of infection impacts the ability of T cells to reach the salivary gland but not their differentiation into T_{RM}.

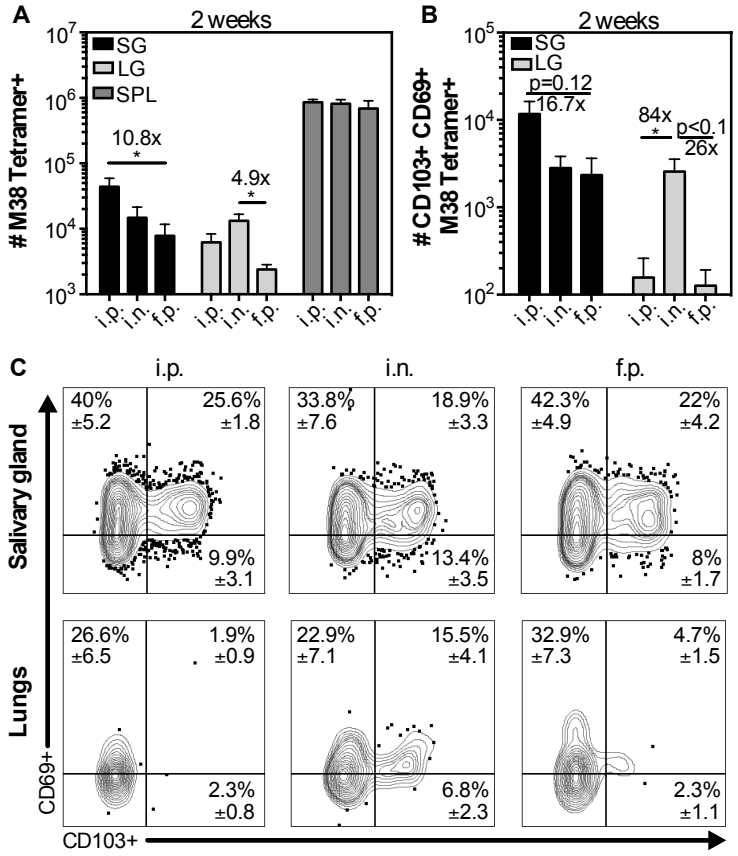


Figure 1. MCMV-specific CD8⁺ T cells accumulate in the salivary gland after several routes of MCMV infection.

Mice were infected by intraperithoneal (i.p.), intranasal (i.n.) and footpad (f.p.) inoculation. Cells in the parenchyma or vasculature of each tissue were distinguished by i.v. staining as described in the materials and methods. **A**) Shown is the absolute number of M38 tetramer⁺ CD8⁺ T cells from the parenchyma of the salivary gland (SG) and lungs (LG), and from the overall CD8β⁺ population of the spleen two weeks after infection. **B**) Absolute number of CD103⁺CD69⁺ M38 Tetramer⁺ cells in the SG and LG from the data shown in (A). Data are from 2 independent experiments (n=6 for f.p. and i.p.; n=5 for i.n.). Error bars represent SEM; statistical significance was measured by one-way ANOVA after $\log_{10}$ transformation of the absolute numbers (*$p<0.05$). **C**) Concatenated FACS plots from one representative experiment (n=2 i.n.; n=3 for i.p. and f.p.) of the CD69 and CD103 expression of M38 tetramer⁺ CD8⁺ T cells from the SG (top panel), and the LG (bottom panel). The mean frequency ±SEM in the indicated quadrant were calculated considering both experiments.

The infectivity and the viral spread associated with each route might influence the $CD8^+$ T cell numbers that reach to the salivary gland and then differentiate into T_{RM}. However, similar T_{RM} formed after infection even when viral replication was compromised (figure 2). These data suggest that viral replication have minor impact in T_{RM} differentiation in the salivary gland.[49] This is in line with previous studies that suggested that activated T cells could enter and reside in naïve, uninfected salivary glands.

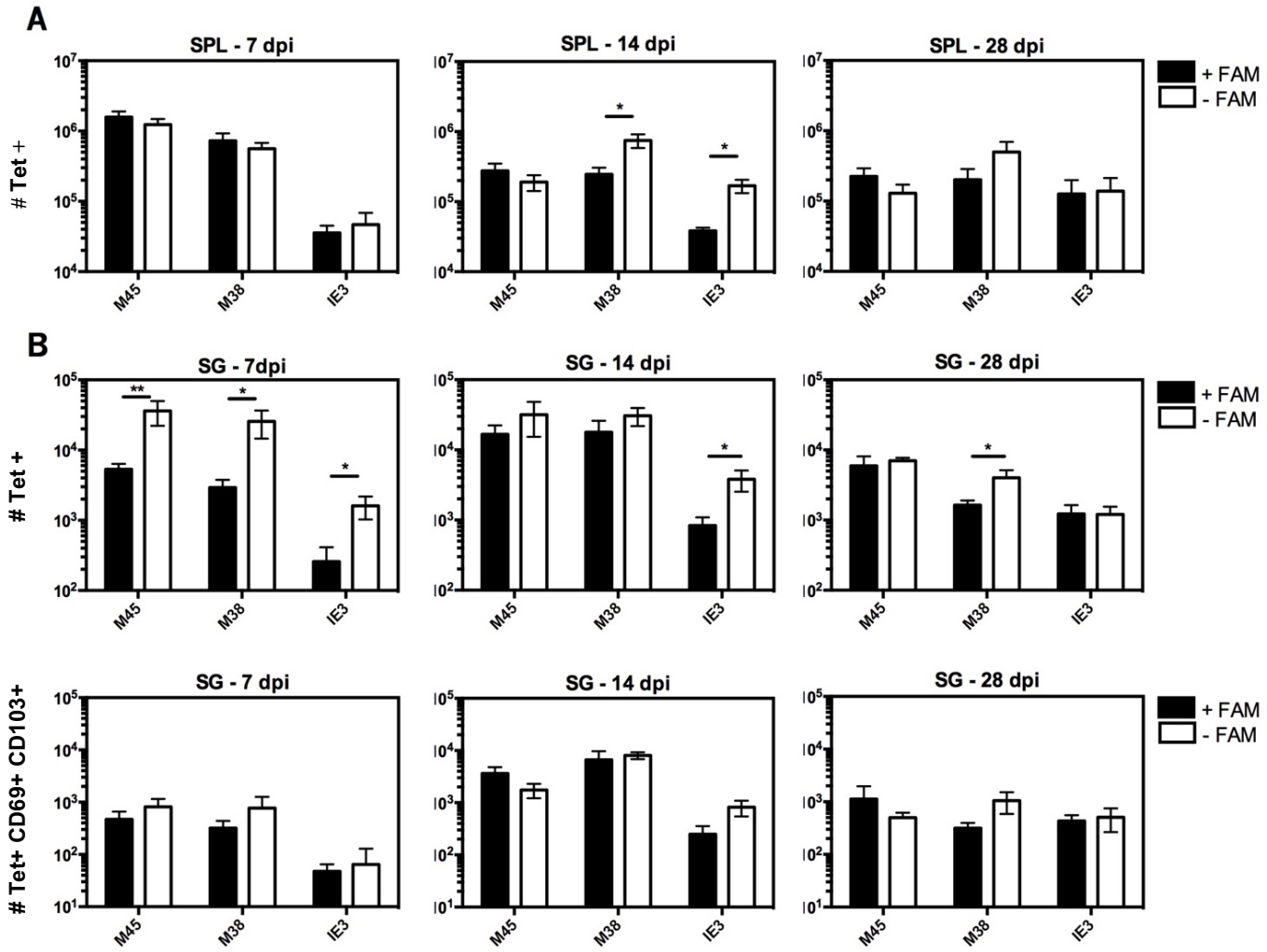


Figure 2. MCMV-specific $CD8^+$ T cells and T_{RM} following i.p. infection with replicative and non-replicative virus.

Mice were divided into 2 groups. One group was treated with famcyclovir (+FAM), which blocks MCMV-TK viral replication, and the other remained untreated (-FAM). Mice were infected with the MCMV-TK virus via i.p. route and sacrificed after 7, 14 and 28 days of infection. **A**) Absolute number of $tetramer^+$ cells in the spleen (SPL). **B**) Absolute number of $tetramer^+$ cells (M45, M38 and IE3) in the salivary gland (SG).

C) Absolute number of tetramer$^+$ CD69$^+$ and CD103$^+$ cells in the SG. Results from 2 independent experiments were combined (n=4 at day 7 and 28; n=6 at day 14). Error bars represent the SEM and statistical significances were measured by unpaired t-test after $\log_{10}$ conversion of the absolute numbers (*p<0.05; **p<0.01).

Activated CD8$^+$ T cells enter the salivary gland and differentiate into T_{RM} phenotype even when a pre-established T_{RM} population is already present

We previously showed that persistent MCMV infection results in continuous recruitment and retention of new MCMV-specific T_{RM}, long after the primary infection had been resolved.[11] To directly compare these two conditions, naïve and latent MCMV infection, we activated OT-I T cells *in vitro* and transferred them to congenic mice that were either naïve, or latently infected with WT MCMV (9 weeks after infection, figure 3A). WT MCMV was used in these experiments to assess the impact of viral infection and the presence of unrelated T_{RM}, and to avoid the complication of antigen-driven T cell expansion of the donor OT-Is. 2 weeks after transfer, the OT-Is had reached the salivary gland in similar numbers in the naïve and latently infected recipients (figure 3B), and similar frequencies of OT-Is expressing CD69 and CD103, the markers of bonafide T_{RM} in the salivary gland, were observed in naïve and infected salivary glands (figures 3C and 3D). Likewise, a pre-established T_{RM} population did not prevent new T_{RM} differentiation when naïve or MCMV-infected mice were infected with Vaccinia virus (VACV). VACV-specific T_{RM} (B8R tetramer$^+$) were present in the salivary glands in similar numbers and the frequency of T_{RM}-phenotype VACV-specific T cells was equivalent between previously naïve and infected groups (data not shown). Thus, our data suggest that naïve, acutely-infected and latently-infected salivary glands are all capable of recruiting activated CD8$^+$ T cells and supporting their differentiation into T_{RM}.

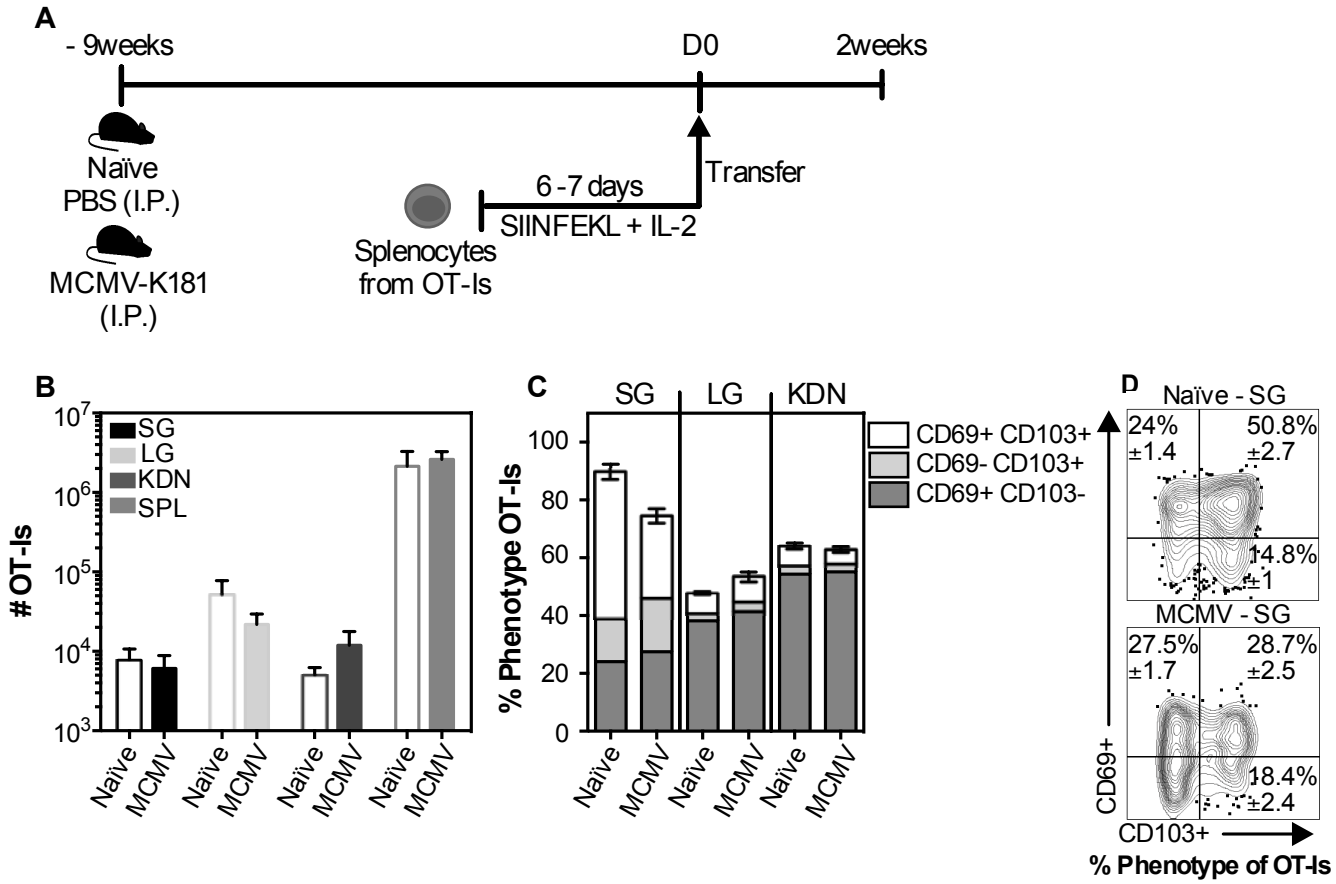


Figure 3. CD8⁺ T cells with a T_{RM} phenotype can form in the salivary glands of naïve mice and mice latently infected with MCMV.

A) Schematic representation of the experimental design. Naïve mice or mice that had been infected (i.p.) for 9 weeks with WT MCMV were seeded with $3x10^6$ *in vitro* activated OT-Is and sacrificed 2 weeks after transfer. **B**) Absolute number of OT-Is from the parenchyma of the salivary gland (SG), lungs (LG), kidneys (KDN) and from the overall CD8β⁺ population of the spleen (SPL). **C**) Frequency of CD103 and CD69 expression on OT-Is from the parenchyma of the SG, LG and KDN. Data combined from 2 independent experiments (n=7). **D**) Concatenated FACS plots from a representative experiment with frequency mean ± SEM values considering all the experiments. An unpaired t-test was used to test for statistical significance.

Acute MCMV infection promotes rapid CD8⁺T cell recruitment to the salivary gland but does not affect the overall numbers of T_{RM} phenotype T cells

Although the above experiments demonstrate that infection and inflammation are not needed for T cell recruitment to the salivary gland and differentiation of the T_{RM} phenotype, MCMV infects the salivary gland directly and our data do not exclude a role for acute inflammation in promoting the process. To

directly compare T cell migration to naïve salivary glands and salivary glands with active MCMV replication, *in vitro* activated OT-Is were transferred to naïve mice or mice infected 11 days previously with MCMV (figure 4), a time when replicating MCMV can be detected in the salivary glands after i.p. infection.[11] For these experiments, we infected mice with WT MCMV (virus that does not express Ova) to test the role of inflammation in the gland, again without the confounding issue of the transferred cells undergoing antigen-driven proliferation in infected mice (figure 4A). An increased recruitment of OT-Is to the salivary gland, but not other organs, was observed in the infected recipients early after transfer (figure 4B). At later time points, 31 days after transfer, there was a reduction in the number of OT-Is in the lung and the kidneys, as expected, due to the lack of antigen, but not in the salivary gland. Remarkably, the early advantage in T cell numbers recruited to the infected salivary gland was not due to higher T cell proliferation rates in the infected glands (figure 4E-G) and was no longer evident at 31 days after transfer (figure 4B). Moreover, at this later time-point, the frequency of OT-I T_{RM} present in the salivary gland was similar in both infected and naïve recipients (figure 4C-D). Thus, surprisingly, our data suggest that although inflammation of the salivary gland, caused by MCMV infection, resulted in an accelerated accumulation of activated OT-Is in the gland shortly after transfer, infection and inflammation provided no long-term advantage for the differentiation or maintenance of $CD8^+$ T_{RM}.

MCMV infection of the salivary glands results in an influx of $CD4^+$ T cells in the salivary gland that can contribute to T_{RM} differentiation, even in an antigen-independent way. $CD4^+$ T cells can impact the outcome of $CD8^+$ T cells in multiple ways.[50,51] $CD4^+$ T cells have been shown to be crucial for $CD8^+$ T_{RM} differentiation in the brain and in the lungs.[52,53] Interestingly, $CD4^+$T cell depletion did not impact the number of MCMV-specific T_{RM} seen in the salivary glands at 14 days after MCMV infection (sup. figure 2), also suggesting that the MCMV infection and the resulting increased presence of $CD4^+$ T cell have a minor impact in T_{RM} differentiation in the glands.

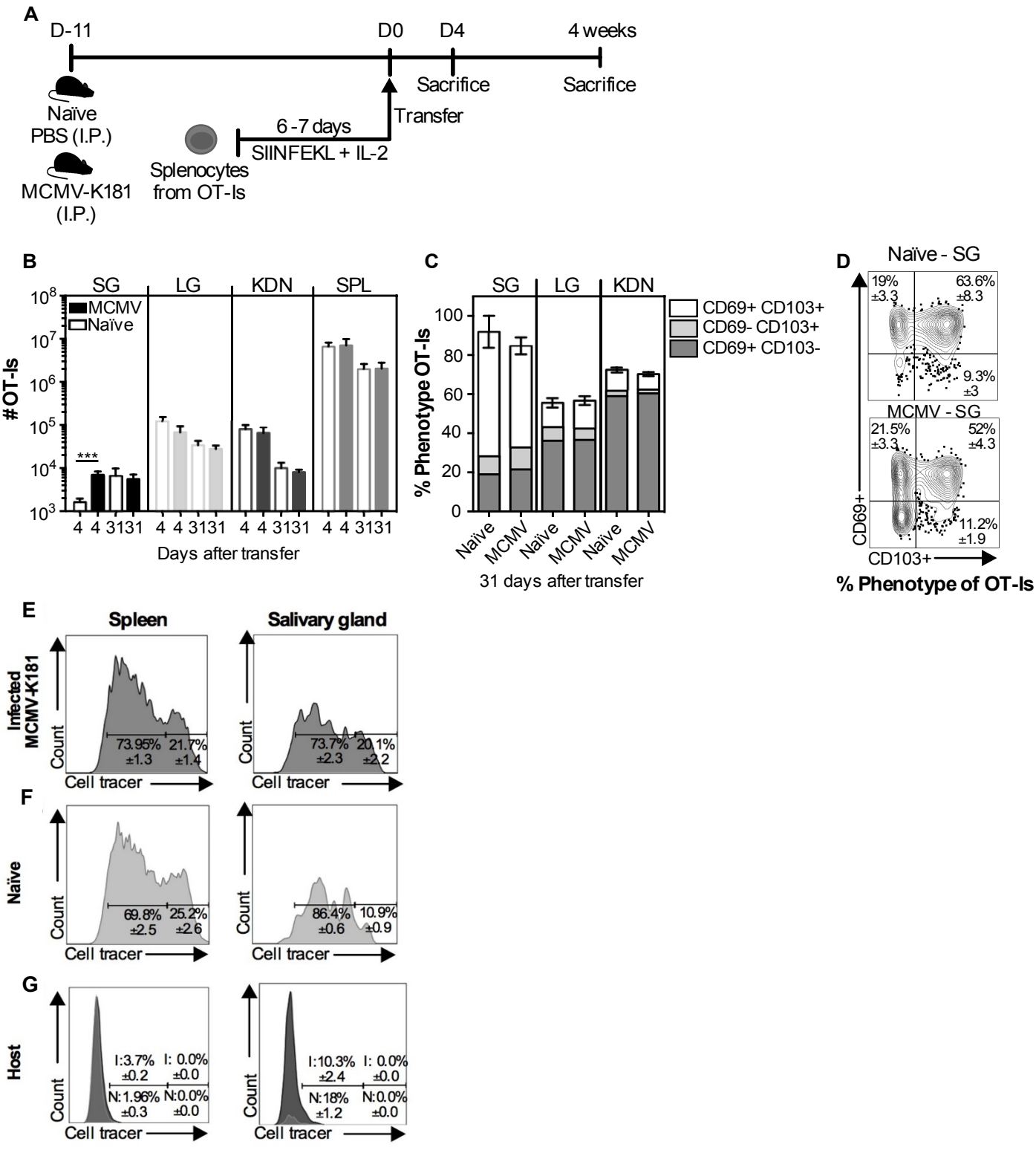


Figure 4. CD8⁺ T_{RM} phenotype cells can form and persist at similar numbers in salivary glands from MCMV infected and naïve mice.

A) Schematic representation of the experimental design. Naïve mice, or mice infected 11 days earlier with MCMV (via the i.p. route) were seeded with $3x10^6$ *in vitro* activated OT-Is. The recipients were sacrificed at 4

or 31 days after transfer. **B**) Absolute number of OT-Is in the parenchyma of the salivary gland (SG), lungs (LG), kidneys (KDN) and from the CD8β$^+$ cells of the spleen (SPL). **C** and **D**) Frequency of CD103- and CD69-expressing OT-Is in the parenchyma of the SG, LG and KDN at 31 days after transfer. Data (A-D) are combined from 2 experiments (n= 7 naïve and 6 infected recipients at day 4; n=6 naïve and 6 infected at day 31). **D**) Concatenated FACS plots, of CD103 and CD69 expression of the OT-I T cells in the SG from one representative experiment (n=3 naïve and n=3 infected recipients) with mean ± SEM values considering all the experiments. Error bars represent the SEM and the statistical significance in (B) was measured by unpaired t-test after $\log_{10}$ transformation of absolute numbers (***p<0.001). **E-G**) *In vitro* activated OT-Is dilute cell tracer dye similarly in both naïve and MCMV infected mice. The experiment was performed as described before but the OT-I T cells were labelled with cell tracer violet before transfer to naïve mice or mice infected with MCMV for 11 days. Concatenated FACS plots from the OT-I T cells in the infected (E) or naïve (F) recipients. **G**) Data from host CD8β$^+$ T cells, as a control. Plots show host cells in the infected (I- Dark grey) and naïve (N - Light grey) recipients. Frequencies ± SEM were represented from one experiment (n=2-3). No differences between infected and naïve animals were observed in a second experiment using CFSE labeling (data not shown).

2.4.2 Mechanisms involved in CD8$^+$ T cell migration to the salivary gland

CD8$^+$ T cell homing to the salivary gland is mediated by α4β1 and chemokines at steady state

Recent data published by Woyciechowski *et al.* (2017) have suggested that the integrin α4β1 plays a critical role in T cell recruitment to the salivary gland during systemic inflammation induced by intravenous poly (I:C) treatment.[32] We wondered whether this mechanism would also contribute to T cell recruitment to naïve salivary glands. To test this, naïve WT OT-Is were transferred into congenic mice and driven to expand with MCMV-Ova infection. 5 days after infection, splenic T cells containing activated OT-Is were transferred to MCMV-K181 infection-matched or naïve recipients in the presence or absence of antibodies to block the α4-integrin or the α4β7 integrin. As expected, the α4 blockade greatly reduced the number of OT-Is in the salivary gland of infected recipients 2 days after transfer (figure 5A).[32] Likewise, in naïve recipients, the α_4 blockade reduced the numbers of OT-Is in the salivary gland and in the lamina propria of the small intestine compared to the group that received the isotype control antibody, differences that were not observed in the spleens of the same animals (figure 5B). In contrast, the blockade of α4β7 had no significant impact on T cells in the salivary gland, despite inhibiting OT-I migration to the lamina propria of the small intestine (figure 5B), as expected.[30,32,54] In addition, pre-incubation of the OT-I T cells with retinoic acid, which strongly induces the α4β7 integrin, had no effect on salivary gland migration

(data not shown). [55] The α4 integrin is known to pair with either β1 or β7 chain. Therefore, these data suggest that α4β1 is important for the migration of activated CD8+ T cells to the salivary gland regardless of infection. These data also imply that the salivary gland expresses sufficient VCAM-1, the ligand for α4β1, independently of local MCMV infection.

Chemokines are important for activating integrins and promoting lymphocyte transendothelial migration. However, several recent reports have implicated chemokine-independent mechanisms of T cell recruitment (e.g. CD44, IL-33, or antigen).[56–58] To test whether chemokines are mediators of CD8+ T cell recruitment to the naïve salivary gland, OT-I T cells were activated *in vitro* and treated, or not, with PTx, which irreversibly inhibits signalling through G-coupled protein receptors like chemokine receptors.[59–61] Untreated, activated OT-I T cells migrated into all organs, including the salivary gland, within 4 days of transfer as expected (figure 5C). In contrast, PTx treatment substantially reduced the numbers of OT-I T cells that migrated into the lymph nodes (figure 5C), as previously shown [60,61] as well as the salivary gland and kidney (figure 5C). These data suggest that activated CD8+ T cells migrate to naïve salivary glands in response to a chemokine signal.

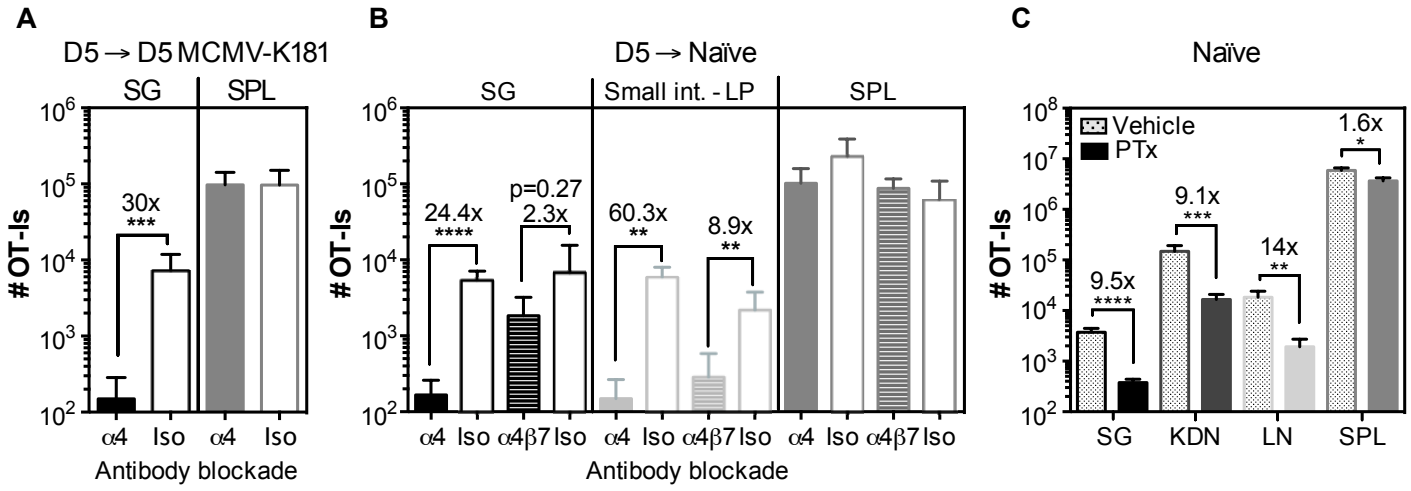


Figure 5. CD8+ T cell accumulation in infected and uninfected salivary glands is dependent on α4 integrin and chemokines.

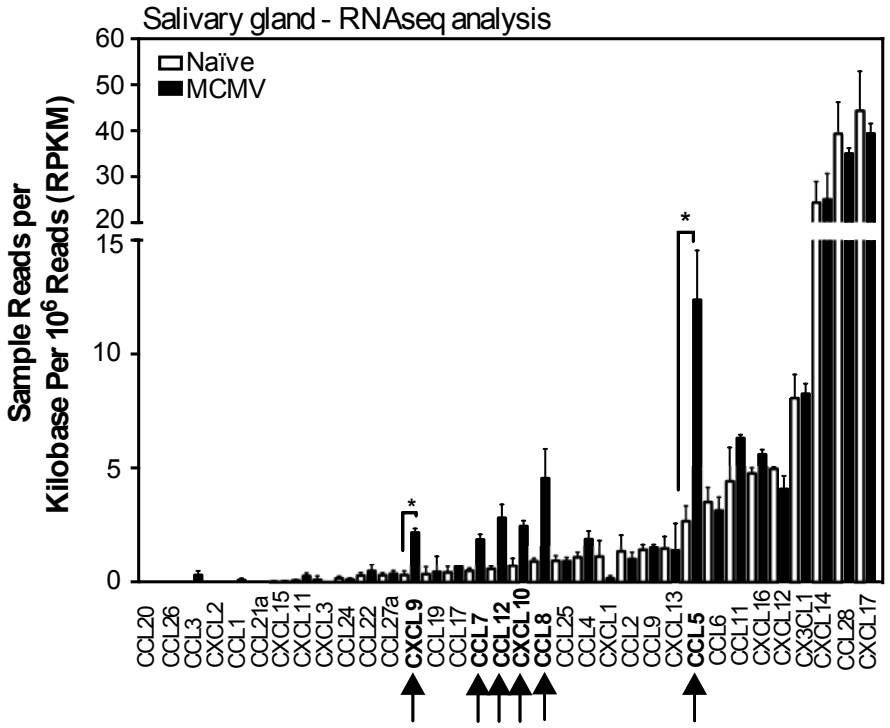
A and **B**) Naïve OT-Is (5x10[4]) were transferred into congenic mice that were infected 1 day later, with MCMV expressing Ova via the i.p. route. Splenocytes containing expanded OT-Is were recovered from the spleen 5 days later and transferred to (A) mice infected with WT MCMV (lacking Ova) or (B) naïve mice. Recipients were either treated or not with anti-α4 blocking antibody on the day of the transfer and mice were sacrificed 2 days after the transfer. Data show the absolute number of OT-I T cells recovered from the parenchya of salivary

gland (SG), small intestine lamina propria (LP) and from the CD8β$^+$ fraction of the spleen (SPL). Results were combined from 2 independent experiments (n= 5-6 mice per group). **C)** Naïve B6 mice were seeded with 8x10^6 *in vitro* activated OT-Is that had been treated with PTx or vehicle as a control before the adoptive transfer. Shown are the absolute numbers of OT-Is recovered from the parenchyma of the SG, kidneys (KDN) lymph nodes (LN) and from the overall CD8β$^+$ T cell population of the SPL 4 days after the adoptive transfer. Results from 2 independent experiments were combined (n=7). Error bars represent the SEM and statistical significances in (A-C) were measured by unpaired t-test after $\log_{10}$ conversion of the absolute numbers (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Chemokines expressed in the salivary gland with or without MCMV infection

To explore the chemokines expressed by the salivary gland in the presence or absence of MCMV infection, we used the RNA-Seq data described before. Interestingly, there were no significant changes in expression of integrins or cell adhesion molecules that survived correction for multiple testing errors (FDR < 0.05), although expression of the α4, αL (CD11a), αX (CD11c) and β2 integrins was increased in the infected salivary glands when the gene set was filtered by a raw p value of lower than 0.05 (table S1B).

Of the chemokines, expression of CCL5 and CXCL9 were significantly increased by infection (FDR < 0.05) and CCL7, CCL8, CCL12, and CXCL10 were also detected when we used a raw p-value cutoff of 0.05 (figure 6 and tables S1A and S1B). In contrast, several chemokines were abundantly expressed in the salivary gland, regardless of infection, including CCL28, CXCL12, CXCL14, CXCL16, CXCL17 and CX3CL1 (figure 6 and table S1B). All of these chemokines, with the exception of CXCL17, have been described to recruit T cells in various settings.[29,62–74] These data show that MCMV infection of the salivary gland induces a dominant inflammatory response centered on IFN-stimulated gene expression. However, several chemokines were abundantly expressed in the salivary gland regardless of infection.



| Figure 6. Chemokine profile of the salivary gland after MCMV infection.

The array of chemokines expressed in the salivary glands of uninfected mice, or 14 days after MCMV infection. Shown are the RPKM of chemokines to illustrate the relative abundance of different transcripts. The complete gene list of genes that were significantly differentially expressed is shown in table S1A. The complete gene list sorted by fold change, regardless of significance, is shown in table S1B. Data are from one experiment (n=3 mice per group). Error bars represent the SEM (↑=p< 0.05; *=FDR<0.05). |

MCMV-specific T cells express multiple chemokine receptors and are able to migrate towards multiple chemokines

To test whether MCMV-specific T cells could migrate to some of the chemokines that were present in the salivary gland independently of infection, we conducted Transwell migration assays *in vitro* with the top 6 most abundantly expressed chemokines, along with CCL5, CXCL9, CXCL10 and CCL19. T cells were harvested from the spleens of mice 7 days after MCMV infection, which represents the peak of T cell clonal expansion and a time at which splenic T cells migrate readily to the salivary gland in adoptive transfer experiments (not shown). [35,75] In multiple experiments, CCL28 and CXCL16 failed to induce any migration of splenic $CD8^+$ T cells (data not shown). In contrast, robust $CD8^+$ T cell migration was induced by CCL19 (approximately 9-fold increased over background, not shown), but most of these cells were $CD44^{low}$ as expected (not shown) and there was no increase in tetramer-binding MCMV-specific T cells

among the migrated cells (figure 7A). All the other tested chemokines induced significant migration of MCMV-specific T cells (figures 7A-B). These included chemokines that were expressed at high levels constitutively (CXCL12, CXCL14, CXCL17 and CX3CL1), as well as chemokines induced in the salivary gland by infection (CXCL9, CXCL10 and CCL5) (figure 7). These data show that MCMV-specific T cells can migrate *in vitro*, toward several chemokines that are either induced by MCMV infection or constitutively expressed in the salivary gland.

Next, we used flow cytometry to explore chemokine receptor expression by OT-I T cells in the spleen and salivary gland, 7-9 days after MCMV-Ova infection. Cells were further differentiated by KLRG1 expression since our previous data showed that most KLRG1 expressing T cells failed to accumulate in the parenchyma of the salivary gland.[11] 7 to 9 days after MCMV-Ova infection, the KLRG1 expressing splenic OT-Is were mostly CX3CR1^{+} CXCR3^{-}, which is consistent with recent work. [76,77] Additionally, we found that these KLRG1^{+} cells expressed CXCR6, but mostly lacked CXCR4 (figure 8A). In contrast, the KLRG1^{-} portion of the splenic OT-Is contained subsets expressing or lacking CX3CR1 and a higher frequency of KLRG1^{-} cells expressed CXCR3, CXCR4 and CXCR6 (figure 8A). Notably, these phenotypes were consistent: at 7 months after MCMV infection, KLRG1 expression on MCMV-specific T cells still correlated with the expression of CX3CR1 and a lack of CXCR3 (data not shown).

Within the parenchyma of the salivary gland, the majority of cells lacked CX3CR1 and KLRG1 (figure 8B, left). In sharp contrast, T cells found in the vasculature of the salivary gland (i.e. stained by the intravascular antibody) were almost entirely CX3CR1^{+} and most also expressed KLRG1 (figure 8B, right). We have had difficulty getting consistent staining of any of the other chemokine receptors on T cells extracted from the salivary glands. In our hands, CXCR3 has been sensitive to the collagenase used to extract T cells from the salivary gland and attempts at extracting T cells without collagenase have failed to result in sufficient T cell recovery. Therefore, we measured the expression of chemokine receptors on T cells sorted from the spleen and salivary gland using qRT-PCR.

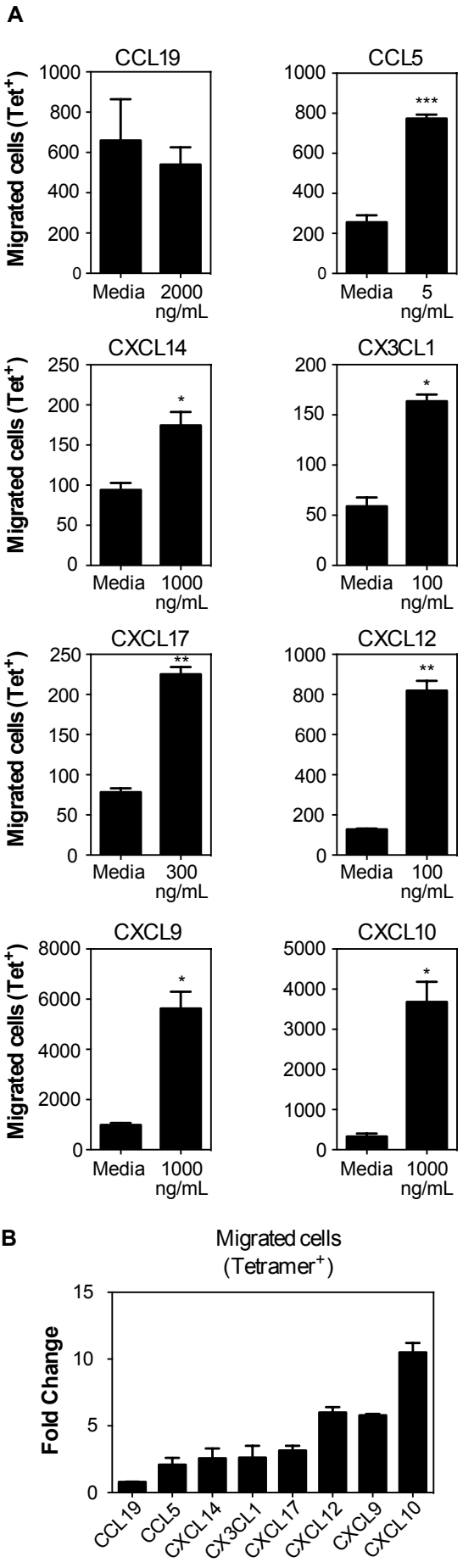
Naïve, WT OT-Is were transferred into congenic mice and driven to expand with a MCMV- Ova infection. 7 days later, OT-I T cells sorted from the spleen and salivary gland expressed CX3CR1, CCR5, CXCR3, CXCR4 and CXCR6 (figure 8C-D). In contrast, CCR10 (receptor for CCL28) and CXCR7 (one receptor for CXCL14) were undetectable on either spleen or salivary gland localized OT-I T cells (data not shown). Interestingly, while salivary gland and spleen-localized T cells expressed comparable amounts of CXCR3 and CCR5, salivary gland-localized T cells expressed more CXCR4 ($p=0.016$) and CXCR6 ($p=0.023$) than spleen-localized T cells. In contrast, spleen-localized T cells expressed much more CX3CR1 than salivary gland localized T cells ($p<0.0001$, figures 8C-D), consistent with our FACS data.

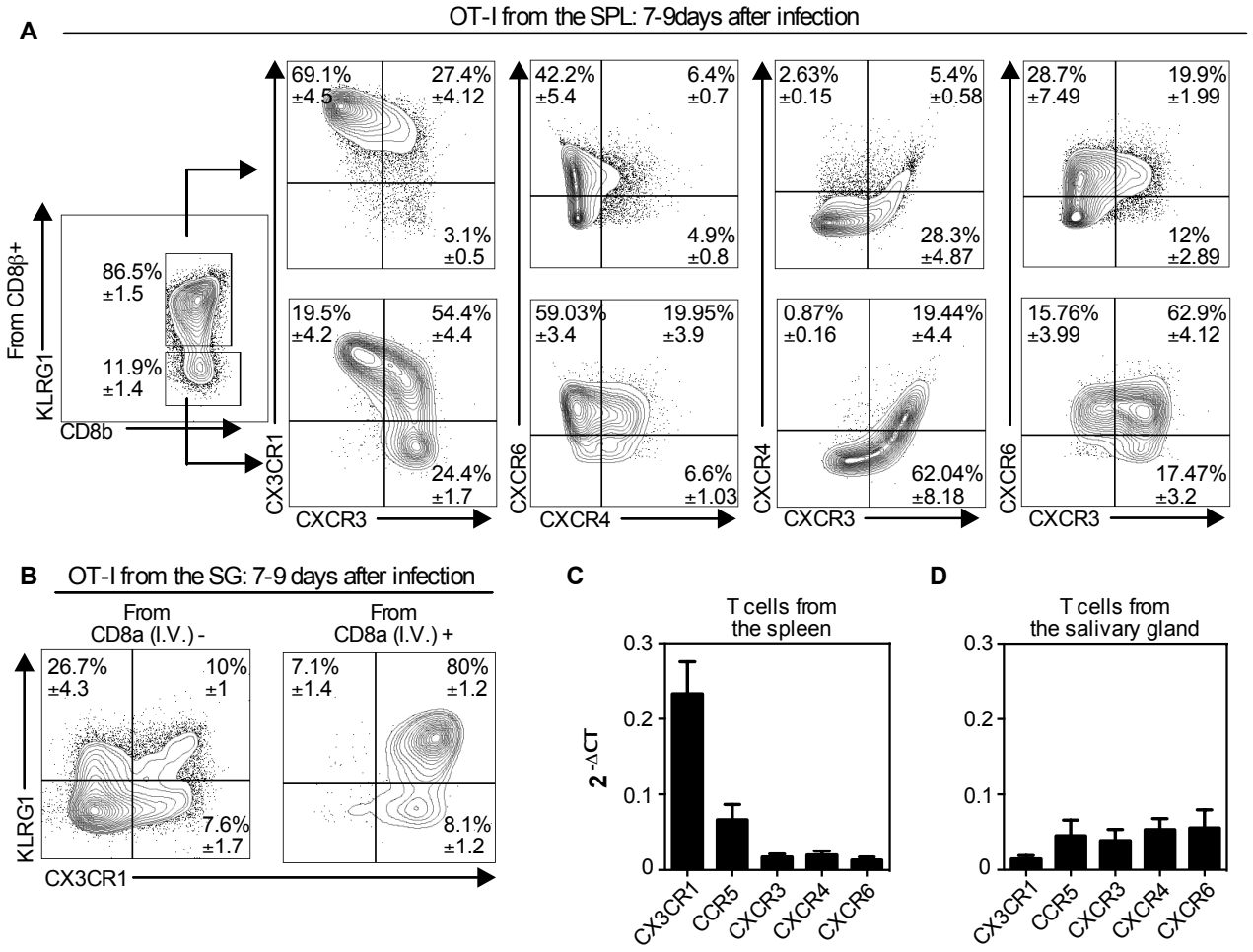
Figure 7. MCMV-specific CD8$^+$ T cells migrate towards multiple chemokines.

Mice were sacrificed 7 days after MCMV infection (i.p.). CD8$^+$ T cells were enriched and used to performed transwell migration assays with multiple chemokines. Assays were performed with replicates and media control was included in each assay to account for plate-to-plate variation.

A) Absolute numbers of MCMV-specific T cells (M45-Tetramer$^+$ plus M38-Tetramer$^-$) that migrated towards the indicated chemokine in comparison to control wells with media alone. Data are from a single experiment performed in triplicate or quadruplicate, and representative of 2 to 5 experiments per chemokine. Statistical significance was determined using paired t-test (*p<0.05; **p<0.01; ***p<0.001).

B) Fold change of migration in comparison to media. Pooled data from 2 to 4 independent experiments indicating the fold change of migration of MCMV-specific cells (M45-Tetramer$^+$ plus M38-Tetramer$^-$) in response to the indicated chemokines at the concentrations shown in (A).





| Figure 8. Most CD8⁺ T cells in the parenchyma of the salivary gland lack KLRG1 and CX3CR1, but express multiple other chemokine receptors.

Naïve OT-Is ($2x10^3$) were transferred to naïve B6 mice that were infected via the i.p. route 1 day later with MCMV expressing Ova. Mice were sacrificed 7 or 9 days after infection. **A**) Chemokine receptor expression within the KLRG1⁺ and KLRG1⁻ subsets of the OT-I T cells in the spleen. **B**) KLRG1 and CX3CR1 expression of OT-Is from the vasculature (i.v.+) and parenchyma (i.v.-) portions of the salivary gland. In (A) and (B), the data show concatenated FACS plots from one representative experiment at day 9 (n= 3) with mean ± SEM values in each quadrant derived from all experiments (total n=8). **C** and **D**) Naïve OT-Is were transferred as in (A) and sorted from the spleen (B) and the salivary gland (C) 7 days after infection with MCMV expressing Ova. Chemokine receptor expression was assessed on sorted T cells by RT-qPCR. Data were combined from 2-5 independent RT-qPCR assays per sample, with cDNA from OT-I T cells sorted from 2-3 independent mice. Error bars represent SEM. |

CXCR3 is critical for T cell migration to uninfected salivary glands, but is dispensable after MCMV infection

Since MCMV infection induced chemokines that bind CCR5 and CXCR3, we directly tested whether these receptors were critical for the accumulation of MCMV-specific T cells in the salivary gland. To this end, OT-I T cells that expressed or lacked either receptor were mixed with their WT counterparts and co-transferred to congenic B6 recipients. Recipient mice were infected on the following day with MCMV virus expressing Ova (figure 9A-D). Two weeks after the infection, there were only small reductions in the numbers of CXCR3 KO or CCR5 KO OT-Is in the salivary gland (figures 9A and 9C) and these small differences were mirrored in the spleens of the same animals, implying no impairment in the recruitment of T cells lacking either CXCR3 (figure 9A) or CCR5 (figure 9C) receptor. Moreover, there were no differences in the absolute numbers of OT-Is that expressed the tissue-resident markers CD69 and CD103 in the gland (figures 9B and 9D).

It was possible that CCR5 and CXCR3 played redundant roles in migration of T cells to the salivary gland during MCMV infection. To address this possibility, WT and CCR5 KO OT-I T cells were activated *in vitro* and transferred into infected mice, with or without an antibody specific for CXCR3 that has been reported to block CXCR3-dependent cell migration.[78,79] For infection, we used the K181 strain of MCMV lacking Ova, again to avoid the influence of antigen and additional T cell expansion after adoptive transfer (figure 9E). However, donor OT-I T cells still migrated to the salivary gland in all cases and the absolute number of OT-Is in the gland was similar in both groups independently of the CXCR3 blockade (figure 9F). There was a subtle difference on the overall number between the WT OT-Is in the unblocked group and the CCR5KO OT-Is in the CXCR3 blocked group, possibly suggesting a combined impact of CXCR3 and CCR5 on T cell migration. However, the effect was subtle and the CXCR3 blockade had an impact in the spleen in all mice. Therefore, it is difficult to distinguish if this effect was partially due to differences on T cells in circulation (figure 9F). There was no impact of CCR5 deficiency with or without CXCR3 blockade on T cell migration to lungs or kidneys. Although it is possible that the CXCR3 blockade was poorly effective *in vivo* in infected mice, these data suggest that CCR5 and CXCR3 are not required for T cell accumulation in infected salivary glands.

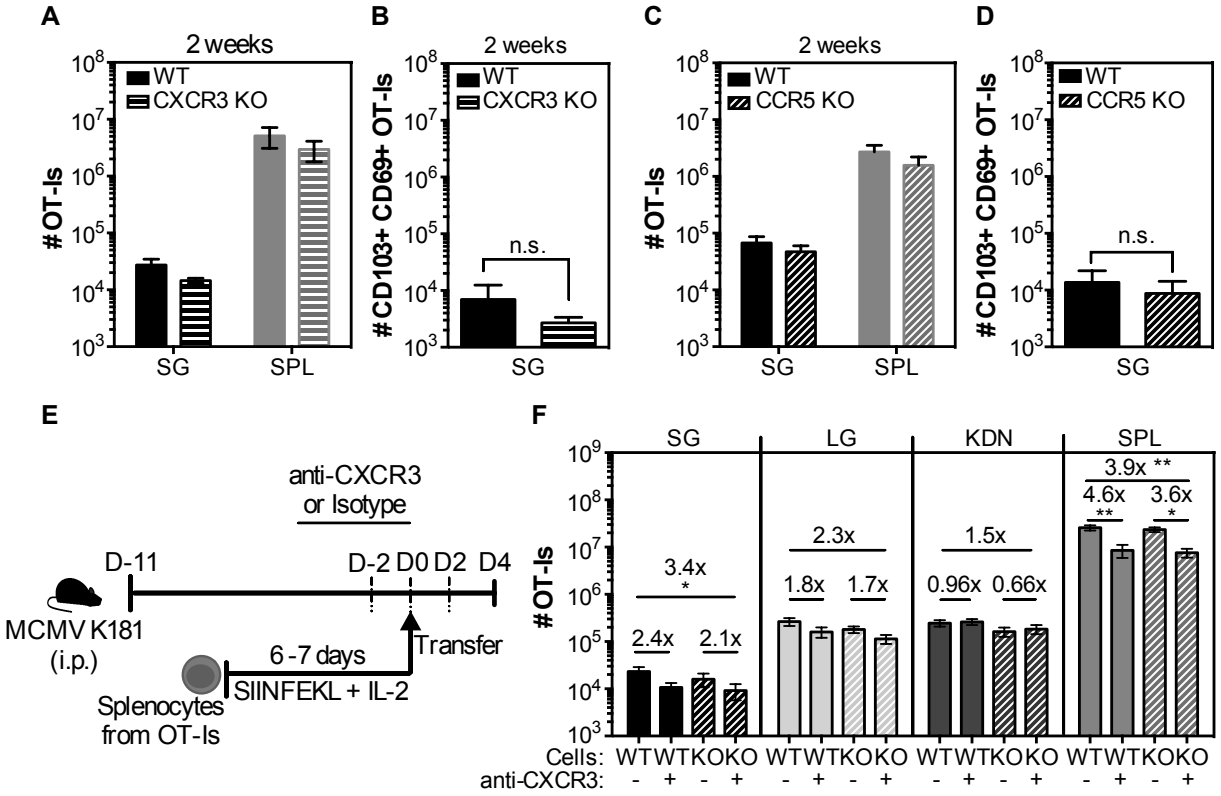
As a control for these experiments, we had transferred *in vitro* activated WT OT-Is to naïve recipients, with or without CXCR3 blockade (figure 10A). Surprisingly, in naïve mice, the CXCR3 blockade had a striking impact on T cell accumulation to the salivary gland resulting in approximately a 9-fold reduction in the numbers of OT-Is that reached the parenchyma (figure 10B). Although the blockade had an impact on the numbers of OT-I T cells in the spleen, as in infected mice (figure 9F), it was lower than the impact on the salivary gland in naïve mice and there was no effect on the T cells recovered from the

lungs or kidneys (figure 10B). These data imply that the CXCR3 blockade was effective in naïve mice and suggest that the CXCR3 blockade was having a specific impact on T cell accumulation in the salivary glands of naïve mice, despite the absence of an effect in infected mice.

To confirm these surprising results, and determine whether CXCR3 was needed on T cells specifically, CXCR3 KO and WT OT-Is were activated *in vitro*, mixed together, and co-transferred to naïve mice. At 4 days after transfer, the absolute number of each OT-I population was assessed and the ratio of KO to WT cells was calculated within the parenchyma and the circulation (figure 10C). In line with our blocking antibody data, accumulation of CXCR3 KO OT-Is in the salivary gland, but not the kidneys or spleen, was markedly impaired, in two separate experiments (figures 10D-E). Together, these data strongly imply that T cell migration to and accumulation in uninfected salivary glands depends critically on CXCR3.

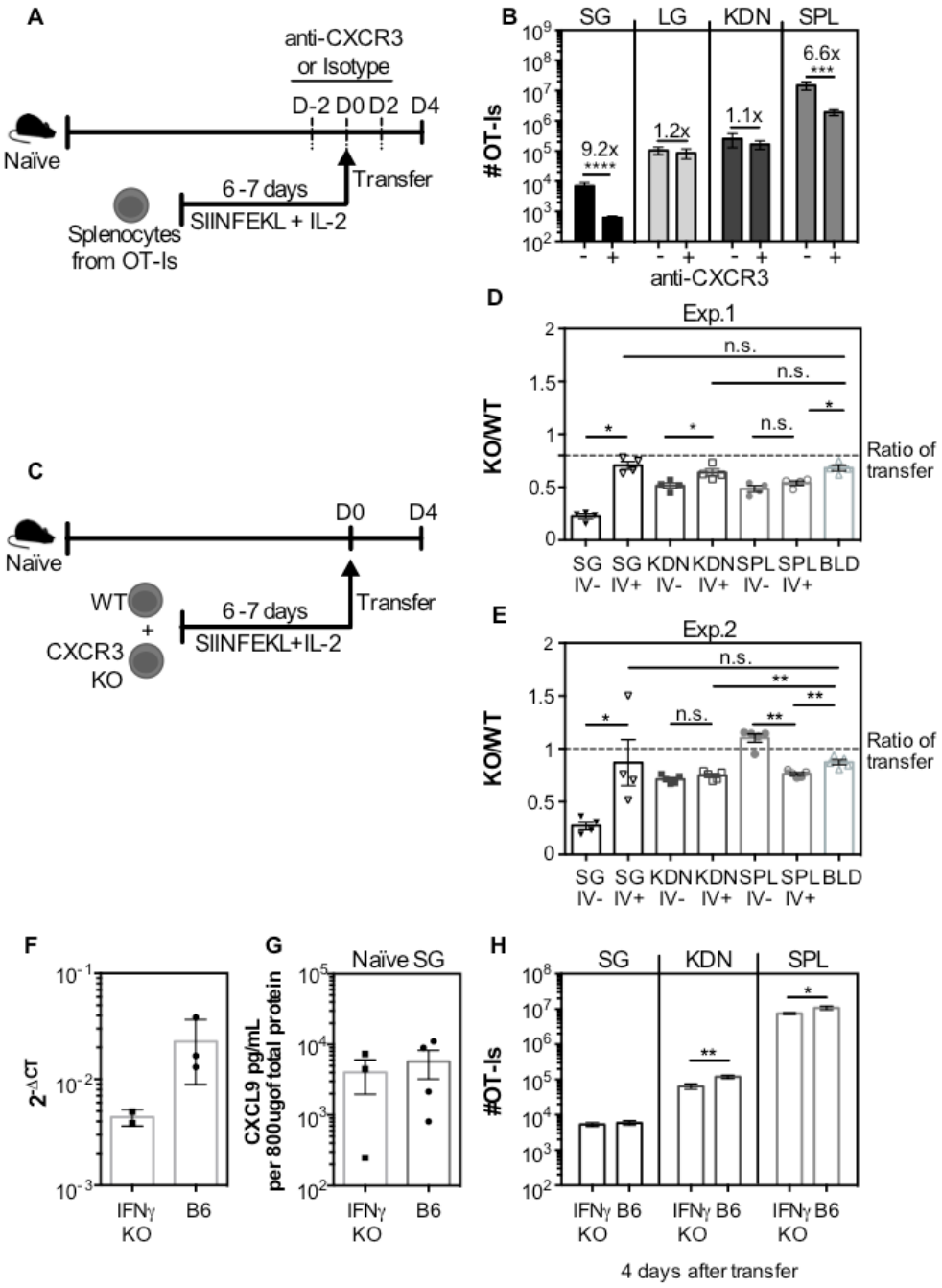
CXCL9 is expressed in the salivary gland at steady state even in the absence of IFN-γ

Because the ligands for CXCR3 (CXCL9 and CXCL10 in B6 mice) are strongly induced by IFN-γ and upregulated by MCMV infection (figure 6), we wished to confirm that these ligands were present in naïve mice and test whether IFN-γ was required for T cell migration to the salivary gland. The chemokine CXCL9 was readily detectable by ELISA and qRT-PCR in the salivary glands of naïve B6 and was also evident in IFN-γ KO mice (figures 10F-G). In addition, when *in vitro* activated OT-Is were transferred to naïve IFN-γ KO or B6 mice they reached the salivary gland at similar numbers (figure 10H). Thus, CXCL9 is available for T cell recruitment to uninfected salivary glands with or without infection or IFN-γ. Collectively, these data suggest that the integrin $\alpha 4\beta 1$ and the chemokine receptor CXCR3 play critical roles in the recruitment of T cells to uninfected or non-inflamed salivary glands, but that CXCR3 is redundant during inflammation induced by MCMV infection.



| Figure 9. Lack of CXCR3 or CCR5 does not impact the accumulation of CD8⁺ T cells in the salivary gland after MCMV infection.

A and **C**) WT OT-Is and OT-Is that lacked either the chemokine receptor CXCR3 or CCR5 were mixed ($1x10^3$ of each) and co-transferred to naïve B6 mice that were then infected with MCMV-Ova. Shown are the overall numbers of CXCR3 KO (A) or CCR5 KO (C) versus WT OT-Is from the spleen (SPL) and the parenchyma of the salivary gland (SG) 14 days after infection. **B** and **D**) Shown are the absolute numbers of $CD103^+$ $CD69^+$ OT-I T cells from the SG. Data are combined data from 2 independent experiments for each KO (dashed bars)/WT (filled bars) pair (n=7 for the WT/CXCR3 and n=5 for the WT/CCR5 experiments). **E**) Experimental design of the OT-I adoptive transfer for (**F**). WT and CCR5 KO OT-Is were activated *in vitro*, and $4x10^6$ of each were mixed. Mixed cells were treated with either anti-CXCR3 antibody or an isotype control and co-transferred to MCMV infected mice that were treated with anti-CXCR3 (+) or an isotype control antibody (-) via i.p. injections every other day starting 2 days before the adoptive transfer. **F**) Shown are the absolute numbers of WT (filled bars) or CCR5 KO (dashed bars) OT-I T cells in the parenchyma of the SG, lungs (LG), kidneys (KDN) and from the overall $CD8\beta^+$ T cells from the spleen (SPL) of CXCR3 blocked (+) or isotype control-treated (-) mice. Data from 2 independent experiments (n=7 for the isotype treated group and n=8 for the CXCR3 treated group). Error bars represent that SEM. The statistical significance was measured by unpaired t-test after log10 conversion of the absolute numbers (A-D) and One-way ANOVA (F). **|**



| Figure. 10. CXCR3 blockade reduces the recruitment of CD8⁺ T cells to salivary glands in uninfected mice independently of IFN-γ.

A) WT OT-I T cells were activated *in vitro*, treated with either anti CXCR3 antibody or isotype control and $4x10^6$ cells were transferred to naïve mice. The recipients were also treated with anti CXCR3 antibody or isotype control every other day starting 2 days before transfer until sacrifice. **B**) The absolute numbers of OT-I T cells that reached the parenchyma of the salivary gland (SG), lungs (LG) and kidneys (KDN) and from the CD8β⁺ population of the spleen (SPL) are shown. Data are from 2 independent experiments (n=7 for the isotype

treated group and n=8 for the group treated with anti-CXCR3). **C**) Experimental design for figures D and E. WT and CXCR3 KO OT-Is were activated *in vitro*, and $4x10^6$ of each were mixed and co-transferred to naïve mice. **D** and **E**) Shown is the ratio of KO/WT OT-Is in the vasculature (i.v.-) and parenchyma (i.v.+) of the SG, KDN, SPL, and from the overall $CD8\beta^+$ cells in the blood. Two independent experiments are shown [n=4 in (D) and n=5 in (E). The dotted lines represent the ratio of KO to WT cells in the transferred pool (as assessed by FACS on the day of transfer). Error bars represent SEM. Statistical significance was measured by unpaired t-test after $\log_{10}$ conversion of the absolute numbers (B) and ratio paired t-test in (D-E). The levels of CXCL9 in the SG of naïve B6 and IFN-γ KO mice were determined by qPCR (**F**) and ELISA (**G**). Data from one experiment [n=2-3 in (F); n=3-4 in (G)]. **H**] WT OT-I were activated *in vitro* and $4x10^6$ T cells were transferred to naïve B6 or IFN-γ KO mice [similarly to (A) and (B)]. The organs were collected 4 days after transfer and the overall number of OT-I in the SG, KDN and SPL are shown. Data are pooled from 2 independent experiments (n=6 B6 mice and 7 IFN-γ KO mice). Error bars represent the SEM and the statistical significance was measured by unpaired t-test after $\log_{10}$ conversion of the absolute numbers (*$p<0.05$; **$p<0.01$).

2.5 Discussion

The salivary gland is an important site of viral infection for transmission to new hosts. Indeed, all β- and γ-herpesviruses infect the salivary gland and are shed into saliva. Moreover, herpesviruses like CMV establish latency in the salivary gland and reactivate readily in this site during periods of immune suppression, particularly when $CD8^+$ T cells have been depleted.[9] Therefore, it is important to understand the mechanisms that promote and establish $CD8^+$ T cell residency in the salivary gland.

Recent studies have shown that salivary gland localized T cells can become T_{RM} that reside near or within the epithelial layer.[10,11] Interestingly, the mechanisms governing T_{RM} differentiation and maintenance differ in different tissues.[80] While transforming growth factor beta (TGF-β) is described as critical for promoting CD103-expressing T_{RM} in most tissues tested to date, including the salivary gland, a role for antigen and inflammation is more variable.[10,11,15,21,25,81] Antigen seems to be required for the differentiation of $CD103^+$ T_{RM} in the central nervous system and lungs.[19,20,82] In the skin, the efficiency of T cell recruitment is poor without local inflammation and antigen improves the T_{RM} maintenance as well as the selection of T_{RM} specificities. [16,24] In contrast, T_{RM} can form in the small intestine independently of antigen, infection, or commensal microbiota. In fact, antigen in the small intestine may reduce expression of $CD103^+$ on T cells that reach this tissue.[28] Remarkably, like the small intestine, the salivary gland seems capable of recruiting activated T cells in the absence of a specific infection or inflammation.[10–12] In fact, our data show that naïve, uninfected salivary glands and those with an active MCMV infection were equally capable of recruiting activated T cells over a month of time (figure 4). These data suggest that the salivary gland and small intestine may represent a set of tissues that are continuously surveyed by activated T cells and capable of inducing their retention through constitutive TGF-β expression. T cell recruitment to both naïve and infected salivary glands depended on the α4 integrin (figure 5) while recruitment to naïve salivary glands depended on the chemokine receptor CXCR3 (figure 10). These results imply that sufficient levels of VCAM-1 and CXCR3 ligands are expressed at steady state in the salivary gland for T cell recruitment. Indeed, CXCL9 was detectable in the salivary glands of naïve B6 mice and its expression was not dependent on IFN-γ (figure 10). These data establish a mechanism for the recruitment of activated T cells to the salivary gland, regardless of infection.

Given that CXCL9 is classically thought of as IFN-γ-induced chemokine, it is interesting that it was constitutively expressed in the salivary gland. It is possible that animal colony conditions or husbandry practices within our animal colony contribute to the constitutive expression of CXCL9 that we observed (figure 10) and the constitutive ability of naïve salivary glands to recruit T cells (figure 4). However, the

recruitment of T cells to the salivary gland in naïve mice has been demonstrated in 2 other laboratories besides our own, suggesting that our results cannot be explained by housing conditions in the Thomas Jefferson University animal facility.[12,10] It must be noted that the salivary gland ducts are open to the oral cavity and therefore likely to be colonized by the oral microbiome. Thus, it is possible that CXCL9 expression is a direct response to oral microbiota. Interestingly, several chemokines including CXCL9, CXCL10 and CXCL11, can have antimicrobial functions. [83–85] Therefore, it is possible that T cell recruitment is secondary to the anti-microbial role of chemokines in the salivary gland, and perhaps other digestive tissues. It will be fascinating in future work to determine whether CXCL9 levels, and the steady-state recruitment of activated T cells to the salivary gland, are controlled by the oral microbiome.

Although CXCR3 appears to play a crucial role in the recruitment of T cells to naïve salivary glands, it is important to note that our experiments did not test whether CXCL9 and CXCL10 were required to recruit activated T cells to the naïve or infected salivary glands. Indeed, it is possible that CXCR3 was being activated by alternative ligands in naïve mice or that CXCL9 and CXCL10 could use alternative chemokine receptors in infected mice. Moreover, heterodimerization of chemokines as well as heterodimerization of receptors have been described and can increase the breadth of potential ligand/receptor interactions. [86,87] Therefore, extensive future work will be needed to tease apart the specific ligand/receptor interactions involved in T cell recruitment in different settings.

Although infected salivary glands recruited activated OT-Is more rapidly, we observed a plateau in recruitment (figure 4). It is possible that the ultimate numbers of T_{RM} that accumulated in the salivary gland may have depended on the potential of the T cells induced by *in vitro* activation. In our hands, a subset of *in vitro* activated OT-Is expressed CXCR3, CXCR4 and/or CXCR6, and the population, as a whole, almost completely lacked CX3CR1 (not shown), much like our MCMV-specific T cells in the salivary gland (figure 8). However, we cannot exclude the possibility that only a subset of activated OT-I T cells was capable of migrating to the salivary gland and becoming T_{RM}, which could simulate a plateau in OT-I T_{RM} cell numbers in the salivary gland over time. If such limit in cell numbers existed however, it was unlikely to be caused by competition for space or environmental cues in the salivary gland. New OT-I or VACV-specific T_{RM} were able to form in the salivary glands of mice previously infected with MCMV (figure 3 and data not shown), despite the fact that infected salivary glands contained 20 to 30-fold more T cells than naïve salivary glands (data not shown). Together, these experiments suggest that the salivary gland can accommodate more T_{RM} than were induced in our experimental systems. Nevertheless, it is possible that pre-existing T_{RM} in the salivary gland would be reduced over time in animals exposed to repeated infections. Thus, it will be exciting to learn how salivary gland-localized T_{RM} populations would evolve over

time in response to multiple different infections.

Our data showed that most KLRG1 expressing MCMV-specific T cells upregulated CX3CR1, but lost CXCR3 expression and failed to migrate into the salivary gland, even during MCMV infection (figure 8). However, CXCR3 deficiency did not preclude MCMV-specific T cells from the salivary gland during infection (figure 9). Thus, presumably the loss of CXCR3 alone was not responsible for the inability of these T cells to migrate into the salivary gland. Moreover, a lack of the $\alpha 4$ integrin cannot explain the failure of these cells to enter the salivary gland as we have seen comparable expression of $\alpha 4$ integrin on $KLRG1^{+}$ and $KLRG^{-}$ MCMV-specific T cells (data not shown). These results were surprising to us, but also broadly consistent with work from Woyciechowski, S. *et al.* (2017), which suggested that CXCR3 was dispensable for $CD8^{+}$ T cell migration to the salivary gland after LCMV-WE infection.[32] LCMV-WE is reported to not infect the salivary gland directly [12] and it is unclear whether CXCR3 ligands in the salivary gland are increased by LCMV-WE infection. Regardless, it is certain that other changes induced by infection will contribute to the efficiency and speed of T cell recruitment. Indeed, LCMV-WE infection was associated with an increase in VCAM-1 on the salivary gland vascular endothelium that likely facilitated the recruitment of T cells. Thus, it is possible that MCMV infection reduced the burden on CXCR3 by increasing additional molecules involved in recruitment of T cells. Another possible explanation is that MCMV infection induced an array of chemokines that could redundantly recruit T cells. We tested whether CCR5 and CXCR3 were acting together to recruit T cells by blocking CXCR3 on CCR5 KO T cells. However, these results should be interpreted cautiously. Although the CXCR3 blockade reduced T cell recruitment to the salivary gland in naïve mice, it is unclear whether the antibody was fully able to block the CXCR3 receptor in infected mice, which likely have many more CXCR3-expressing cells. Future work will need to explore the specific mechanisms used by T cells to enter the salivary gland during acute MCMV infection.

In conclusion, our data show that the salivary gland is able to constitutively recruit $CD8^{+}$ T cells in a $\alpha 4$ integrin and CXCR3-dependent manner, and subsequently induce and sustain T_{RM} populations in the absence of infection, antigen or inflammation. Thus, it is plausible to think that the T_{RM} populations in the salivary gland, and perhaps other tissues in the digestive tract, will retain a record of previous infections and their specificities, regardless of whether those infections were related to the salivary gland. Moreover, the fact that CXCR3 was critical for recruitment of T cells to naïve salivary glands should be useful for guiding the future development of vaccines that aim to establish or boost the mucosal immune responses in the salivary gland.

2.6 References

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3 Discussion

Tissue-resident memory T cells (T_{RM}) are increasingly becoming relevant players in different scenarios. Having specialized residing immune cells within the tissues seems to be the ideal mechanism to effectively improve the local immune response to subsequent events. A great effort has been done to manipulate these cells and take advantage of their unique characteristics to promote a better immune response to infection and tumors. From prime-boost to repeated vaccination, multiple strategies are able to differentiate T_{RM} in different organs.[1–5] However, the requirements for T_{RM} differentiation and maintenance vary according to the organ and the strategy used.[6] Therefore, to successfully promote and modulate T_{RM} it is crucial to understand the cascade of events that leads to T_{RM} differentiation, its characteristics and modifiers. Consequently, this work was focused on further characterizing the T_{RM} differentiation in mucosal tissues with clinical relevance. Salivary glands are essential for replication and shedding of herpesviruses.[7–10] Human Cytomegalovirus (HCMV)'s high prevalence and morbidity/mortality worldwide makes it an interesting target and tool to study T_{RM}.

Briefly, our results showed that Murine Cytomegalovirus (MCMV) infection alters the gene expression profile in the salivary glands, however activated $CD8^+$ T cells could differentiate in T_{RM}, regardless of viral infection or viral replication. Moreover, $CD8^+$ T cell homing to the salivary gland seems to be promoted by CXCR3 (in naïve mice) and $\alpha4\beta1$ expression (in both naïve and MCMV infected mice).

The succeeding discussion will be divided into 3 main parts. Initially, the influence of MCMV infection in the salivary gland environment and its impact in $CD8^+$ T cell recruitment and T_{RM} differentiation will be discussed (Part 3.1). This will be followed by a reflection about the mechanisms involved in T_{RM} differentiation in naïve salivary glands (Part 3.2). Finally, the possible implications of the prompt ability for T_{RM} to differentiate in the salivary glands will be discussed (Part 3.3).

PART 3.1 What does MCMV do to the salivary glands?

3.1.1 Differences in gene expression between salivary glands from naïve and MCMV infected mice

MCMV infection of the salivary gland induces, in the mouse model, tissue inflammation, which recreates some of the Sjögren's syndrome clinical findings.[11,12] Although commonly accepted that Cytomegalovirus (CMV) infection promotes an inflammatory status of the glands, few studies have addressed the specific inflammatory changes that occur after MCMV infection. Cavanaugh *et al.* (2003) have shown that interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), CCL1, CCL3, CCL4 and CCL5 were enriched in the salivary gland of MCMV infected mice compared to the secondary lymphoid organs at 2 weeks after infection.[13] In line with previous findings, our data (Table 2 and Table S1A-B) show that, after MCMV infection, an inflammatory state is seen in the salivary gland, with those molecules being detected in infected salivary glands along with up regulation of IFN-γ related genes, such as CXCL9 chemokine. Additionally, the RNAseq analysis performed identified novel changes between naïve and infected salivary glands 2 weeks after MCMV infection. Looking at the top 50 differently expressed genes, MCMV infection mainly promoted interferon (IFN)-induced genes as well as genes related to antigen processing/presentation/recognition. Nonetheless, it is important to note that these results should be interpreted carefully since post transcriptional and (post) translational modification, as well as protein interactions may occur and are not considered within our analysis, therefore limiting the strength of transcript comparisons. However, some of these genes are interesting to consider in the context of MCMV infection, thus motivating a brief discussion.

Interferon induced genes

Following infection, IFN signaling is mediated by Janus and tyrosine-specific kinases that phosphorylate signal transducers and activators of transcription (STAT) proteins.[14,15] The translocation of STAT proteins to the cell nucleus results in expression of multiple IFN-associated genes. As described before, both type I and II IFN (IFN-I and IFN-II) are induced after MCMV infection and are mediators in the antiviral response to CMV.[14,16–18] Therefore, it is not surprising that multiple IFN-induced genes were increased in the salivary gland following MCMV infection.

Multiple IFN-induced GTPases were identified in our RNAseq analysis. IFN can induce four families of GTPases: the guanylate-binding proteins (Gbp), the immunity-related GTPases (Irg), the Mx-proteins and the very large inducible GTPases.[19,20] Although most of these GTPases have a low expression profile in mice, their transcription is promoted in response to interferon alpha (IFN-α), interferon beta (IFN-β)

and/or by IFN-γ.[20] Therefore, it was not surprising that the transcription of GTPases such as Interferon gamma-induced GTPase (Igtp); Gbp2; Gbp3; Interferon gamma inducible protein 47; Immunity-related GTPase family M (Irgm) 1 and 2 were significantly increased in the salivary glands by MCMV infection.

These GTPases play an important role in resistance to intracellular pathogens such as *Toxoplasma gondii*, rabies virus and *Mycobacterium tuberculosis* through different mechanisms from lysosome fusion and acidification and autophagy.[21–26] Although the role of these GTPases in viral infection is not as striking, some reports using human samples suggest that they can be linked to protection against hepatitis C virus and Influenza virus.[27–29] Previous reports suggest that GTPases such as Irgm1 and Irgm3 are not critical for CMV control.[30,31] Although the expression of these GTPases may only reflect the increased IFN-signal in the glands without significant antiviral role, this is still an interesting question to test since most of the functions of GTPases in CMV infection remain to be determined. For further studies it is important to note that the GTPases family and function differs in different species, being more extensive in mice in comparison to humans.[26]

IFN signal also induces other immune mediators such as IFN-induced transmembrane proteins (IFITM). IFITM proteins are constitutively expressed in multiple tissues, especially barrier epithelial cells and its expression is significantly promoted by both type I and type II interferons.[32] Due to the increased IFN expression following MCMV infection, Not surprisingly, IFITM3 was significantly increased in infected salivary glands.

IFITM proteins are antiviral mediators preventing entry, endosomal fusion and/or viral replication of several viruses such as: Influenza A virus, Vesicular stomatitis virus, West Nile virus, Dengue virus and Human immunodeficiency virus (HIV), but this effect is not universal.[32–36] Most of the viruses, which entry is restricted by IFITM are thought to fuse depending on pH or cathepsin mechanisms, whereas non-sensitive viruses tend to fuse at the plasma membrane.[37,38] Since HCMV entry in epithelial cells depend on both endocytosis and pH dependent fusion, it was surprising that IFITM3 expression cannot prevent viral entry in epithelial cells.[39] Although HCMV is able to bypass IFITM3 to enter the cells and the fact that neither MCMV nor HCMV replication impair its expression, it is important to note that IFITM3 has other antiviral roles. IFITM3 expression in mice limits MCMV induced lymphopenia and the production of cytokines such as TNF-α and IL-6, which modulates T cell and Natural killer cell (NK) responses.[40] Interestingly, following influenza virus infection IFITM3 expression in T_{RM} in the lungs was antigen-driven and prevented these cells from being infected.[41] Remarkably, IFITM3 promoted T_{RM} survival and increased protection following subsequent viral challenges. Notably, the same protection conferred by IFITM3 expression in T_{RM} occurs in the brain following vesicular stomatitis virus infection.[42] As a parallel, MCMV-

specific T_{RM} in the salivary gland persist at high numbers long after MCMV infection, which implies that this population is also maintained and survives during infection. It is thus natural to wonder if this could be as well due to increased IFITM3 expression. Accordingly, it would be interesting to explore which cells are contributing to this increased IFITM3 expression in the salivary gland and test the role of IFITM3 in T_{RM} survival. Moreover, the way IFITM3 could be shaping the immune response against MCMV infection still needs to be characterized, which could be tested using IFITM3 knock-out (KO) mice.

IFN signals also promote the expression of 2'–5' oligoadenylate synthetases **(**OAS) that produce 2',5'-linked oligoadenylates (2-5A) and activate a ribonuclease L (RNaseL) leading to RNA degradation.[43,44] In mice, there are several OAS1 genes, OAS2, OAS3 and 2 OAS-like (Oasl) genes that do not have the OAS function.[45] Differently in humans, 3 functional genes exist (OAS1-3) and a single Oasl gene.[46,47] Besides IFN signal, OAS are also induced by dsRNA, which allow for the antiviral role of the OAS/RNaseL axis against RNA viruses. Although less obvious, the expression of 2-5A have also been described in several infections caused by DNA viruses. The antiviral activity of OAS in infections by DNA viruses like vaccinia virus (VACV) and herpes simplex virus (HSV)-1 can be linked to the RNaseL activation and destruction of viral mRNA.[43] Interestingly however, following VACV and HSV infection the levels of RNaseL do not follow the increase of 2-5A, which could reflect the RNaseL-independent antiviral activity of OAS proteins or, alternatively a viral inhibitory mechanism towards the OAS/RNaseL.[48] In line with this last hypothesis, HCMV ORF94 (UL126a) has been suggested as a mediator of OAS blockade.[49]

According to our results, MCMV infection leads to Oasl2 expression in the salivary gland. Oasl2 exhibits OAS enzyme activity and is upregulated by both IFN-I and IFN-II.[50] Remarkably, some studies suggest that Oasl2 in response to DNA viruses acts differently than RNA viruses. Following infection with DNA viruses, such as VACV and vesicular stomatitis virus, Oasl2 tend to decrease the cGAS-mediated IFN expression, which in turn promotes viral replication.[51] Therefore, Oasl2 induces a negative-feedback on IFN expression and a higher susceptibility to viral replication. Similarly, *in vitro* Oasl2$^{-/-}$ fibroblast produced reduced MCMV infectious particles than wild-type (WT) cells.[51] If a similar mechanism happens *in vivo*, it would be curious to see if Oasl2 expression promotes MCMV infection in the salivary glands. Additionally, it is interesting to consider that Oasl2 expression may function as a regulatory mechanism preventing chronic inflammation, tissue destruction and auto-immunity.

Schlafen 2 (Slfn2) is another IFN-α induced gene which was differently expressed in the salivary glands of naïve and MCMV infected mice. The Slfn2 resulting protein is one of the ten members of its

family and although there is no human homolog, Slfn2 is closely related to the Slfn12 and Slfn12L in humans.[52,53] Slfn2 modulates the immune response in multiple ways and it is mostly known for its role in the maintenance of a quiescent state in naïve T cells.[53–55] Slfn2 prevents both apoptosis of $CD8^+$ T cell and monocytes following activation and expansion signals.[53,56] Interestingly, mice that lack Slfn2 are more susceptible to MCMV infection and although the mechanisms remain obscure, it is thought to be due to its impact on inflammatory monocytes.[53] Moreover, Slfn2 negatively regulates the NF-kB activation following IFN-β signal and thus preventing the *in vitro* expression of IFN-induced genes such as CXCL10, Interferon regulatory factor (Irf)7 and Oasl2 by mouse embryonic fibroblasts, which may impact different branches of the immune system that react to these signals.[57] Interestingly, most of these IFN-induced genes were up-regulated in MCMV infected salivary glands probably suggesting a difference in the study design, timing, as well as a possible overpower of the IFN-I signal following infection. Like Oasl2, it is possible that Slfn2 expression functions as a compensatory mechanism to control the immune and inflammatory response to prolonged viral replication and latent infection in the gland. Nonetheless, further studies are needed to determine the role of Slfn2 as a regulator of the IFN-associated immune response to CMV infection.

Cathepsin S (Ctss) is a member of cathepsins that act mainly as cysteine proteases usually activated within the lysosome.[58] Nonetheless, cathepsins play multiple roles in the immune system promoting apoptosis, antigen processing and tissue remodeling.[59–63] Ctss is mostly expressed by antigen-presenting cell (APC) cells and is linked to Toll-like receptor 9 signaling and acts as one of the predominant regulators of the peptides presented in major histocompatibility complex (MHC)-II complexes.[64,65] Moreover, Ctss can be secreted by immune cells such as macrophages and contributes to extracellular matrix degradation promoting pathology such as atherosclerosis, arthritis and tumor metastasis.[66–68] In the lungs, Ctss expression promotes pulmonary epithelial cell injury and apoptosis that might be linked with the expression of caspase 3, 8, and 9, Fas, Fas ligand, TNF-related apoptosis-inducing ligand, TNF-α, BH3 interacting-domain death agonist and Bcl2-like protein 11.[69] Interestingly, Ctss inhibitors have been used as an effort to prevent emphysema, Th1 inflammation and to control atherosclerosis.[69,70]

Ctss has a restricted expression within the tissues, which is potentiated by different signals such as IFN-γ.[71,72] Therefore, it is not surprising that Ctss is expressed in an IFN enriched environment such as the salivary gland following MCMV infection. However, this result contradicts what was previously seen in dendritic cells (DC) infected with HCMV. Kessler *et al.* (2008) have shown that HCMV infection *in vitro* leads to a reduction of MHC-II, Ctss, cathepsin Z, B, H and L expression in DC, which promotes viral replication and pathology.[73] On the other hand, Lee *et al.* (2006) suggested that Ctss levels remained

stable independently of HCMV infection (TB40/E) in lysates of mature Langerhans cells.[74] These differences might be due to the cells, CMV strain, timing and overall methodology used. Nonetheless, the Ctss expression and its function after CMV infection still needs to be clarified.

Due to Ctss´ destructive ability, it is possible that the high expression of Ctss in the salivary gland may also be related to tissue destruction as seen in Sjögren's syndrome. In fact, Ctss was highly detected in tears of both patients and in a Sjögren's syndrome mouse model and has been proposed as a marker for Sjögren's syndrome disease.[75,76] Intriguingly, these studies have not addressed the levels of Ctss in saliva samples. Remarkably however, in Sjögren's syndrome mouse models, besides the increased levels of Ctss in the lacrimal gland samples, there was also a parallel increase in IFN-γ, TNF-α, MHC II expression and T cell infiltrates. Although these molecules are commonly expressed in an inflammatory environment, it is interesting to note that lacrimal glands in Sjögren's syndrome mouse models present a similar profile that MCMV infected salivary glands present. Therefore, it would be interesting to address the levels of Ctss also in the saliva of Sjögren's syndrome models and patients and see if, as cathepsin D, Ctss can also be proposed as a salivary marker of Sjögren's syndrome.[77,78]

Nucleotide-binding oligomerization domain-like receptors (NLRs) are an example of multiple classes of receptors that detect pathogens or dangerous signals initiating innate immune responses.

The NLR caspase recruitment domain containing protein 5 (Nlrc5) expression was increased in salivary glands after MCMV infection. Nlrc5 is induced by IFN-I and IFN-II and is predominantly expressed by lymphocytes and epithelial cells at a lesser extent.

Nlrc5 modulates $CD8^+$ T cells in multiple ways, being therefore interesting to consider in this work. Nlrc5 is important for MHC-I expression on both T and NK cells and it is also involved in preventing T cells from NK-cell-mediated elimination in inflammatory settings.[79] These mechanisms may justify the reduced $CD8^+$ T cells responses following several infections in the absence of NLRs.[80] Therefore, it is possible that dampening Nlrc5 expression represents an evasion mechanism for some viral infections. Surprisingly however, the opposite was seen in the salivary gland following MCMV infection. It is likely that following MCMV infection, Nlrc5 was induced by the IFN signal in the gland and it would be pertinent to test if Nlrc5 expression acts as a MHC-I promoter in the gland, opposing the evasion mechanisms induced by the MCMV. Indeed, the forced expression of Nlrc5 can promote the immune response as demonstrated by a study that used B16-F10 melanoma cell lines that also tend to downregulate MHC-I expression, in which the overexpression of Nlrc5 increased the rate of T cell-mediated killing.[81]

Z-DNA binding protein 1 (Zbp1) expression was also increased after MCMV infection. Zbp1 is an IFN-induced protein that recognizes both double-stranded Z-form DNA and RNA in the cytoplasm acting as an

antiviral mechanism.[82] Similarly to our results, Zbp1 transcripts were also increased in fibroblast cells following HCMV infection and led to an increase in IFN-β and thus having an antiviral effect.[83] Zbp1 signal also induces RIPK3-dependent necroptotic cell death, which could be another antiviral mechanism. Although necroptosis can limit MCMV replication and dissemination of MCMV, the M45 viral protein is able to prevent the ZBP1-RIPK3 association and therefore contradict this mechanism contributing to the viral replication within the cell.[84] Nonetheless, it is interesting to consider if Zbp1 overexpression would benefit the antiviral response. Some studies have suggested that Zbp1 overexpression can act as a DNA vaccine enhancer boosting both the innate and adaptive T cell responses including the frequency of IFN-γ $CD8^+$ T cells.[85] Similarly to some of the discussed genes, the increased expression of Zbp1 transcript in MCMV infected salivary glands can result from the IFN-rich environment in the salivary gland and be part of the resulting antiviral response.

Genes involved in antigen processing, presentation and recognition were differently expressed in the sali vary gland of naïve and MCMV infected mice

Recognition of infected cells by the adaptive immune cells is crucial for activation, differentiation and magnitude of the immune response. Antigen process and presentation are crucial steps to mount a successful immune response following viral infections. Accordingly, several genes related to antigen processing (Tap1; Tap2) and antigen presentation (H2-ab1; H2-D; H2-Eb1; H2-K1; H2Aa; H2Q4; H2T23) were highly expressed in the salivary gland after MCMV infection.[86]

Proteasome subunit beta (Psmb) type 8 and 9 are curious examples of genes related to antigen processing that were increased in MCMV infected salivary glands. Psmb8 and Psmb9 genes both encode subunits of the 20S immunoproteasome complex, which degrades intracellular proteins into peptides that are posteriorly presented by MHC-I molecules.[87] The resulting IFN-γ signaling after several viral infections, such as MCMV induces different expression of proteasome subunits forming the immunoproteasome. This leads to changes in the efficiency and specificity of the proteolytic activity and the resulting peptides.[88,89] In both human and mouse fibroblasts, CMV infection prevented the IFN-γ induction of immunoproteasome at a pre-transcription level, which resulted in failed expression of the immunoproteasome *in vitro.*[90] These results may seem contradictory to our increased expression of immunoproteasome subunits in the salivary gland following MCMV infection. Nonetheless, these differences may be related to the conditions used (*in vitro* vs *in vivo*) and the fact that the RNAseq analysis was performed using whole salivary glands, where the infected cells represent a minority, possibly weakening the effect of infection. Moreover, although MCMV infection may favour the inhibition of some

of the proteasomes´subunits in specific cells, the resulting inflammatory environment seen in the gland with the expression of IFN-γ, Irf1 and Irf7 may successfully induce the immunoproteasome assembly in other cell types and even in uninfected cells.

Not only genes related to antigen processing, but also genes related to antigen presentation were significantly increased after MCMV infection. Beta 2 microglobulin (B2m) is part of the MHC-I molecule, found in nucleated cells, and its expression is promoted by molecules that are also up-regulated by CMV infection such as TNF-α, IFN-γ and IFN-α.[91–94] So, it is not surprising that its expression is high in infected tissues as seen in the salivary glands of MCMV infected mice. B2m further leads to the expression of pro-inflammatory cytokines such as IL-6 and IL-8, however these cytokines were not up-regulated in the infected salivary glands (table S1A).[95–97] In line with our results, B2m was also increased in blood samples from HCMV infected individuals under different contexts.[98,99] It is interesting to consider that this induction of B2m expression following MCMV infection can be used as an immune evasion mechanism, since CMV is able to use B2m as a receptor and as a coating molecule, therefore B2m can also promote viral infectivity.[100,101]

Additionally, regulators of T cell receptor (TCR) expression in $CD8^+$ T cells were also promoted by MCMV infection of the salivary glands. An example is the Lysosomal protein transmembrane 5 (Laptm5) that is mainly expressed by immune cells in lymphoid organs. In $CD8^+$ T cells, Laptm5 has been shown to promote CD3ζ-chain intracellular degradation and thus negatively regulate the TCR expression after CD3 stimulus.[102,103] Consequently, increased Laptm5 levels after T cell activation act as control mechanism that prevents new TCR expression and an exaggerated T cell response.[102] In fact, in $Laptm5^{-/-}$ mice, increased TCR levels, prolonged T cell responses, increased proliferation and cytokine production by T cells occurred after CD3 stimulation in comparison to WT cells.[102] The increased expression of Laptm5 in infected salivary glands can merely reflect the previous activation of immune cells but can also contribute to the reduced ability of $CD8^+$ T cell to control acute MCMV infection in this organ. It would be interesting to compare the Laptm5 expression in the gland and other organs where $CD8^+$ T cells are crucial in CMV control such as the spleen. Additionally, it is interesting to consider Laptm5 (or its reduced expression) as a tool to increase the responsiveness of $CD8^+$ T cells in the salivary glands following CMV infection.

Although several interesting hits were detected by the RNAseq analysis, surprisingly however only approximately 1% of the 24000 genes detected were differentially expressed in the salivary gland after MCMV infection. Besides the intrinsic limitation of the sensitivity of the RNAseq technique used, it is important to ponder that whole salivary glands were used. Therefore, the slight differences might result

from the evaluation of the whole organ instead of isolated infected versus uninfected cells. Nevertheless, it still is interesting to consider the reasons for the lack of greater differences in gene expression between MCMV infected and naïve glands. It is possible that viral-induced responses seen in infected salivary glands might dampen the immune response and the associated inflammatory changes. Indeed, CMV pathology in the salivary glands has its particularities, as the reduction of MHC-I molecules in infected epithelial cells and the limited cross-presentation, which restricts $CD8^+$ T cells function after infection.[104,105] This compromised recognition of the infected cells by $CD8^+$ T cells in the salivary glands may prevent significant destruction of infected cells and possibly contributing to less inflammation in the gland in comparison to other infected organs where the $CD8^+$ T cells significantly contribute to the viral control.

As another example of regulatory mechanisms that might be preventing bigger differences in gene expression, Campbell *et al.* (2008) have shown that IL-10 RNA (an anti-inflammatory cytokine) is increased in the salivary glands of MCMV infected mice.[16,106] Additionally, it was recently suggested that NK cells following MCMV infection, express IL-27b, which leads to IL-10 expression and dampens the immune response against MCMV infection, resulting in prolonged viral persistence.[107] This IL-10 increase can impact the $CD4^+$ T cell and APC cells response in the gland. In fact, IL-10 has been shown to prevent dysregulated pro-inflammatory signals in the liver following MCMV infection.[108] Therefore, it is plausible that the same occurs in the salivary glands.

Similarly, TNF-related apoptosis-inducing ligand expression by NK cells limits the action of activated $CD4^+$ T cells contributing for viral persistence but preventing further damage of the gland.[109] These protective anti-inflammatory responses antagonize other pro-inflammatory stimuli induced by infection and can possibly prevent the expression of more striking differences such as inflammatory cytokines and chemokines between MCMV infected and naïve glands. An interesting variety of responses were also seen in our results since both genes related to an antiviral response as well as genes that facilitate CMV infection were increased in the infected salivary glands. It would be fascinating to understand how this balance evolves during the acute infection and latency.

Although our results characterize the changes seen in the salivary gland after 2 weeks of MCMV infection, further studies are crucial to elucidate the role of some of these molecules in the immune response and pathology of MCMV. Prior to testing the specific role of some of the hits, the protein levels in the glands, its source and a comparison to other organs such as the spleen and blood would be essential. Nonetheless, the characterization done of the genes, which expression was promoted by MCMV infection in the salivary gland is an initial tool to design new hypotheses regarding the pathology and immune response to CMV.

Finally, it is relevant to mention that the changes caused by MCMV infection detected by the RNAseq and their potential roles will continue to be discussed in future sections.

Besides the impact on the local gene expression, MCMV infection also modulates the cellular composition of the salivary glands. Subsequently, we will discuss the impact that MCMV infection has in T cell recruitment to the salivary gland and T_{RM} differentiation.

3.1.2 Impact of MCMV infection in $CD8^+$ T cell recruitment to the salivary glands and T_{RM} differentiation

For most tissues, inflammation and/or antigen are important for $CD8^+$ T cell homing and T_{RM} differentiation.[110–115] Even in organs such as the skin, where T_{RM} can develop in response to inflammation, antigen tend to promote enhanced T_{RM} differentiation.[113] Infection and the resulting inflammatory environment impact $CD8^+$ T cells in many ways since antigen sensitivity, proliferative capacity, and their trafficking abilities.[116–118] All of these can promote $CD8^+$ T cell maintenance in the tissue and T_{RM} differentiation.

Variances in the infection, such as the different routes, resulted in different $CD8^+$ T cell numbers in the salivary gland, which can indicate a role for the inflammatory status or for the presence of local antigen in the recruitment of $CD8^+$ T cells. Therefore, based on what happens in most organs, it was tempting to think that local CMV viral infection and the resulting inflammation, could promote T_{RM} differentiation in the salivary glands.

3.1.2.1 $CD4^+$ T cells are not required for the differentiation of M38-specific T_{RM}

One key difference upon MCMV infection is the arrival of many different immune cells in the salivary gland. As referred, $CD4^+$ T cell are crucial in controlling MCMV infection in this organ. Therefore, it is not unexpected that $CD4^+$ T cells are present in significant numbers in the gland after MCMV infection.[17,104,119]

$CD4^+$ T cells are key in licensing APC cells, promoting APC-$CD8^+$ T cell-interaction and thus $CD8^+$ T cell activation.[120,121] $CD4^+$ T cells also improve $CD8^+$ T cell proliferation, cytotoxic activity and survival.[122–124] Naturally, $CD4^+$ T cells impact multiple $CD8^+$ T cell subsets and have been shown to be crucial in $CD8^+$ T_{RM} differentiation, maintenance and antiviral activity in the brain in the context of CMV infection.[125,126] Help provided from $CD4^+$ T cells additionally promotes $CD8^+$ T cell migration to the female reproductive tract and T_{RM} differentiation in the lungs.[127,128] Therefore, a role for $CD4^+$ T cells in promoting T_{RM} could also be expected in the salivary gland. Surprisingly however, $CD4^+$ T cell depletion did not impact the number of T_{RM} in the salivary gland after MCMV infection (sup. figure 2). Although still not completely understood, it

seems that the dependence of $CD4^+$ T cell diverges according to the organ studied. In fact, $CD4^+$ T cells were also not required for T_{RM} accumulation in the skin.[129] It is likely that other homing molecules overcome the help provided by $CD4^+$ T in migration of $CD8^+$ T cells to other organs. Moreover, other immune cells such as DC and macrophages could provide differentiation signals such as transforming growth factor beta (TGF-β) and TNF-α, that would promote $CD8^+$ T differentiation and retention, as happens in the intestine, and thus reducing the impact of $CD4^+$ T cells.[130] Even though our studies focused on a specific $CD8^+$ T cell population (M38-specific), our data suggest that $CD4^+$ T cell help is not crucial for MCMV-specific T_{RM} differentiation in the salivary gland. Interestingly, $CD4^+$ T_{RM} were found in the salivary gland after MCMV infection and the interaction and dependence between these two subsets would also be a relevant topic to explore both in acute and latent times of infection.

It is also curious to consider that reduced $CD4^+$ T cells increase the MCMV viral load in the salivary gland and therefore the antigen burden in the organ.[119,131] Intriguingly, neither significantly influenced the overall number of T_{RM}.

3.1.2.2 MCMV replication impacts $CD8^+$ T cell accumulation in the salivary glands but not the T_{RM} differentiation

Using Famcyclovir to prevent MCMV-TK virus replication, we further asserted that viral replication in the salivary gland was unnecessary for T_{RM} differentiation (figure 2). Curiously, viral replication promoted early MCMV-specific $CD8^+$ T cell accumulation in the salivary gland.

Surprisingly however, the number of T_{RM} in the salivary gland was comparable between mice infected with replicative and non-replicative virus (figure 2), which suggests that viral replication impacts $CD8^+$ T cell accumulation in the salivary gland but did not significantly contribute to T_{RM} differentiation. It is important to reinforce that these experiments did not exclude a role for antigen since viral DNA was still detectable in the salivary gland of some mice that were infected with a replicative-deficient virus (data not shown). This can possibly be explained by the migration of infected cells to the salivary gland. DC have been previously described as MCMV carriers, allowing for viral spread to the salivary glands, therefore it is possible that DC were initially infected with MCMV-TK and then migrated to the salivary glands.[132,133] Even though the Famcyclovir treatment has been shown to avert viral replication, the presence of viral DNA in the salivary gland could provide antigen to drive T_{RM} differentiation in this organ.[134] For this reason, we chose the OT-I system to further investigate the role of tissue inflammation in T_{RM} differentiation independently of antigen.

3.1.2.3 MCMV infection promotes early OT-I T cell recruitment to the salivary gland

When we compared OT-I T cell recruitment in the salivary gland of naïve and MCMV infected mice in an antigen-independent system, OT-I T cell migration and early accumulation was promoted by MCMV infection (figure 4). This result is an interesting parallel with the increased accumulation of MCMV-specific $CD8^+$ T cells in the salivary glands in the presence of virus replication (figure 2). Combined, these results suggest that MCMV viral replication transiently promotes $CD8^+$ T cell accumulation in the salivary gland regardless of antigen recognition.

Importantly, the augmented number of OT-I T cells in infected salivary glands at acute time points after infection was not due to cell proliferation (figure 4). Instead, the increased recruitment of OT-I T cells to infected salivary glands may reflect an enhanced ability of T cells to enter the salivary gland when MCMV infection is present.

Woyciechowski, S. *et al.* (2017) have suggested that inflammation can increase the expression of vascular cell adhesion molecule (VCAM)-1 on the salivary gland´s vasculature, enhancing the recruitment of T cells expressing the corresponding receptor, $\alpha4\beta1$ integrin.[135] The mechanism by which infection induces VCAM-1 expression was not explored but it is possibly mediated by IFN-γ since it induces the expression of integrin ligands such as VCAM-1 in endothelial cell in other infections.[136] This is a possible explanation for how infection promotes recruitment of immune cells in an antigen-independent way.

Another factor that may help justify the increased recruitment of OT-I T cell to infected salivary glands is the tissue damage and the resulting production of inflammatory cytokines such as IL-1 and TNF-α. These cytokines can induce P- and E-selectin expression by the vascular endothelium, both in human as in mice.[137–140] Curiously however, no differences in integrin ligands or selectins were detected by the RNAseq between MCMV infected and naïve glands (table S1A-S1B), which makes these factors less likely to explain the difference in OT-I T cell recruitment between naïve and infected recipients. Nonetheless, it would be interesting to determine the levels of integrin ligands (such as VCAM-1) and selectins on the vasculature of the salivary glands early following MCMV infection using other techniques such as immunofluorescence.

Alternatively, the environment that the OT-I T cells were exposed to before reaching the salivary gland differs in infected and naïve mice and can also contribute to the expression of selectin ligands by the T cells. Activated $CD8^+$ T cells express enzymes required to fully produce the core 2 O-glycans, which allow for P- and E-selectin binding and the initial contact between T cells and the endothelium.[141] Interestingly, cytokines such as IL-12 and IL-15, that have been detected in different tissues after MCMV infection, promote O-glycans expression in $CD8^+$ T cells *in vitro* and thus enhance the ability of T cells to

bind to the vasculature.[18,142,143] These different mechanisms, help hypothesize that the resulting cytokine production following MCMV infection may promote the vasculature-binding activity of $CD8^+$ T cells and explain the initial increase of OT-I T cells seen in infected salivary glands.

Besides integrins and selectins, the inflammatory status following MCMV infection also changes the chemokine profile in the salivary gland which can modulate $CD8^+$ T cells recruitment in an antigen-independent way. CXCL9 and CXCL10 mRNA levels were increased in infected salivary glands in comparison to naïve salivary glands (figure 6) and therefore likely candidates to contribute to the improved recruitment of activated OT-Is.

Surprisingly, the main chemokine receptor for CXCL9 and CXCL10 (CXCR3) had no significant impact in $CD8^+$ T cell migration to infected salivary glands. This result proves even more astounding since all these chemokines have been implied in the trafficking and maintenance of T cells to different infected organs such as the liver, lymph nodes, genital mucosa and the skin in melanoma models.[144–148] Nonetheless, the absence of a significant effect of CXCR3 deficiency in $CD8^+$ T cell accumulation in infected mice can have several explanations. It is possible that MCMV infection of the glands triggers a broader expression of chemokines, which can diminish the singular importance of each chemokine/receptor in $CD8^+$ T cell homing during infection. This hypothesis is even more likely if we consider that both MCMV-specific $CD8^+$ T cells and OT-I T cell express multiple chemokine receptors after activation that were able to induce migration *in vitro*. Additionally, MCMV infection may not only alter chemokine expression in the organs, but also the expression of these chemokine receptors by the $CD8^+$ T cells. In fact, IFN-γ is important for the induction of CXCR3 on T cells.[149] Therefore, it is plausible that MCMV infection and the increased levels of IFN-γ in multiple tissues induced changes in CXCR3 expression by the OT-I T cells occurred after the adoptive transfer. This may have limited the effectiveness degree of the CXCR3 blocking antibody in the infected mice (figure 9). Consequently, it is important to note that early $CD8^+$ T cell recruitment to infected salivary glands was not tested using CXCR3 KO OT-I T cells (experiments were performed using blocking antibodies). Therefore, a more significant difference in $CD8^+$ T cell recruitment to infected salivary glands could have been seen comparing WT or CXCR3 KO OT-I T cells at early time-points after the adoptive transfer.

Although the role of CXCR3 in T cell recruitment in naïve and infected salivary glands were not compared directly, it is unlikely that CXCL9 and CXCL10 explain the increased recruitment in infected salivary glands. Nonetheless, it is important to note that the chemokine receptor was the variable tested, thus the role of these specific chemokines in promoting $CD8^+$ T cell recruitment was not directly verified. This becomes relevant since dimerization and promiscuous recognition of ligands by other chemokine

receptors has been described.[150,151] If so, other chemokine receptor(s) besides CXCR3 may alternatively recognize CXCL9 and CXCL10. Therefore and although unlikely, we cannot exclude a minor role for these chemokines in $CD8^+$ T cell recruitment to MCMV infected salivary glands.

Another facilitator of $CD8^+$ T cell recruitment and differentiation in the tissues is the presence of antigen.[152–154] Although using the OT-I system makes it unlikely for OT-I T cell to recognize antigen in the recipient mice, since OT-I T cells express RAG genes we cannot exclude the impact of antigen in both OT-I recruitment and T_{RM} differentiation. However, this is very unlikely as the recipient mice do not express ovalbumin. Additionally, the presence of OT-I T cell clones that express a self-reactive endogenous α-chain are unlikely to have interfered with the results due to the rarity of these cells and due to the low variability among experiments. Additionally, prior to the transfers, over 80% of the OT-I T cells presented the classic $V\alpha2^+$ $V\beta5^+$ phenotype. Moreover, other groups have also shown the differentiation of T_{RM} in $RAG^{-/-}$ models using other antigen-independent models.[155,156] New data from the Snyder´s group (not shown - personal communication with Corinne Smith) replicated the differentiation of T_{RM} in naïve mice after adoptive transfer of $RAG^{-/-}$ OT-I T cells. Thus, although it would be interesting to use $RAG^{-/-}$ OT-I T cells to further compare the cell accumulation and T_{RM} differentiation in the gland of naïve and MCMV infected mice, we believe that the differences seen between these groups are more likely due to the virus-induced inflammatory environment than due to antigen recognition.

Although we were unable to define which mediator (integrins, selectins and/or chemokines) promotes OT-I migration to the salivary gland early after MCMV infection, it is possible that these mechanisms have a synergic contribution. Further work will be needed to clarify the impact of MCMV in promoting the expression of homing molecules both in the $CD8^+$ T cells and in the vasculature of the salivary glands. Defining these mechanisms would highlight important clues about $CD8^+$ T cell trafficking and possibly allow us to improve $CD8^+$ T cell migration and immune surveillance in the salivary gland.

3.1.2.4 MCMV infection does not impact the differentiation of a non-cognate T_{RM} population

It was surprising that the greater initial accumulation of OT-I T cells in infected salivary glands was not maintained in later time points of infection and that no differences were seen in the overall OT-I T_{RM} between naïve and MCMV infected mice (figure 4). It is conceivable that after the peak of viral replication in the salivary gland, the inflammatory cues and factors that promoted the early OT-I T cell accumulation in the infected salivary glands are no longer present explaining the lack of differences in

OT-I T cell numbers at a later time after adoptive transfer. This loss of the early advantage may also imply differences in retention and survival cues for $CD8^+$ T cells.

CD69 expression promotes $CD8^+$ T cell retention and maintenance within non-lymphoid organs by decreasing the S1P signal.[157,158] According to our RNAseq data, CD69 inducers such as TNF-α and IL-33 are expressed in the salivary glands of both naïve and infected mice. These cytokines could promote CD69 expression in both groups and contribute to similar late accumulation of OT-I T cells and T_{RM} differentiation. However, the RNAseq data does not exclude local expression niches within the infected glands that may promote this process.

Another possible explanation for the similar number of T_{RM} in both naïve and infected glands relies on the expression of survival modulators. An interesting factor is the P2X purinoceptor 7 (P2RX7) that has been described in Lymphocytic choriomeningitis virus (LCMV)-specific T_{RM} in the salivary gland and is induced by IL-12.[159] The lack of P2RX7 did not significantly alter the frequency of LCMV-specific $CD8^+$ T cells in the gland (most of which presented a T_{RM} phenotype). However, by recognizing NAD^+ and extracellular nucleotides ATP produced upon tissue damage and inflammation, P2RX7 was able to decrease T_{RM} survival *in vitro*.[159] Therefore, it is conceivable that the inflammation caused by MCMV increases local NAD^+ and extracellular nucleotide, which could represent a disadvantage for P2RX7 expressing T_{RM}. This could help to explain the similar number of T_{RM} in the infected and naïve mice. It is possible that this effect is intensified in an antigen-independent model, since TCR signal and antigen recognition reduces the expression of P2RX7.[159] The restriction of P2RX7 expression caused by TCR signaling may function as a safety mechanism preventing the establishment of unrelated T_{RM} that could compete with the differentiation of cognate-specific T_{RM} within the tissue.

Interestingly, and contrarily to what was described above, $CD8^+$ $P2RX7^{-/-}$ cells were reduced in the salivary gland in comparison to WT cells after LCMV infection.[160] Similarly, after LCMV infection P2RX7 was important for the generation of $CD103^{hi}$ T_{RM} in organs such as the small intestine.[160] Due to multiple and distinct described roles of this receptor in T_{RM}, it would be interesting to measure the P2RX7 expression in T_{RM} over time in the salivary gland in both naïve and MCMV infected mice (in the presence and absence of cognate antigen).

Moreover, survival cues can be dependent on the nutrition and blood supply available to the salivary gland. It is likely that acute MCMV infection increases the vessel permeability allowing for extra blood supply and nutrients to the glands. As for the epidermis, it is possible that the OT-I survival and the T_{RM} differentiation in the salivary gland are shaped by the blood supply and nutrients available.[161] If that is the case, it is possible that MCMV infection increases the recruitment of OT-I cells to the salivary glands

initially in infected recipients, though, at later times with less inflammation, the cells that remain in the gland are the limited cells that are able to survive under more hypoxic conditions and differentiate into T_{RM}.

It is important to mention that this plateau in OT-I T cells and T_{RM} in the salivary glands can also represent a limitation of our model since a restricted number of OT-I T cells were transferred. Within the transferred cells, it is plausible that a certain proportion of T_{RM} precursors were transferred limiting the resulting T_{RM} number. The transfer of subsequent populations of activated OT-I cells might help us determine the limit of T_{RM} differentiation. Nonetheless, it is crucial to understand which precursor cells become T_{RM} under certain conditions. This might be accomplished by further *in vitro* characterization of the cells that can become $CD103^+$ and $CD69^+$ associated with subsequent *in vivo* depletion studies.

Our data suggest that MCMV replication and the resulting inflammatory environment may promote $CD8^+$ T cell recruitment to the salivary gland but does not seem to impact T_{RM} differentiation. It is important to note that, although antigen is not required for T_{RM} differentiation, our work cannot exclude a role for antigen in promoting this process. One way to further test the importance of antigen would be to use OT-Is from $RAG^{-/-}$ mice as explained before. It would be interesting to compare naïve recipients as well as MCMV and MCMV-Ova infected mice and the resulting differentiation of T_{RM} with the proper normalization for transferred cells, expansion and proliferation.

PART 3.2 Mechanisms involved in T_{RM} differentiation in naïve salivary glands

3.2.1 Mediators of $CD8^+$ T cell homing to naive salivary gland

Neither a certain route of immunization (figure 1) or MCMV infection (figure 3 and 4) significantly increased the ability of the activated $CD8^+$ T cells that reached the salivary glands to differentiate into T_{RM}. Then, the migration of $CD8^+$ T cells and specifically T_{RM} precursors to the salivary glands seems to be an important determining factor for T_{RM} differentiation and therefore worth exploring and characterizing.

$\alpha4\beta1$ mediates $CD8^+$ T cell recruitment to salivary gland

Our data demonstrated that, as happens in infected salivary glands, $\alpha4\beta1$ expression also contributes to $CD8^+$ T cell migration to naive salivary glands (figure 5). This suggests that $\alpha4\beta1$ ligands (as VCAM-1) are expressed in the salivary gland at sufficient levels at a steady state to promote $CD8^+$ T cell entry. Although it was already shown that LCMV infection and inflammation induce VCAM-1 expression in the salivary glands' vasculature, previous reports fail to identify VCAM-1 in the salivary gland of control C57BL/6 (B6) mice.[135,162]

To further complement our results, the VCAM-1 expression in the vasculature of naïve mice needs to be assessed. This could be achieved using immunofluorescence and its function tested using blocking antibodies or mouse models with VCAM-1 conditional deletion. The same evaluation should be performed to other $\alpha4\beta1$ ligands, especially because epithelial cells in the salivary glands produce other ligands under steady state conditions such as fibronectin, which have not been tested in the context of T cell homing to the salivary glands.

Role of chemokines in $CD8^+$ T cell migration to the salivary gland

Chemokines are other crucial mediators of $CD8^+$ T cell migration to the organs, as seen by the crucial role of CCR5 in a rapid recruitment of memory $CD8^+$T cell to the airways following some viral infections.[163–166] Besides recruitment, chemokines are also critical for the localization within the tissue. This is important for T cells to receive the appropriate signals and resident cues that mediate T_{RM} differentiation. These two chemokine roles are evident by the CXCL17-CXCR8 interaction that promotes mobilization of $CD8^+$ T cells to and within the female genital tract, leading to protection against HSV-1.[167]

Inflammation and infection is thought to promote chemokine expression in the affected tissues allowing for an increased recruitment of immune cells and consequent protection.[168–171] CMV is thought to induce chemokines in different organs such as the spleen and liver where CXCR3-CXCL9/CXCL10

interactions promote CD8+ T cell infiltration and IFN-γ responses.[147] Surprisingly however, our RNA-Seq data showed minor changes in chemokine expression between the salivary glands of naïve and MCMV infected mice (figure 6). Several chemokines were indeed constitutively expressed in the salivary gland of naïve mice at a steady state (figure 6). Interestingly, these chemokines expressed in naïve glands can be recognized by receptors expressed by CD8+ T cell and had the potential to induce CD8+ T cell migration *in vitro* (figure 7 and figure 8). Since chemokines are usually prominent upon infection or tissue damage, it is fascinating to think of explanations for the lack of more striking differences in chemokine expression between naïve and MCMV infected salivary glands. We believe that the presence of food antigens and microbiota in the salivary glands might contribute to this surprising basal expression of chemokines.

The impact of food antigens in the inflammatory and chemokine profile in the tissues is still not fully characterized. In fact, the recognition of food antigen tends to be associated with food allergies and increased IgE and Th2 responses.[172,173] However, multiple changes occur in response to food antigens, especially in the gastrointestinal (GI) tract.[174] For example, dietary nucleotides increase the proportion of γδ+ IEL in the gut, while alkylamine antigens found in mushrooms, apples and edible plants promote γδ+ T cells expansion.[175,176] γδ+ T cells have also been described in the salivary glands and if a similar mechanism promotes their presence and expansion, γδ T cells can shape the gland environment and indirectly induce CD8+ T cell recruitment, by producing CCL3, CCL4 and CXCL10.[177–180]Therefore, it is possible that, under certain conditions, food antigens can be a sufficient trigger to promote local immune responses and chemokine production. This is appealing to consider in organs of the GI tract that have contact with food-derived antigens such as the salivary gland.

Nevertheless, food is not the only source of antigens that can promote an immune response in the gland. Salivary glands are in communication with the oral cavity and retrograde migration of bacteria from the oral cavity through the salivary ducts has been proposed (retrograde theory of sialolithiasis formation).[181,182] Therefore, it is tempting to think that the contact with microbiota may contribute to the basal chemokine and pro-inflammatory levels in naïve salivary glands.[183–185] Microbiota has indeed been linked to T cell homing modulation.[186] Microbial association to germ-free mice resulted in increased CD8+ T cells in the intestine.[187] Microbiota was also associated with the differentiation of residing γδT cells in the intestine.[188,189] Furthermore, some reports suggest that probiotics such as *Lactobacillus acidophilus* can modify the inflammatory status of the intestinal epithelial cells, leading to cytokine and chemokine production.[185] The mechanisms by which microbiota induce immune cell recruitment are not totally clear but might be due to the antimicrobial function of some chemokines such as CXCL9, CXCL10 and CXCL11.[190,191] Therefore, it is possible that organs that contact regularly with microorganisms, such as the

GI tract and the salivary glands, constitutively express chemokines with anti-microbial properties that further promote the recruitment and maintenance of immune cells. This hypothesis possibly helps in explaining the constitutive expression of chemokines in the salivary gland (figure 6) and the surprising role of CXCR3 in $CD8^+$ T cell migration to the salivary gland in naïve mice (figure 10). The characterization of the ligand present in naïve salivary glands that promotes CXCR3-dependent $CD8^+$ T cell migration still needs to be performed. Although only CXCR9 and CXCL10 are classical CXCR3 receptor ligands in B6 mice, chemokine receptors have been shown to heterodimerize with other chemokine receptors as referred before. CXCR3, in particular, can heterodimerize with other receptors such as CXCR4.[151,192,193] Since CXCR4 was also expressed by $CD8^+$ T cells in the salivary gland, it is still formally possible that non-classical receptor/ligand combinations are responsible for recruiting T cells to the salivary glands. Thus, due to heterodimerization of chemokines and their receptors, there may be some variability in the ability/affinity of ligands to bind CXCR3, which shapes the OT-I T cell recruitment. Our results are also limited since the role of CXCR3 receptor in $CD8^+$ T cell localization within the salivary gland was not systematically done. Although our preliminary results using immunohistochemistry did not show differences in location between CXCR3 WT or KO cells in the salivary gland parenchyma (data not shown), additional experiments comparing several sections of the gland and different times after transfer are needed to surely answer this question.

Although CXCR3 expression was not required for T_{RM} differentiation in MCMV-infected salivary glands (figure 9), it is still exciting to question if CXCR3 overexpression could promote $CD8^+$ T cell migration, especially to naive salivary glands and thus T_{RM} differentiation. This would be an advantage for the development of vaccines against infections that target the gland. To test this hypothesis CXCR3 expression could be achieved by either modulating the *in vitro* activation conditions (antigen and IFN-γ levels); by using a vector systems or preferentially using an inducible set-up such as Cre-inducible mice, which will allow for a controlled CXCR3 expression.[149] That way, it would be possible to determine the role of this chemokine in $CD8^+$ T cell migration but also in different steps of T_{RM} differentiation.

3.2.2 Cues for T_{RM} differentiation in naïve salivary glands

More surprising than activated $CD8^+$ T cells being recruited to naïve salivary glands, was the fact that similar proportions of T_{RM} differentiated in salivary glands of naïve and MCMV-infected mice (figure 4). Although this study does not define the *in vivo* inducers of T_{RM} differentiation, according to our results, the salivary gland at a steady state must have the necessary cues for T_{RM} differentiation. The signals that promote T_{RM} differentiation are not clear for all the organs but TNF-α, IL-33 and TGF-β have been implied

in this process.[154,157,194] Interestingly, these cytokines were detected in the salivary gland of both naïve and MCMV infected mice in the RNAseq analysis. Large amounts of the three TGF-β transcripts are present in both infected and naïve salivary glands (TGF-β2 > TGF-β3 > TGF-β1). Even though we have not directly measured the protein levels of these cytokines in naïve salivary glands, several studies have detected IL-33, TGF-β and TNF-α expression in the salivary glands of non-pathological/control subjects.[195–198] It is exciting to consider reasons for the presence of these cytokines in naïve salivary glands. In fact, TGF-β and IL-33 can also be induced by tissue damage and inflammation, limiting the expansion of T and B cells and promoting T_{REG} cells.[196,199–202] Thus, production of such cytokines could be promoted by contact with microbiota, food antigens, and act as a defense mechanism to prevent excessive inflammation and damage of the gland. Additionally, TGF-β is required for salivary gland development and branching morphogenesis and, therefore, is expressed since early development stages of the salivary gland, which could explain the constitutive expression in this organ.[203,204] The constitutive presence of these cytokines in the salivary glands might explain the singular ability of $CD8^+$ T cells to differentiate in T_{RM} in this organ under steady state conditions. Nonetheless, it is likely that the activated OT-I cells express cytokines such as TNF-α, which may also contribute to the T_{RM} differentiation after the adoptive transfer in both groups of mice. Moreover, it is not clear when these cytokine stimuli are received by $CD8^+$ T cells allowing for T_{RM} differentiation. Therefore, we cannot exclude that during our activation protocol cells were exposed to cytokines that may contribute to the subsequent T_{RM} differentiation pathway. An inducible KO system of the cytokine receptors can be helpful in dissecting the timing and their contribution in T_{RM} differentiation *in vivo*.

PART 3.3 Implications of the prompt ability for T_{RM} differentiation in the salivary glands

3.3.1 Possible downsides of this prompt ability for T_{RM} differentiation in the salivary glands

A striking feature of our results is the ability for activated $CD8^+$ T cell to differentiate into T_{RM} in the salivary glands under multiple conditions (different viral infections, after non-productive MCMV infection and even in naïve salivary glands). Similarly, studies from Pircher and Oxenius' laboratories, demonstrate that activated cells can enter uninfected salivary glands and differentiate into T_{RM}.[155,156] It is easy to hypothesize about the advantage of the salivary glands' prompt ability to differentiate T_{RM} from activated T cells when considering a sialotropic infection. Nonetheless, some disadvantages might also emerge, especially in gland-unrelated infections. This ability to differentiate T_{RM} from activated cells in the gland even in the absence of local infection may imply that the disease history/record of specificities of the activated $CD8^+$T cells can be mirrored in the gland and impact the resulting T_{RM} profile.

Competition between T_{RM} subsets

Firstly, if multiple unrelated infections trigger CXCR3 and α4β1 expression on T cells, promoting their ability to enter and differentiate into T_{RM} in the salivary gland, the space, local differentiation cues and survival factors may exhaust leading to a limit and competition between T cell subsets. Although a previous infection did not prevent new T_{RM} differentiation in the salivary gland (figure 3), other studies support this limit idea. In fact, some of our previous reports show that although a peptide-based approach leads to an increase in $CD8^+$ T cells in the salivary gland, a comparable increase in T_{RM} does not occur.[205] Similarly, Muschaweckh A. *et al.* (2016) has shown that $CD8^+$ T cells in the skin undergo antigen-dependent competition which shapes the T_{RM} repertoire and localization.[206] Therefore, further work is needed to explore and define the limit of T_{RM} differentiation in the gland, as well as the impact of competition between T_{RM} subsets. Both scenarios could have clinical implications in the resulting immune response in the salivary gland. As an example, HIV infection of the central nervous system results in $CD8^+$ T cell recruitment partially due to α4β1 and CXCR3 expression.[207] According to our results, it is possible that these HIV-induced $\alpha4\beta1^+$ $CXCR3^+$ cells could simultaneously enter the salivary gland and differentiate in T_{RM} possibly competing with other relevant T_{RM} subsets.

Exaggerated immune response and the role of T_{RM} in autoimmunity

Besides considering competition between $CD8^+$ and T_{RM} subsets, the fact that activated $CD8^+$ T cells can migrate and differentiate into T_{RM} in the salivary gland under steady state conditions can also result in an unrelated and exaggerated immune response in this organ. In fact, T_{RM} are not always related with good outcomes. In the study of colorectal cancer, the presence of $CD103^+$ TIL did not correlate with survival and was associated with a poor prognosis in some groups.[208] This is an interesting result since, like the salivary glands, T_{RM} in the gut can form independently of antigen. If intestinal T_{RM} could form at a steady state as the salivary gland, then maybe the additional stimuli and inflammation caused by the disease might increase the recruitment of cells to an extent that would be beyond a beneficial response. It is thus fascinating to hypothesize that this propensity for T_{RM} differentiation seen in the gland can lead to a deleterious response and autoimmunity.

The link between T_{RM} and autoimmunity is not new. In fact, T_{RM} have been suggested as important players in multiple diseases with an autoimmune component such as contact dermatitis, multiple sclerosis and psoriasis.[209–211] Indeed, when non-lesioned skin from patients with psoriasis were engrafted in mice, it resulted in resident T cell expansion and the development of lesions.[212] Due to the cytotoxic and immune enhancing abilities of T_{RM} it is also tempting to explore and think about the role of T_{RM} in autoimmune diseases in the salivary gland, such as the Sjögren's syndrome.

The role of T_{RM} in Sjögren's syndrome

Sjögren's syndrome is thus an autoimmune disease that mostly affects the salivary and lacrimal glands resulting in poor secretory function.[213] It is believed that the pathological mechanism involves, at least partially, the increased lymphocytic infiltration, IFN-γ production and autoantibodies.[214,215] $CD8^+$ T cells have already been linked to Sjögren's syndrome pathology, however based on the prompt ability of $CD8^+$ T cells to infiltrate the salivary gland and differentiate into T_{RM}, it is enticing to think that T_{RM} specifically be involved in IFN-γ production and tissue damage.[216–218] Recently, T_{RM} started to be characterized in labial salivary glands from Sjögren's syndrome patients and were found in significant numbers, outnumbering the $CD4^+$ T cell.[219] This presence of $CD8^+$ T cells can be related with the disease, especially since the lack of $CD8^+$ T cells restored the excretory function in salivary glands in a Sjögren's syndrome mouse model.[219] Both IFN-γ KO or $CD8^+$ T cell depletion were related with decreased $CD8^+$ T cells in the gland, pathologic manifestations and tissue destruction.[219] Another interesting mediator to consider is IFN-γ, which is a well-established contributor to the Sjögren's pathology and disease.[220,221] Although our data suggest that chemokines such as CXCL9 can be produced in the gland independently to IFN-γ, it is likely that the IFN-

γ production in the presence of the disease, further promotes the expression of chemokines such as CXCL9 and CXCL10 and therefore the recruitment of $CD8^+$ T cells. In fact, in other studies the IFN-γ mRNA levels were correlated with lymphocyte infiltration in the salivary glands of both human patients and Sjögren's syndrome mouse model.[219,222] This can either suggest an increased IFN-γ production by the lymphocytes in the gland and/or a role in T cell recruitment. Interestingly, the levels of CXCL9 mRNA were increased in salivary glands from Sjögren's syndrome mouse models, which was not seen after depletion of $CD8^+$ T cells or IFN-γ. [219] These data suggest that in Sjögren's syndrome models $CD8^+$ T cells contribute to IFN-γ and the resulting chemokines in the gland. Although these studies focused on the role of $CD8^+$ T cells the role of T_{RM} were not defined. Therefore, it is possible that T_{RM} in the gland contribute to the IFN-γ and chemokines production, which promotes further immune cell recruitment and tissue damage.

Similarly to IFN-γ, TNF-α blockade also resulted in CXCL9 reduction in the salivary glands, which was associated with T cell reduction and remission of the symptoms in a mouse model of Sjögren's syndrome.[223] Since TNF-α promotes T_{RM} differentiation this can also indicate a role for T_{RM}. Interestingly, CXCR3 blockade diminished the number of $CD8^+$ T cells in the salivary gland of non-obese diabetic mice used as a Sjögren's syndrome model, which resulted in similar remission of symptoms.[224] Besides demonstrating the impact of $CD8^+$ T cells in Sjögren's syndrome pathology, these data highlight the role of CXCR3 in recruiting cells to the gland in a different context than our experiments. It would be equally interesting to address if α4β1 would have a similar effect in Sjögren's syndrome pathology since it is also involved in T cell recruitment to the salivary gland. Additionally, $CD4^+$ T cells also promoted $CD8^+$ T cell accumulation in the glands of a Sjögren's syndrome mouse model.[219] Although these results were obtained in completely different conditions than our results, it is possible that $CD4^+$ T cells impact the overall $CD8^+$ T cell population in the gland, but not the resulting T_{RM}. These data suggest that although different factors modulate the recruitment and presence of $CD8^+$ T cells in the salivary glands under different contexts, $CD8^+$ T cell migration and residency in the salivary gland might be a crucial factor in Sjögren's syndrome pathology.

It would be interesting to further conclusively distinguish the role of effector $CD8^+$ T cells and T_{RM} in Sjögren's syndrome. Furthermore, it will be important to determine the $CD8^+$ T cell´s specificity and the mechanisms involved in tissue damage, all of which could contribute to the development of a targeted therapy.

3.3.2 Possible benefits of the prompt ability for T_{RM} differentiation in the salivary glands

Although risks associated with the propensity of T_{RM} to differentiate in the salivary gland exist, advantageous outcomes with clinical implications can also be envisioned. This prompt ability for T_{RM} differentiation in the salivary glands may promote mucosal immunity and it is interesting to consider in the context of multiple aggressions and as a preventive measure.

The protective role of T_{RM} has been shown in different conditions such as respiratory, ocular and cutaneous infections.[2,167,225,226] Interestingly, T_{RM} have also been able to induce heterotypic protection.[225,227] Therefore, it is possible that promotion of T_{RM} in the salivary gland using activated $CD8^+$ T cells could confer protection from a variety of sialotropic pathogens such as HSV, Rubulaviruses, HIV and Epstein-Barr besides CMV. Though more studies are required to test the role of T_{RM} in CMV immunity, $CD8^+$ T cells have been suggested to improve protection against CMV reactivation and intraglandular infection.[156,228] Consequently, it is plausible that T_{RM} differentiation contributes to protection against viral reactivation and transmission. Our results suggest that T_{RM} differentiation can be induced in both infected or uninfected subjects, which is beneficial for the development of a vaccine strategy or for improving the mucosal immunity in immunocompromised subjects.

Several strategies have been used to induce T_{RM} differentiation in different organs. The use of vectors and prime and pull strategies have been common approaches used to drive T_{RM} differentiation.[3,229–233] The last method requires a primary vaccine/exposure to antigen (prime) followed by a secondary inflammatory trigger (pull) to promote migration and residency in the desired organ. Recombinant chemokines, such as CXCL10 can act as the pulling cue.[234] This prime-pull approach was able to induce T_{RM} in the female reproductive tract and lungs.[110,234–237] Although effective in inducing T_{RM}, the vaccine strategies developed so far struggle to induce an uniform T_{RM} population along the organ and to establish enough T_{RM} to induce protection (a threshold that itself is hard to calculate). Moreover, the prime and pull approach has the disadvantage of requiring multiple procedures and easy access to the tissue. Our data suggest that some organs, such as the salivary gland, might not require the "pull" trigger to induce significant T_{RM}. This is advantageous since it may imply a reduction in the number of interventions needed as well as overcoming the difficulty of reaching some organs.

According to our results T_{RM} differentiation in the salivary gland can be easily achieved even in the absence of antigen/inflammation in a $\alpha4\beta1$ and CXCR3 dependent way. Our data also suggest that a potential vaccine strategy using either activated transferred cells or through the *in vivo* induction of activated cells that express these homing receptors may induce T_{RM} differentiation in the salivary gland. This approach could be significantly beneficial, not only in reducing CMV replication and latency in the

salivary gland, but especially preventing virus reactivation and shedding. Considering that scenario, induction of protective T_{RM} in the salivary gland could possibly reduce horizontal and vertical transmission through saliva. Additional clarification of the role of T_{RM} in CMV control is crucial and this work may contribute to the development of new approaches to test T_{RM} function in the salivary glands.

Additionally, similar strategies could be used to induce T_{RM} in other areas such as oncology. Interestingly, $CD103^+$ tumor infiltrating lymphocytes improved survival in melanoma, lung, breast and ovarian cancer.[3,238–242] Similarly, a vaccination approach was able to induce T_{RM} and decrease tumor growth in a head and neck cancer model.[243,244] Therefore, it is conceivable that this application can also be noteworthy in salivary glands' tumors. Remarkably, in the most common salivary gland tumors, the mucoepidermoid carcinomas, CD8 expression correlated inversely with the tumor size.[245] As happens with most salivary gland pathologies, the role of $CD8^+$ T cells in salivary glands has not been extensively studied and it would be fascinating to determine the role of T_{RM} in tumor progression and survival.

In the future, the translational application of these results can have implications from autoimmune diseases to anti-tumor therapeutics and vaccines. However, more studies are needed to completely understand the processes involved in T_{RM} differentiation in the salivary glands and their function before trying to make conclusions with clinical implications. Studies using other animal models and the study of $CD8^+$ T cells specificity from human salivary glands can provide vital clues about the differentiation of T_{RM} and unveil potential parallels. Meanwhile, looking at our results, it is important to consider that the salivary glands in mice and humans exhibit different characteristics.[246–248] While in mice the largest gland is the submandibular gland, the parotid gland is the major salivary gland in humans.[246] Moreover, the submandibular gland and the sublingual glands are fused in mice, unlike humans.[246] Besides the anatomical changes, both species present similar gland histology and function. Saliva production is the main function of the salivary glands. Saliva is mainly composed of water, that serves as a vehicle for all the electrolytes, lipids, enzymes and a wide variety of other proteins with antimicrobial activity, all of which promote digestion. Nonetheless, some components in saliva are not shared between humans and mice.[247] Furthermore, in mice, saliva is also important component in grooming and as rodents, it is plausible that salivary glands partake a more significant role in mice than in humans. Therefore, the translation of our results must be done carefully.

3.4 Final considerations

In sum, our results provide further evidence that a portion of activated $CD8^+$ T cells differentiate into T_{RM} in both uninfected and MCMV infected salivary glands. Although differences in gene expression were seen in the salivary glands after MCMV infection, these differences did not correlate with changes in T_{RM} differentiation. Moreover, the characterization of homing receptors and mediators of $CD8^+$ T cell migration to the salivary glands was conducted. $CD8^+$ T cell migration to the gland was promoted by the expression of CXCR3 (in naïve mice) and $\alpha 4\beta 1$ (both naïve and MCMV infected mice). Regardless of its limitations, this work is an additional contribution in studying T_{RM}. However, many relevant and exciting questions remain: the definition of the precursor cells; the factors triggering the basal inflammatory state in the salivary glands that allow for T_{RM} differentiation, the requirements for T_{RM} maintenance in this organ, the role of competition between subsets and ultimately the function of T_{RM} in the salivary gland especially after MCMV infection. Knowledge truly is a never-ending process.

3.5 References

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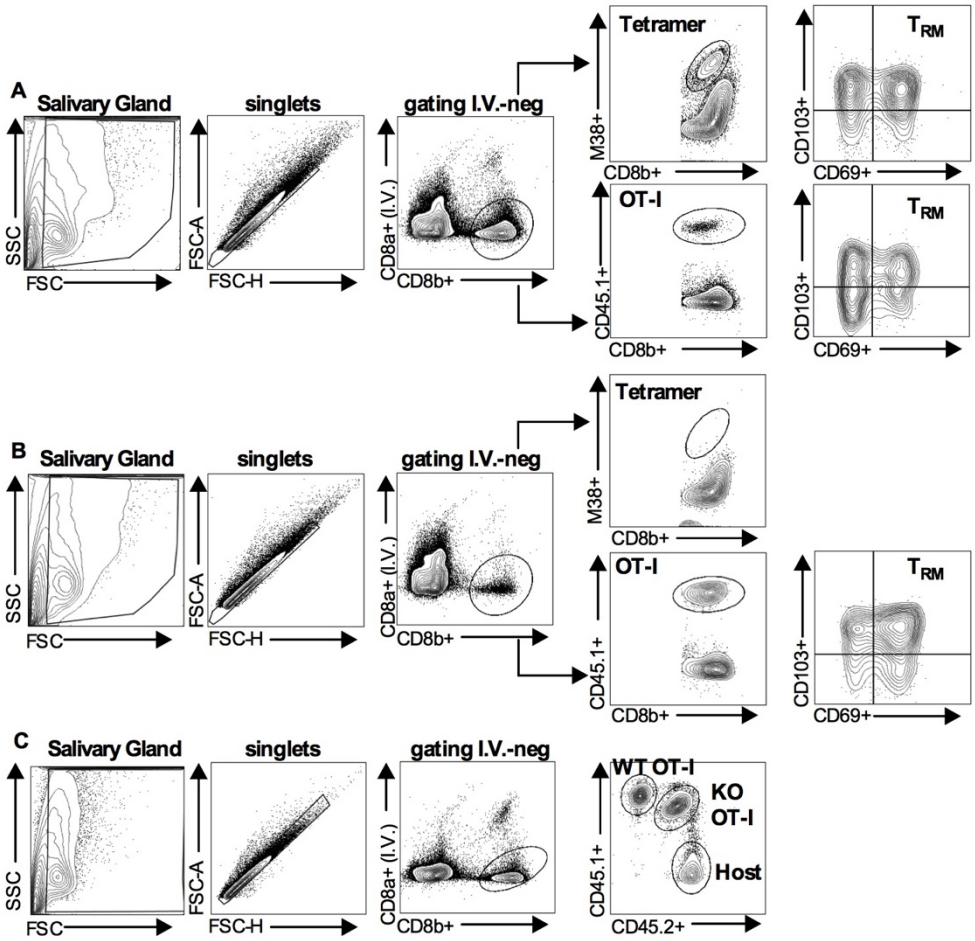
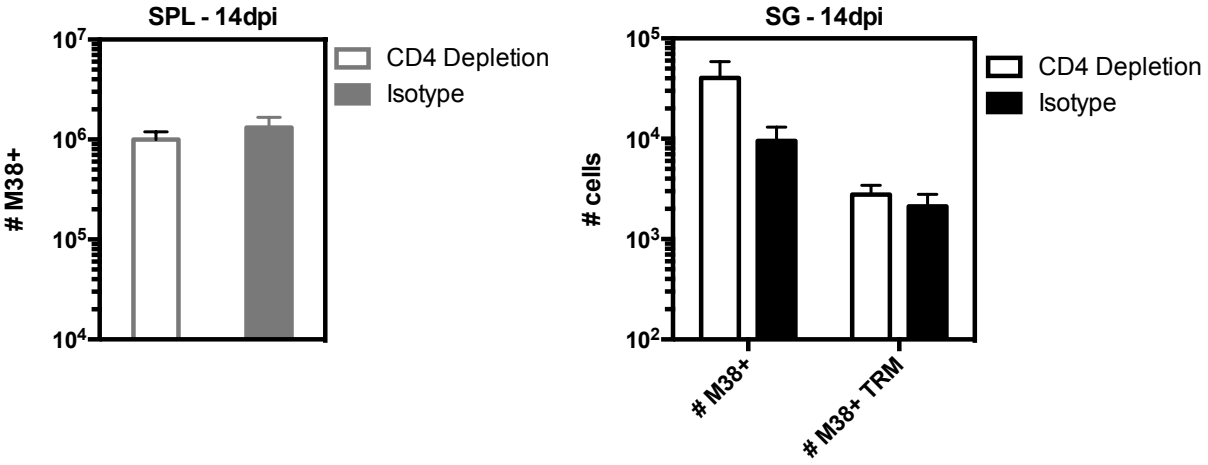
Supplemental data



Sup. figure 1. Representative Gating

A) Gating strategy used to identify $CD8^+$ T cells in the parenchyma of the salivary gland. After a broad lymphocyte gate, singlets were selected by FSC-H vs FSC-A and the $CD8\beta^+$ T cells that were not intravenous (i.v.) CD8α antibody were selected. From the i.v.- $CD8\beta^+$ population, MCMV-specific T cells were identified by tetramer-binding (shown are M38-specific T cells) and OT-Is were identified by expression of the congenic marker (CD45.1, CD45.2 or Thy1.1). The T_{RM} $CD8^+$ cells were characterized by the expression of CD69 and CD103. (**B**) Representative gating strategy for detection of OT-I T cells in naïve mice as in **A**. **C**). Representative gating (as in **A**) of two congenically-marked OT-I populations in the same recipient.



Sup. figure 2. CD4⁺ T cells are not required for T_{RM} cell development in the salivary gland.

Within 7 days, anti-CD4 antibody (open bars) or isotype control (filled bars) were injected i.p. every other day until the day of infection with MCMV (confirmation of depletion was done prior to infection). The antibody depletion injections were maintained (1-2 days interval) until sacrifice at 14 days of infection. The absolute number of M38 tetramer⁺ cells in the spleen (SPL) are represented on the left (grey); Absolute number of M38 tetramer⁺ cells in the salivary gland and the absolute number of tetramer⁺ CD69⁺ and CD103⁺ cells in the salivary gland (SG) are represented on the right (black). Combined results from 3 independent experiments were combined (n=9 for depleted animals and n=7 for the group that received the isotype control). Error bars represent the SEM and statistical significances were measured by unpaired t-test after $\log_{10}$ conversion of the absolute numbers.

Sup. tables S1A-S1C: RNAseq analysis of MCMV infected and uninfected salivary glands

Tables available at:

https://www.jimmunol.org/content/suppl/2017/12/29/jimmunol.1701272.DCSupplemental

Table S1A – Gene set with all the genes that significantly increased or decreased with MCMV infection (FDR<0.05);

Table S1B - Gene set was filtered by a raw p value lower than 0.05;

Table S1C - Gene Set Enrichment Analyses (GSEA) using the Gene Ontology Biological Process (GOBP) database.