Doctoral Thesis

Immune Control of Cytomegalovirus in the Salivary Glands

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Immune Control of Cytomegalovirus in the Salivary Glands

A thesis submitted to attain the degree of
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1 General Summary

1.1 English Summary

Cytomegaloviruses (CMVs) are herpesviruses that establish life-long infection with alternating periods of active virus replication and latency, a state of viral dormancy. 60 – 90% of the world population is infected with human CMV (HCMV). While HCMV infection runs an asymptomatic course in immunocompetent individuals, it is a frequent cause of severe morbidity and mortality among the immunocompromised.

HCMV persists selectively in mucosal tissues and exploits their secretions for horizontal spread. The saliva is the most relevant vehicle of viral transmission and CMV persists in the salivary glands (SG) far beyond the time point at which viral latency has been enforced in other organs by the host’s immune response. CMVs outstanding ability to hide from the immune system at mucosal sites builds the foundation of the virus's epidemiological success. In infected cells, CMV compromises the surface expression of MHC I molecules, which are essential for recognition by activated CD8+ T cells. This process is most effective in the SG, where MHC I expression is fully suppressed in saliva producing epithelial cells, rendering CD8+ T cells entirely inoperative in the SG despite their crucial contribution to CMV control in all other organs. Instead, persistent CMV replication in the SG is contained by CD4+ T cells, which play a subordinate role in CMV control in other organs.

While experimental infection of mice with murine cytomegalovirus (MCMV) has led to a profound understanding of MCMV-specific T cell responses in the SG, studies dedicated to antigen presenting cells (APCs) in this organ are scarce and inconsistent. Since APCs are important activators and regulators of CD8+ and CD4+ T cell responses, we focused in the first part of this thesis on the ontogeny and function of APCs in the SG. We found that SG-resident APCs are a network of CD11c+CD11b+ tissue macrophages that are unable to cross-present endocytosed MCMV-derived antigen via MHC I molecules, thus failing to activate CD8+ T cells. To
our surprise, classical dendritic cells as well as inflammatory monocytes were absent from the SG at the evaluated time points. We therefore suggest that the SG-specific composition of APCs is a contributor to the severely limited efficacy of CD8$^+$ T cells in this organ. Said shortcoming of CD8$^+$ T cells in the SG is a universal paradigm of CMV immunity, but so far, it has been verified sufficiently only in the acute, not in the latent phase of infection. The principle mechanisms and effectors that restrict local viral reactivation and shedding during latency remain an active field of inquiry. Therefore, the second focus of this thesis was on the ability of MCMV-specific CD8$^+$ and CD4$^+$ memory T cells to control locally introduced MCMV in the SG. We found that the SG was immensely potent to induce CD8$^+$ and CD4$^+$ tissue-resident memory T (T$_{RM}$) cells. The generation of T$_{RM}$ cells depended on cognate antigen for CD4$^+$ but not CD8$^+$ T cells, indicating important differences in T cell subset-specific demands within the same organ. We furthermore found that CD8$^+$ T$_{RM}$ cells were able to control locally introduced MCMV. Our data therefore suggest a novel role for CD8$^+$ T cells to restrict viral reinfection and reactivation periods in the SG, an organ that has so far been deemed resistant to CD8$^+$ T cell-mediated virus control.
1.2 German Summary

Cytomegalieviren (CMV) gehören zu den Herpesviren und verursachen lebenslange Infektionen, welche geprägt sind durch abwechselnde Phasen der aktiven Virusreplikation und der Latenz, einem inaktiven Ruhezustand. 60-90% der Weltbevölkerung ist mit dem humanen Cytomegalievirus (HCMV) infiziert. Während die HCMV Infektion bei immunkompetenten Individuen zumeist asymptomatisch verläuft, stellt sie für immunsupprimierte Patienten einen der häufigsten Auslöser lebensbedrohlicher Komplikationen dar.


In infizierten Zellen beeinträchtigt CMV die Oberflächenexpression von MHC I Molekülen, welche essenziell für die Erkennung durch aktivierte CD8⁺ T-Zellen sind. Diese Massnahme der Immunevasion ist besonders wirksam in der Speicheldrüse, wo sie zur vollständigen Abwesenheit von MHC I Molekülen auf infizierten speichelproduzierenden Epithelzellen führt. Letztere sind folglich unsichtbar für CD8⁺ T-Zellen, welche in allen anderen Organen massgeblich zur Viruskontrolle beitragen. In der Speicheldrüse hängt die Viruskontrolle nun von CD4⁺ T-Zellen ab, welche in anderen Organen wiederum eine untergeordnete Rolle spielen.

Während T-Zellantworten in der Speicheldrüse zumindest in der akuten Phase der CMV Infektionen bereits ausführlich untersucht wurden, ist über die antigenpräsentierenden Zellen (APZ) in diesem Organ nur wenig bekannt. APZ spielen für die Aktivierung und Funktion von T-Zellen eine wichtige Rolle. Ein Fokus der Dissertation lag daher auf der Untersuchung der ontogenetischen Herkunft sowie der Funktionalität der APZ in der Speicheldrüse. Wir konnten zeigen, dass das Netzwerk der APZ ausschliesslich von Makrophagen gespannt wird, welche nicht


2 General Introduction

Viruses are infectious agents whose replication relies on the reproductive machinery of a cellular host. The human body is constantly exposed to viral attempts to hijack vital cellular pathways, thereby threatening the host’s metabolic integrity. Most of these efforts fail in the face of non-permissive barriers, such as epidermal sheets, mucus layers, or unfavourable acidic conditions. Viruses that breach these barriers evoke immune responses that usually achieve complete virus clearance several days after initial encounter. These infections are called acute viral infections. Some viruses, however, can never be cleared by the immune system and engage in life-long co-existence with their hosts to establish so-called persistent viral infections. Clinically relevant examples of persistent viruses are HIV, Hepatitis B Virus, and all herpesviruses. The latter form the large and ubiquitous taxonomic family of Herpesviridae, which are exceptionally prevalent in the population owing to their extraordinary ability to evade immunity and persist in tissues. An excellent model pathogen to study the interaction of herpesviruses with their hosts' immune system is cytomegalovirus.

2.1 Cytomegalovirus and its Pathogenesis

Cytomegalovirus

Based on differences in their host ranges as well as in the lengths of their reproductive cycles, Herpesviridae are divided into the subfamilies Alpha-, Beta-, and Gammaherpesvirinae. Cytomegaloviruses (CMVs) belong to the slowly replicating Betaherpesvirinae and infect mainly primates and rodents. The infectious unit of CMV, the CMV virion, is composed of large double-stranded DNA genomes packed in an icosahedral nucleocapsid, which is surrounded by matrix proteins and a lipid bilayer envelope. CMV genomes span approximately 200 kbp DNA encoding for 200 open reading frames. Productive infection initiates sequential viral gene expression in three stages, starting with regulatory immediate early genes (0-2h), followed by early genes (<24h), which initiate viral DNA
synthesis, and concluded by *late genes* (>24h), which encode structural proteins that form the CMV virion.

**Pathogenesis**

Depending on the geographic area, 60% - 90% of the population is infected with human CMV (HCMV). Despite being clinically silent in immunocompetent individuals, HCMV is a leading opportunistic pathogen that causes severe morbidity and mortality among the immunocompromised including AIDS patients, transplant recipients, and congenitally or perinatally infected infants. CMV infects a broad range of organs and therefore clinical manifestation of CMV disease is diverse and comprises pneumonitis, hepatitis, enterocolitis, encephalitis, or severe mental retardation and hearing defects in congenitally infected children.

The main gateways of CMV entry are the epithelia of the gastrointestinal and the upper respiratory tract. HCMV is furthermore transmitted to infants transplacentally during pregnancy, childbirth, or through breast-feeding. Following entry, CMV spreads haematologically to all organs and encounters a large range of permissive cell types, including epithelial and endothelial cells, fibroblasts, macrophages, as well as bone marrow stromal cells. CMV infection is life-long and sequentially subdivided into an *acute phase, persistence*, and *latency*. The *acute phase* lasts several days and is characterised by vigorous lytic virus replication in all tissues. Cessation of virus replication in visceral organs marks the transition to viral *persistence*, where CMV specifically proceeds to replicate in mucosal tissues, allowing for prolonged horizontal spread via mucosal secretions. During the persistent phase, CMV is most productive and virulent in the saliva producing cells of the salivary glands (SG) – the acinar glandular epithelial cells (AGECs) [1]. The saliva functions as a vehicle of viral dissemination and paves the most efficient route to the non-infected population. Finally, CMV enters *latency*, in which viral genomes are maintained in the absence of infectious virion production. Myeloid cells and secretory glands are carriers of latent CMV genomes and clinically silent
viral reactivation occurs routinely from those tissues [2, 3], allowing for infectious shedding to new hosts. The mechanisms that allow CMV to reactivate in immunocompetent hosts are not entirely described but evidence suggests that physical trauma, inflammatory stimuli, and temporary immune suppression are involved.

2.2 Immune Control of CMV Infection
The clinical importance of HCMV has prompted the investigation of immunological mechanisms and players that are required for virus control. Murine CMV (MCMV) infection is the leading animal model to study HCMV infection and both viruses share many features including their tropism for glandular tissue and sustained shedding of infectious particles via the saliva. Both non-specific, innate as well as CMV-specific, adaptive immune effectors are required to control CMV replication and dissemination.

Innate Immune Responses
A large body of evidence indicates that Natural Killer (NK) cells and mononuclear cells are the main innate players that limit the duration and the disease severity of acute CMV infection. More recent data furthermore suggests a role for neutrophils in the termination of MCMV infection in peripheral tissues [4].

NK cells distinguish between healthy and infected cells by integrating signals that they receive via activating and inhibitory surface receptors. A cell that provides sufficient inhibitory signals to outbalance activating signals is accepted as healthy. Activating signals are induced by cell stress as it occurs in tumour transformation, cell damage, or viral infection. Inhibitory signals are mainly provided by MHC I molecules, which are expressed on every nucleated cell but are frequently downregulated in virus-infected cells. Thus, the activating signals provided by a virus-infected cell outweigh inhibitory signals, initiating NK cell-mediated killing of the infected cell by release of cytotoxic molecules. Furthermore, activated NK cells
secrete the cytokines IFNγ and TNF, which induce an antiviral state in uninfected cells. In the antiviral state, the cell’s reproductive machinery shuts down while the processing and presentation of viral antigen to virus-specific T cells is supported. The importance of NK cells in HCMV immunity is highlighted by the pronounced susceptibility of humans with NK cell deficiencies [5]. In the mouse model, strains that mount effective NK cell responses are more resistant towards MCMV and depletion of NK cells abrogates this resistance [6]. Murine NK cells primarily engage the activating receptor Ly49H that recognises the MCMV gene product m157 [7]. Ly49H is expressed in C57BL/6 mice but not in 129/J or BALB/c strains, rendering the latter two more susceptible to MCMV.

Mononuclear cells include monocytes, macrophages, and dendritic cells (DCs), all of which are targeted by CMV and serve as vehicle for viral spread via the circulation. DCs carry out two essential functions in CMV immunity: (1) They produce the type I Interferons IFNα and IFNβ, which activate NK cells and induce an antiviral state. (2) In their function as professional antigen presenting cells (APCs), DCs prime the adaptive immune response and regulate its magnitude.

Adaptive Immune Responses in Visceral Organs

In contrast to innate effectors, which sense virus infections by evolutionarily conserved, pathogen- or stress-associated patterns, adaptive effectors rely on their highly pathogen-specific surface receptors. Both, T cell and B cell responses are involved in the control of CMV.

B cells produce CMV-specific antibodies that are dispensable for the control of acute viral infection [8, 9], but have been correlated with the prevention of congenital virus transmission and the limitation of viral reactivation from latency [10, 11].

CD8+ T cells are the dominant effectors in CMV immunity and are involved in termination of productive CMV infection with subsequent establishment of latency. CD8+ T cells use their T cell receptor (TCR) to recognise viral antigen that is
presented in the context of MHC I molecules. The latter are loaded with cell endogenous antigens that are routinely generated during proteasomal degradation. Thus, MHC I molecules function as a constitutive display of a cell’s internal protein pool, which consists of self-antigens in healthy cells and additionally contains viral antigens in CMV-infected cells. If a CMV-specific CD8⁺ T cell recognises its cognate antigen on the surface of an infected cell, it engages in direct killing of the latter via cytotoxicity.

Evidence for the importance of CD8⁺ T cells in CMV immunity comes from both human and mouse studies: Prophylactic and therapeutic transfer of MCMV-specific primed CD8⁺ T cells enabled infected and immunodepleted recipient mice to control MCMV infection [12]. In humans, immunotherapeutic T cell transfer to patients with HCMV disease that occurred in bone marrow transplant recipients led to disease control [13]. As acute CMV infection resolves, memory CD8⁺ T cells are formed and patrol tissues to mount rapid recall responses upon pathogen re-encounter. While most CD8⁺ T cells die in the process of memory T cell formation, CD8⁺ T cells specific for certain viral epitopes continuously accumulate over time as a direct result of their activation from sporadic viral recurrence, giving rise to an inflationary pool of memory CD8⁺ T cells [14-16]. The phenomenon of memory inflation dominates the adaptive immune response to an extent where 20-50% of the CD8⁺ T cell repertoire in elderly HCMV-experienced individuals is occupied by CMV-specific inflationary T cells [17].

T cells of the CD4⁺ subset produce cytokines to regulate adaptive immune responses. They recognise viral antigen presented in the context of MHC II molecules, which are expressed on professional APCs, such as DCs, macrophages, and B cells and serve to display exogenous antigen that was actively internalised by the APC. In CMV infection, CD4⁺ T cells contribute to virus control by the production of pro-inflammatory cytokines such as IFNγ and TNF. In immunocompetent hosts, CD4⁺ T cells were long considered dispensable for the control of lytic virus replication in visceral organs, mainly due to the lack of tools to properly interrogate these cells for their protective capacity. Several studies, however, argue in favour
of a protective role for CD4+ T cells: A robust HCMV-specific CD4+ T cell response was correlated with long-term protection of CMV in transplant recipients [18] and a delayed CD4+ T cell response was associated with prolonged shedding of virus in urine [19]. Moreover, mice depleted of CD8+ T cells eliminated MCMV from tissues indicating that CD4+ T cells can take over antiviral activity in hosts devoid of CD8+ T cells [20, 21]. This phenomenon shows how the immune system is able to compensate for the functional lack of one of its effectors. Yet, important exceptions apply for some functions: Mice depleted of CD4+ T cells are unable to eliminate virus from the SG despite strong influx of CD8+ T cells [22].

**Adaptive Immune Responses in the Salivary Glands**

CMV spreads by persistently shedding from the SG far beyond the time point of virus control in visceral organs. How well a virus persists in its tissue of dissemination determines its evolutionary success and exceptionally high selective pressure applies in the arms race between viral immune evasion and host immune defence. These processes have led to unique immunological signatures in the SG. In contrast to visceral organs, virus replication in the SG is resistant towards CD8+ T cell-mediated control specifically in the saliva producing AGECs. Instead, CD4+ T cells achieve virus control by the production of IFNγ, which acts on non-hematopoietic cells to establish an antiviral state [23, 24]. The reason why CD8+ T cells fail to control CMV in the SG is the virus’s most extraordinary capacity to downregulate MHC I molecules specifically on the surface of infected AGECs [24, 25].

**Viral Immune Evasion**

Both HCMV and MCMV encode immune evasion proteins that enable them to reactivate and spread in the face of a fully primed immune response. These so-called *immune evasins*, primarily target CD8+ T cell responses by suppressing the MHC I antigen presentation pathway, which is redundantly achieved by the three
gene products m06, m04, and 152. m06 binds to correctly folded MHC I molecules in the endoplasmic reticulum (ER) and redirects them to lysosomal degradation [26]. m152 retains assembled MHC I complexes in the ER-Golgi intermediate compartment [27]. In contrast to m06 and m152, m04 does not lead to MHC I molecule degradation but binds to MHC I molecules in the ER to form a complex that is subsequently transported to the cell's surface where it blocks MHC I interaction with TCRs and protects from NK cell attack [28, 29].

The immune system counteracts virally mediated MHC I deprivation by two measures: (1) As described above, the absence of MHC I molecules activates NK cells as a consequence of lacking inhibitory signals and leads to the elimination of the infected cell. (2) While MHC I molecules are loaded with cell endogenous antigen, MHC II molecules present external antigen that was taken up via endocytosis. Usually, these two pathways do not cross, however, some DC subsets are capable of so-called cross-presentation. In this process, viral antigen is taken up by endocytosis from remnants of infected cells and channelled into the MHC I presentation pathway. Thus DCs circumvent the necessity of direct infection to present viral antigen to CD8+ T cells and furthermore avoid virally mediated MHC I downregulation. In the SG, however, NK cells are functionally suppressed [30] and cross-presenting DCs are absent [24, 31], ruling out these two immunological countermeasures against viral immune evasion in this organ.
3 Aim of the Thesis

In the defence against pathogens, exocrine glands are faced with special challenges that arise from their exposed anatomic location in combination with their function as a secretory organ. CMV is a highly prevalent virus that persists in the SG and abuses its secretions for viral spread during primary infection and upon viral reactivation in latency. Identifying the players that enforce viral latency and restrict reactivation builds the foundation for efforts to curtail CMV’s ubiquity. It is the aim of this thesis to contribute to this objective.

The first part of the results section is dedicated to the ontogeny and function of APCs in the SG. We show that SG-resident APCs are constituted by tissue macrophages that are unable to cross-present MCMV-derived antigen and thereby fail to activate CD8+ T cells. Conventional DCs and inflammatory monocytes are absent from the SG. Thus, the specific composition of APCs in the SG contributes to the organ’s resistance toward CD8+ T cell-mediated CMV control.

In the second part of the results section, we addressed the capacities of CD8+ and CD4+ memory T cells to protect the SG from reinfection and viral reactivation. We found that the SG induces CD4+ and CD8+ tissue-resident memory T cell populations that are excluded from the circulation. Using intraglandular virus infection, we show that CD8+ T cells afford immediate protection against localised MCMV infection, thus highlighting a yet unappreciated role of CD8+ T cells in CMV immunity in the SG.
4 Results Section I

Salivary Gland-Resident APCs are Flt3L- and CCR2-Independent Macrophage-Like Cells Incapable of Cross-Presentation

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4.1 Abstract

Cytomegaloviruses (CMVs) disseminate within the human population via mucosal excretions, e.g. from the salivary glands (SG), which represent a privileged site of viral immune evasion and persistence. The murine CMV (MCMV) model has served to identify factors that maintain a unique virus-host relationship in this organ. In contrast to all other organs, the SG is resistant to CD8$^+$ T cell-mediated control of MCMV replication due to virally induced MHC I downregulation, which is exceptionally efficient in acinar glandular epithelial cells (AGECs). Uniquely to the SG, IFN$\gamma$-producing CD4$^+$ T cells are required for virus control. While T cell responses have been extensively characterised in the SG, the ontogeny and function of APCs in this organ remain to be assessed. This study shows that macrophage-like cells constitute the population of SG-resident APCs in steady state and during MCMV-induced inflammation. Inflammatory monocytes, monocyte-derived DCs as well as conventional, Flt3L-dependent DCs do not contribute to this population. Despite supporting contact formation to CD4$^+$ and CD8$^+$ T cells in principal, SG-resident APCs fail to activate the latter due to their inability to cross-present MCMV-derived antigen.
4.2 Introduction

Cytomegaloviruses (CMVs) are large double-stranded DNA viruses of the β-herpesvirus subfamily that have closely co-evolved with their mammalian hosts to become highly efficient in establishing life-long latent infection. Human CMV is highly prevalent in the population and maintains its existence through intermittent and asymptomatic mucosal secretions from infected genital tissue, mammary glands, or salivary glands (SG) to the non-infected population. Thus, the virus's ability to resist immunity and exploit tissue-specific features to persist in those mucosal tissues is crucial to its epidemiological success. In fact, the SG supports chronic virus replication for months after virus replication is controlled in all other target organs, offering the virus an extended time frame to spread within its host population. Persistent infection of the SG is also observed in murine CMV infection (MCMV), validating it as a tool to explore the relationship between this epidemiologically relevant pathogen and its tissue of dissemination. Persistent virus specifically sequesters to vacuoles of the SG's acinar glandular epithelial cells (AGECs), from which it is shed via the saliva [22]. Unique to the SG, CD8+ T cells fail to control lytic virus replication due to exceptionally efficient virus-mediated MHC I downregulation in AGECs [25]. Instead, virus control is eventually mediated by IFNγ-producing CD4+ T cells [23, 24], which are largely dispensable in other organs. CD4+ T cells rely on antigen-presentation by a cell type capable of MHC II expression, a quality that is mainly restricted to professional antigen presenting cells (APCs) such as macrophages and DCs. Both cell types are highly phagocytic but the quality of other functions differs substantially, i.e. despite both cell types being generally capable of cross-presentation, DCs have been shown superior in performing this function [32]. Previous studies performed in our laboratory indicate that SG derived APCs lack the capacity to cross-present LCMV-derived antigen, thus contributing to the SG's resistance towards CD8+ T cell-mediated virus control [24]. Yet, the ontogenetic origin of APCs in the SG has not been addressed and the current definition of APCs in the SG is inconsistent with regards to their origin, phenotype, and dynamics. While data from our laboratory do not indicate an
increase of CD11c+ MHC II+ cells in the SG after MCMV infection [24], previous studies describe a massive recruitment of mononuclear cells to the SG but disagree on whether the major infiltrating APCs are CD8+ DCs [1] or inflammatory monocytes [33]. The present study reassesses the ontogeny, phenotype, and function of APCs at this privileged site of virus immune evasion. Our data show that APCs in the SG develop independently of fms-like tyrosine kinase 3 ligand (Flt3L) and the C-C chemokine receptor type 2 (CCR2), disqualifying them to be conventional Flt3L-dependent DCs, inflammatory monocytes, or monocyte-derived DCs. Instead, APCs in the SG share features of tissue-resident macrophages and are unable to cross-present exogenous MCMV-derived antigens. Our data provide new insights into the immunological environment of the SG that renders CD4+ T cells crucial to control persistent CMV replication.

### 4.3 Results and Discussion

**Phenotypic Characterisation of SG-Resident APCs**

To gain insight into the composition and distribution of SG-resident APCs in steady state as well as during MCMV infection, we performed flow cytometric and confocal microscopic analyses of MHC II expressing cells in the SG. Under steady state conditions, SG tissue accommodates a CD11c+ MHC II+ cell population which was not increased in total numbers at 4 weeks post MCMV infection, a time point that coincides with peak virus replication (Figure 4.1A and Figure 4.1B). The CD11c+ MHC II+ cell population was B cell, T cell and NK cell lineage negative and will be referred to as SG-resident APCs in this study. To investigate the morphology and distribution of SG-resident APCs, we used confocal microscopy to visualise CD11c+ cells in SG tissue sections of CD11c-YFP reporter mice [34] in steady state and at 4 weeks post MCMV infection. CD11c-YFP expressing cells in the SG consistently expressed MHC II (Figure 4.1C), making these mice a valid tool to investigate the morphology and distribution of SG-resident APCs in situ. YFP+ cells extend dendrites and formed a cellular network in naive and MCMV-infected SG tissue reminiscent
of a DC phenotype. Despite an overall homogenous distribution, APCs sporadically formed clusters both in steady state and the infected SG (Figure 4.1C, arrows). Interestingly, we observed morphological differences between APCs in the mucous sublingual gland and the seromucous submandibular gland with APCs in the sublingual gland appearing to form longer dendrites and being less densely spread than their counterparts in the submandibular gland (Figure 4.1D). This discrepancy might be a morphological adaptation of APCs to differences in these tissues. The distribution and morphology of SG-resident APCs is reminiscent of skin-resident, immobile Langerhans cells and constitutively migratory dermal DCs [35, 36]. Future investigations of whether SG-resident APCs are in constant transit to and from draining lymph nodes or whether they remain immobile within the organ will provide indications on the ability of SG-resident APCs to participate in the initiation of tissue immune responses. We did neither observe an increase in the population size of SG-resident APCs at 4 weeks post MCMV infection nor did we observe an influx of CD8⁺ DCs compared to the steady state, which contradicts previous studies performed in intraperitoneally infected BALB/c mice [1]. This discrepancy might reflect differences in mouse strains, inoculation route, or virus strains. Recent studies indicate that the BAC-derived strain of MCMV used in our experiments is attenuated in SG infection due to a frameshift mutation in the chemokine homologue MCK-2 [37, 38]. The above study uses the Smith strain of MCMV, which is not defective in MCK-2 and therefore is not attenuated in SG infection, thus potentially contributing to the different observations. Furthermore, T cells have not been excluded from the analysis in the aforementioned study but are known to massively infiltrate the SG upon MCMV infection [1]. T lymphocytes are able to upregulate CD11c [39], thereby potentially contributing to the observed increase of CD11c⁺ cells in the SG in this study.
Figure 4.1: Phenotypic characterisation of SG-resident APCs. (A) Gating strategy for flow cytometric analyses of whole SG tissue in steady state and 4 weeks post i.v. infection with 5x10⁶ pfu MCMV-Δm157. Doublets, dead cells, NK cells, T cells, and B cells were excluded from the analysis. (B) Cumulative data with 5 mice per group are shown as mean ± SEM and are representative of 3 independent experiments. Statistical analysis was performed by two-tailed unpaired Student’s t-test. (C, D) Flow cytometric and confocal microscopic analysis of SG tissue of a CD11c-YFP reporter mouse. Arrows indicate APC clusters. Bars: 100 μm (C) and 50 μm (D) green: CD11c, blue: DAPI. Images shown are representative of 10 SG sections of 1 mouse. p.i.: post infection.
The SG does not Contain Phenotypic Correlates of Cross-Presenting DCs

MHC I downregulation is an immune evasion mechanism used by many viruses to avoid CD8\(^+\) T cell recognition of virally infected cells. MCMV encodes the immune evasion genes *m04, m06*, and *m152*, all of which interfere with the MHC I pathway of antigen presentation \([26, 27, 40]\). The immune system is equipped with mechanisms to compensate for virally mediated antigen deprivation via MHC I downregulation, such as cross-dressing and cross-presentation. The latter is a function of CD205 and Clec9a expressing DCs residing in various lymphoid as well as non-lymphoid tissues \([41, 42]\).

Previous studies performed in our laboratory with LCMV-derived antigens indicate that SG-resident APCs are devoid of a cross-presenting function, thus contributing to the organ's resistance towards CD8\(^+\) T cell-mediated control of virus replication [24]. Here, we aim to further characterise SG-resident APCs for surface markers commonly expressed on cross-presenting DCs. In lymphoid tissues, cross-presentation is mainly a function of the CD11b\(^-\) CD8\(^+\) DC subset. Non-lymphoid tissue DCs consist of two major subsets: CD11b\(^+\) and CD103\(^+\) CD11b\(^-\), the latter being generally accepted as the subset superior in cross-presentation in peripheral organs such as the gastrointestinal tract, the lung, and the skin [43-45]. We did not detect a distinct CD103\(^+\) CD11b\(^-\) population in the SG and thus categorised SG-resident APCs into CD11b\(^+\) and CD11b\(^-\) subsets (Figure 4.2A). We have analysed CD11b\(^+\) and CD11b\(^-\) APCs in the SG of MCMV-infected mice for the expression of the endocytic C-type lectin receptors Clec9a and CD205, which are required to deliver antigen to the cross-presentation pathway \([46, 47]\). Consistent with the literature, we identified a population of cross-presenting DCs as CD11b\(^-\) and Clec9a\(^+\) CD205\(^+\) in the spleen (Figure 4.2A). Despite SG-resident APCs showing marginally elevated levels of CD103 expression compared to splenic DCs, the former do not qualify as cross-presenting migratory DCs due to the lack of CD205 and Clec9a. In addition, APCs in the SG do not qualify as Langerhans cells and plasmacytoid DCs as judged by the lack of langerin, B220, and PDCA1 expression (data not shown).
Results and Discussion

Figure 4.2: APCs in the SG are independent of Flt3L and CCR2 and express macrophage markers. Whole SG and spleen tissue of C57BL/6, Flt3L\(^{-/-}\) and CCR2\(^{-/-}\) mice in steady state and at 4 weeks post i.v. infection with 5x10\(^6\) pfu MCMV-\(\Delta m157\) was analysed via flow cytometry. Doublets, dead cells, NK cells, T cells, and B cells were excluded from the analysis. (A) Representative FACS plots and cumulative data of 5 C57BL/6 mice at 4 weeks post infection are shown. Cumulative data are shown as mean + SEM and are representative of 3 independent experiments. (B) SG and spleen tissue of C57BL/6 and Flt3L\(^{-/-}\) mice in steady state and at 4 weeks post infection. Representative FACS plots and cumulative data of 3-5 mice per group are shown. Cumulative data are shown as mean + SEM. (C) SG tissue of C57BL/6 and CCR2\(^{-/-}\) mice at 4 weeks post infection. Gray shaded histograms show CD64 expression of B cells as reference. Cumulative data of 3 mice per group are shown as mean + SEM. Statistical analysis in (A), (B), and (C) was performed by two-tailed unpaired Student’s t-test (*p<0.05, **p<0.01, ***p<0.001). p.i.: post infection.
APCs in the SG Develop Independently of Flt3L and Express Macrophage Markers

High expression levels of MHC II and CD11c are not a unique feature of the DC family but are shared by macrophages in many tissues [48, 49]. To characterise the ontogenetic nature of SG-resident APCs, we investigated the impact of Flt3L-deficiency on their presence and abundance. Flt3L is a key regulator of DC commitment and mice deficient in Flt3L show strongly reduced numbers of classical DCs and plasmacytoid DCs [50], while monocyte-derived subsets remain unaffected [51]. The cellularities of SG-resident APCs in Flt3L−/− mice did not differ from those in C57BL/6 mice as opposed to splenic CD11c+ MHC II+ DCs (Figure 4.2B). Further analyses revealed that SG-resident APCs uniformly and highly expressed F4/80 and the high affinity IgG receptor CD64, indicative of a macrophage phenotype. The dendritic shape of SG-resident APCs (Figure 4.1C and Figure 4.1D) is not in conflict with their macrophage phenotype as F4/80+ macrophages with stellate morphologies have been described in the lung, the lamina propria, and the renal medulla [52].

Macrophages fall into either of the two categories: tissue-resident macrophages, which reside in their respective tissue in steady state fulfilling homeostatic functions, and infiltrating macrophages, which originate from Ly6C+ CCR2+ “inflammatory” monocytes in the context of inflammation [53]. The presence of SG-resident APCs in steady state argues in favour of a tissue-resident macrophage population as does the absence of Ly6C expression at 4 weeks post MCMV infection (Figure 4.2C). Yet, Ly6C can be subject to downregulation in inflammatory monocytes. To exclude a contribution of inflammatory monocytes to the population of SG-resident APCs at 4 weeks post infection, we analysed SG-resident APCs in mice deficient in the chemokine receptor CCR2, which is required for inflammatory monocytes to infiltrate inflamed tissue [54]. We found that APCs in the SG were not affected by the lack of CCR2 at 4 weeks post MCMV infection (Figure 4.2C), excluding a relevant contribution of Ly6C+ CCR2+ inflammatory monocytes to the pool of SG-resident APCs, despite monocyte attracting
chemokines being expressed (data not shown). The contribution of CCR2+ monocytes to the maintenance of tissue-resident macrophages of the SG remains to be addressed in the future and is controversially discussed for other organs as well. The current dogma of tissue macrophages deriving from monocytes is supported by a large number of studies performed in the 2nd half of the 20th century [55, 56] but is currently challenged by more recent studies that indicate no significant contribution of monocytes to tissue macrophages in the steady state in lung, splenic red-pulp, and the peritoneum. Instead, tissue macrophages are proposed to repopulate through local proliferation [57].

SG-Resident APCs are Unable to Cross-Present Antigen to CD8+ T Cells

For T cells to recognise their cognate antigen on APCs, direct cell-cell interactions are required. To rule out the possibility that differential access of CD4+ and CD8+ T cells to APCs contributes to the inability of CD8+ T cells to control virus replication in infected AGECs, we investigated the localisation of CD8+ and CD4+ T cells relative to CD11c+ cells in SG tissue sections of CD11c-YFP reporter mice at 4 weeks post MCMV infection. We observed that both, CD8+ as well as CD4+ T cells had access and extensively co-localised with APCs in the SG (Figure 4.3A) with no significant differences in the amount of T-cell-DC-co-localisations between CD4+ and CD8+ T cells (Figure 4.3B). Further analyses involving live in vivo microscopic approaches will provide insight on the quality of those interactions with regards to longevity and T cell activation potential.

To functionally assess the capacity of SG-resident APCs to process and present exogenous MCMV-derived antigen in the context of MHC I and MHC II molecules, we pulsed isolated CD11c+ APCs of the SG and the spleen with virus like particles (VLPs) encoding either the MCMV-derived MHC I-restricted epitope M38 or the MHC II-restricted epitope M25. VLPs have been previously shown to rely on cross-presentation for MHC I antigen presentation in splenic DCs [58], making them a valid tool to assess the cross-presentation capacity of SG-resident APCs in
comparison to externally loaded peptide. After pulsing with VLPs or peptide, identical numbers of spleen- or SG-derived CD11c⁺ APCs were added to naïve CFSE-labelled TCR transgenic CD8⁺ T cells recognizing the M38 epitope (Maxi cells, [59]), or TCR transgenic CD4⁺ T cells specific for the M25 epitope (M25-II cells, [60]). Splenic DCs were able to present to Maxi cells when pulsed with VLP-M38 or when loaded with the M38_316-323 peptide (Figure 4.3C). On the contrary, SG derived APCs failed to process and present M38 coupled to the VLP but were not per se unable to stimulate CD8⁺ T cells, as peptide loading induced proliferation of Maxi cells. M25-II cells, however, proliferated when stimulated with both SG-derived or spleen-derived APCs pulsed with VLP-M25 (Figure 4.3C), indicating that SG-resident APCs are selectively compromised in presentation of exogenous antigen via MHC I molecules but were capable of processing and presenting exogenous antigen via MHC II. Previous studies indicate that cell-associated antigen is the main source of antigen for cross-presentation in vivo [61]. To exclude that the inability of SG-resident APCs to cross-present is a result of the context in which the antigen is provided, we assessed cross-presentation of SG-derived and splenic APCs with cell-associated Ova. To this end, we provided SG and splenic APCs with UV-irradiated, Ova-pulsed H2-Kᵇ−/− cells as a source of antigen and assessed the proliferation of CFSE-labelled OT-I T cells as a readout for antigen-presentation (Figure 4.3D). While splenic APCs could drive proliferation of OT-I T cells when apoptotic Ova-pulsed cells were provided as antigen, SG derived APCs failed to induce OT-I proliferation. Together, these data indicate that the inability of SG-resident APCs to activate CD8⁺ T cells in the SG is not due to spatial constraints but due to their inability to cross-present antigen, regardless of the molecular and cellular context the antigen is provided in. Thus, even though cross-presentation is not unique to DCs as some macrophages are capable to cross-present antigen [62], our data clearly highlight that SG-resident APCs lack this function.
Figure 4.3: APCs in the SG are unable to cross-present antigen to CD8$^+$ T cells. (A) Sections of SG tissue of a CD11c-YFP reporter mouse at 4 weeks post i.v. infection with 5x10$^6$ pfu MCMV-Δm157 were stained for CD4 and CD8. Scale bars: 20 μm. green: CD11c, orange: CD4, magenta: CD8. (B) Statistical analysis of data shown in (A) was performed by Fischer’s exact test for total cell counts of 6 microscopic sections of 1 mouse (upper panel). The lower panel shows cumulative data of T cell interactions expressed as % T cells interacting with APCs of all T cells detected per microscopic section. Two-tailed unpaired Student’s t-test was performed with data from 6 tissue sections of 1 mouse. (C) Cross-presentation assay with VLPs. CD11c$^+$ APCs were isolated from spleens and SGs at 4 weeks post MCMV-Δm157 infection and pulsed with VLPs cross-linked to either the MCMV-derived M25 or M38. 10$^5$ naive CFSE-labelled M25-specific CD4$^+$ T cells or M38-specific CD8$^+$ T cells were added to equal numbers of splenic and SG derived CD11c$^+$ APCs. After 4 days of incubation, CFSE dilution of the T cells was analysed. CFSE dilutions of T cells co-cultured with peptide pulsed APCs was set to 100% and data were normalised accordingly. Data are shown as mean + SEM and representative of 3 independent experiments. (D) Cross-presentation assay with cell-associated antigen. H-2Kb$^+$ splenocytes were pulsed with Ovalbumin and subsequently UV-irradiated. 10$^5$ naive CFSE-labelled OT-I T cells were added to equal numbers of splenic and SG derived CD11c$^+$ APCs isolated from C57BL/6 mice. After 4 days of incubation, CFSE dilution of the T cells was analysed. CFSE dilutions of T cells co-cultured with peptide pulsed APCs was set to 100% and data were normalised accordingly. Data show mean + SEM. Statistical analysis of cross-presentation assays was performed by two-tailed unpaired Student’s t-test (*p<0.05, **p<0.01, ***p<0.001).
Concluding Remarks

In conclusion, the present study identifies APCs in the SG as Flt3L-independent cells reminiscent of tissue-resident macrophages. SG-resident APCs extend dendrites, extensively interact with T cells and are not constituted and maintained by CCR2⁺ inflammatory monocytes neither in steady state nor at 4 weeks post MCMV infection, the time point at which virus titres start to decline as a consequence of efficient activation of antiviral CD4⁺ T cells. SG-resident APCs are homogenously distributed throughout the tissue allowing them to form contact zones with CD8⁺ and CD4⁺ T cells. Their inability to cross-present particulate antigens prevents CD8⁺ T cell activation in the MCMV-infected SG, as directly infected AGECs are MHC I negative due to MCMV encoded viral immune evasion genes. We cannot formally exclude the presence of Flt3L-independent DCs in the SG. Yet, given that SG-resident APCs express macrophage markers and phenotypic correlates of classical DCs were not detectable, the SG seems to lack a substantial contribution of conventional DCs in steady state as well as during ongoing lytic MCMV replication. Since DCs are recognised as uniquely competent at antigen presentation and T cell stimulation [63], their absence might contribute to the SGs susceptibility to persistent MCMV replication. Therefore, we identify the specific composition of APCs in the SG as a contributive factor to the organ’s resistance toward CD8⁺ T cell-mediated CMV control.
4.4 Materials and Methods

Mice, Viruses, and Peptides

C57BL/6 (Janvier Elevage, Le Genest Saint Isle, France), CCR2\(^{-/-}\) [64], Flt3L\(^{-/-}\) [65], C57BL/6N-Tg(TCRaM25,TCRbM25)424Biat (M25-II) [60] and C57BL/6N-Tg(TcraM38,TcrbM38)329Biat (Maxi) [59] mice were kept under specific pathogen-free (SPF) conditions and were infected at 6-8 weeks of age i.v. with 5x10\(^6\) plaque forming units (pfu) MCMV-Δm157 (Δm157 deletion mutant, described in [66]). MCMV-Δm157 was grown on C57BL/6 embryonic fibroblasts (MEFs) and titrated by standard plaque-forming assays as described in [67]. The MCMV derived M25\(^{410-425}\) and M38\(^{316-323}\) peptides were purchased from NeoMPS (Strasbourg, France). The virus-like particles VLP-M38 (M38ggc-Q\(\beta\)) and VLP-M25 (cggM25-Q\(\beta\)) were generated as described previously [68]. This study was conducted in accordance to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocol was approved by Cantonal Veterinary Office of the canton Zurich, Switzerland (Permit number 110/2011, 109/2011).

Cell Preparation and Flow Cytometry

Lymphocytes were isolated from spleen and SG as previously described [69], with the modification that cell suspensions were not subjected to a percoll gradient. Cells were surface stained with the following fluorochrome conjugated antibodies that were purchased from BD Pharmingen (Allschwil, Switzerland), from BioLegend (Lucerna Chem AG, Luzern, Switzerland), or from Miltenyi Biotec (Bergisch Gladbach, Germany): CD11c (Brilliant Violet 421), I-A/I-E (PerCP), I-A\(^b\) (Alexa Fluor 647) CD103 (FITC), CD205 (Biotin + Streptavidin-APC), CD11b (APC-Cy7), Clec9a (PE), B220, CD49b, CD3, Ly6G (PE-Cy7), CD64 (PE), Ly6C (FITC), PDCA-1 (PE). Dead cells were excluded with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Basel, Switzerland). Multiparameter flow cytometric analysis was performed using a FACS LSRII flow cytometer (BD, Allschwil, Switzerland) with FACS DIVA software...
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(BD, Allschwil, Switzerland). Data were analysed using FlowJo software (Treestar, San Carlos, CA).

Immunofluorescence Microscopy

SG were isolated from infected animals, fixed for 1 h in PBS containing 4% PFA at 4°C and incubated overnight in PBS containing 20% sucrose, followed by tissue embedding in O.C.T. compound (Sakura, Torrance, CA), snap-freezing in liquid N2 and storing at −80°C. Cryosections of 10 µm thickness were prepared, completely air-dried for 2 hours before recovery by a brief rinse in PBS. Sections were permeabilised with 0.1% Triton-X100 (Sigma-Aldrich, Buchs, Switzerland) in PBS for 7 minutes, rinsed with PBS and subsequently blocked in PBS with 10% natural goat serum (NGS, Reactolab SA, Servion, Switzerland) for 1 hour. Primary antibodies against CD4 (clone RM4-5) and CD8 (clone 53-6.7) were purchased from BD Pharmingen (Allschwil, Switzerland) and incubated overnight at 4°C in PBS + 10% NGS in a final dilution of 1:50. Slides were washed with PBS and then incubated with secondary antibodies (anti-rat Cy3, anti-rat Cy5, purchased from Sigma Aldrich, Buchs, Switzerland), diluted 1:200, for 1 hour. Sections were mounted with VectaShield (Vector Laboratories, Burlingame, CA) containing DAPI (Sigma-Aldrich, Buchs, Switzerland) in 1:1000 dilution. Samples were viewed and analysed with an inverted confocal microscope (Axiovert 200, Carl Zeiss, Inc., Zurich, Switzerland), equipped with an oil-phase contrast objective (Plan Neofluar, Carl Zeiss, Inc., Zurich, Switzerland), an CSU-X1 spinning-disk confocal unit (Yokogawa) and a solid state laser unit with 4 laser lines (405, 488, 561, 647, Toptica). Data analysis was done with Volocity (Improvision). Images were subject to contrast enhancement.

In vitro Cross-Presentation Assay with VLP-Associated Antigens

4 weeks post infection with 5x10^6 pfu MCMV-Δm157, 2 spleens and 5 SGs were digested using Liberase TL Research Grade (Roche, Rotkreuz, Switzerland) and DNase I according to manufacturer's instruction, except that SG tissue was
dissociated previous to digestion and in between two incubation periods of 20 minutes. DCs from the spleen and APCs from the SG were purified with CD11c MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s instructions, pulsed with either 1 µg/ml VLPs or $10^{-8}$ M peptide for 3 hours at 37°C. After intensive washing, $10^5$ splenic DCs and SG APCs were added to $10^5$ TCR transgenic CD8+ or CD4+ T cells previously labelled with CFSE (Invitrogen, Basel, Switzerland; final concentration 0.5 µM) and incubated for 4 days. CD8+ and CD4+ T cells were MACS-purified from spleens of Ly5.1+ Maxi or Ly5.1+ M25-II TCR transgenic mice, respectively. CFSE dilution of the TCR transgenic T cells was analysed by flow cytometry and used as a measure for antigen-presentation by APCs.

**In vitro Cross-Presentation Assay with Ova-Loaded H-2Kb+ Cells**

Single cell suspensions were prepared in serum-free medium from a spleen of a H-2Kb+ mouse. Cells were loaded with Ova (Sigma-Aldrich, Buchs, Switzerland) by osmotic shock as described previously [70] with the modification that cells were not γ-irradiated but UV-irradiated. Control cells were also subjected to osmotic shock and UV-irradiation but Ova was omitted. $5 \times 10^5$ Ova-loaded or control H-2Kb+ splenocytes were incubated with $10^5$ MACS-sorted APCs from SG or spleen and $10^5$ CFSE-labelled OT-1 T cells. Antigen-presentation was judged from CFSE dilution of OT-1 T cells.
5 Results Section II

The Salivary Gland Acts as a Sink for Tissue-Resident Memory CD8$^+$ T Cells which Protect Against Localised Cytomegalovirus Infections

Jenny T Thom, Thomas C Weber, Senta M Walton, Nicole Torti and Annette Oxenius

5.1 Abstract

Tissue-resident memory T cells (T$\text{RM}$) reside at barrier tissues and are appreciated as critical guards of peripheral immunity. Here, we show that systemic infection with murine cytomegalovirus (MCMV) very effectively induces CD8$^+$ and CD4$^+$ T$\text{RM}$ cells in the salivary gland (SG). T$\text{RM}$ generation depended on cognate antigen for CD4$^+$ but not CD8$^+$ T$\text{RM}$ cells, revealing important differences in T cell subset-specific demands for T$\text{RM}$ development. The SG completely relies on CD4$^+$ T cells for the control of primary MCMV infection as MCMV-mediated MHC I downregulation in glandular epithelial cells renders CD8$^+$ T cells inoperative. Using intraglandular viral challenge, we reveal that CD8$^+$ T$\text{RM}$ cells confer immediate protection against localised MCMV infection despite viral immune evasion genes, owing to early viral tropism to cells that resist complete MCMV-mediated MHC I downregulation. Thus, we unravel a yet unappreciated role for memory CD8$^+$ T cells in protecting mucosal tissues against CMV infection.
5.2 Introduction
Following infection, antigen-specific T cells are activated in secondary lymphoid organs that drain the infected tissue. Activated T cells expand to large pools of effector cells which can migrate to peripheral tissues to contribute to pathogen clearance. As the infection resolves, the vast majority of effector cells undergoes apoptosis and only a small, heterogeneous population of long-lived memory T cells remains to patrol tissues and mount rapid recall responses upon pathogen re-encounter [71]. Memory T cells are classically divided into two subsets [72]: Central memory T cells (T\textsubscript{CM}) express CD62L and CCR7, localise mainly to lymphoid organs, and are capable of rapid expansion upon antigen re-encounter, and effector memory T cells (T\textsubscript{EM}) which lack CD62L and CCR7 but express homing molecules enabling them to enter inflamed peripheral sites and exert immediate effector functions. While the protective capacity of T\textsubscript{EM} cells towards systemic infection is undisputed, their efficiency to contain localised, peripheral infections was found to be limited in a variety of models [73-75], owing to numerical contraction over time as well as the progressive loss of homing molecules required to access peripheral tissues.

Early studies have established the concept of circulatory immune surveillance where memory T cells are in constant exchange with either lymphoid or non-lymphoid tissues via the circulation. A growing body of recent evidence provides an important addition to this paradigm demonstrating that a proportion of peripheral memory T cells resides at the site of previous pathogen encounter and is disconnected from the circulation [76, 77]. These tissue-resident memory T cells (T\textsubscript{RM}) are readily positioned at barrier tissues prone to pathogenic invasion or reactivation and are superior to circulating T\textsubscript{EM} cells in protecting against local secondary infections. CD8\textsuperscript{+} and CD4\textsuperscript{+} T\textsubscript{RM} cells express specific signature phenotypes: in the lung CD4\textsuperscript{+} T\textsubscript{RM} cells co-express CD11a and CD69 [78], while CD8\textsuperscript{+} T\textsubscript{RM} cells co-express CD103 and CD69 and have been found in many tissues such as the skin, the brain, sensory ganglia, the intestinal mucosa, and secretory glands [77, 79-83].
Secretory glands pose an attractive target tissue for viruses to persist and exploit mucosal secretions as vehicles for dissemination. Human cytomegalovirus (HCMV) is prevalent in 60-90% of the world population and fosters its transmission by prolonged shedding from infected mucosae such as the salivary glands (SGs), which support chronic viral replication for months after virus is controlled in all other organs. Experimental infection of mice with murine CMV (MCMV) has revealed that virally mediated MHC I downregulation renders the SG uniquely resistant to CD8$^+$ T cell-mediated virus control [25]. Instead, CD4$^+$ T cells are required to cease virus replication during primary CMV infection [23, 24]. These findings suggest that CD8$^+$ T cells may also be incapable of curtailing viral reactivation or superinfection events during latency. Yet, the capacities of CD8$^+$ and CD4$^+$ T cells to protect the SG from reinfection and viral reactivation have not been addressed.

Here we sought to address these questions by scrutinising MCMV-specific CD8$^+$ and CD4$^+$ memory T cells in the SG for their migratory potential, their maintenance, and their protective capacity upon localised pathogen encounter. We demonstrate an exquisite ability of the SG to induce CD4$^+$ and CD8$^+$ T$_{RM}$ populations that are excluded from the circulation. While CD8$^+$ T$_{RM}$ induction was completely independent of cognate antigen, CD4$^+$ T$_{RM}$ generation was strikingly dependent on the presence of local antigen. CD103 expression in CD8$^+$ T cells depended on TGFβ, supported tissue retention, and coincided with localisation of CD8$^+$ T cells to epithelial structures of glandular ducts, while CD103$^-$ CD8$^+$ T cells and CD4$^+$ T cells preferentially localised outside epithelial duct structures. Functionally, using intraglandular infection, we demonstrate that MCMV-specific CD8$^+$ T$_{RM}$ cells confer local protective immunity, owing to initial virus replication in non-epithelial cells that withstand complete MCMV-mediated MHC I downregulation. Therefore, our findings establish a yet unappreciated role for MCMV-specific CD8$^+$ T cells in the control of localised virus replication in the SG, thus likely contributing to the containment of CMV transmission episodes.
5.3 Results

**CD8**\(^+\) and CD4\(^+\) T\(_{RM}\) Cells Develop in the SG upon MCMV Infection

T\(_{RM}\) cells in barrier tissues contain local reinfection in a number of infection models. The SG is directly connected to the oral cavity, thus qualifying as a barrier tissue prone to pathogenic invasion and transmission, prompting us to examine the SG’s potential to form and maintain T\(_{RM}\) cells specific for CMV, a virus that persists in and spreads from this organ [22, 84].

To analyse the formation of T\(_{RM}\) cells in the SG, we employed an adoptive transfer model using monoclonal Ly5.1\(^+\) TCR transgenic CD8\(^+\) and CD4\(^+\) T cells which are specific for the immunodominant MCMV epitopes M38\(_{316-323}\) (Maxi CD8\(^+\) T cells [59]), and M25\(_{411-425}\) (M25 CD4\(^+\) T cells [85]). Following systemic MCMV infection, Maxi cells progressively upregulated CD69 and CD103, the signature markers for CD8\(^+\) T\(_{RM}\) cells, exclusively in the SG (Figure 5.1A and Figure 5.1C). The majority of M25 cells in the SG as well as a minor fraction in the lung and spleen co-expressed CD11a and CD69 (Figure 5.1B and Figure 5.1D), the signature phenotype of non-recirculating CD4\(^+\) T\(_{RM}\) cells in the influenza virus-infected lung [78]. Comparable patterns of CD103, CD69, and CD11a expression were also observed in endogenous, non-transgenic CD8\(^+\) and CD4\(^+\) T cells (data not shown).

A key feature of T\(_{RM}\) cells is their disconnection from recirculation, clearly demarcating T\(_{RM}\) cells from their circulating effector or central memory counterparts [86]. To confirm that T cells with a T\(_{RM}\) phenotype in the SG exhibit limited accessibility to the circulation, we performed intravascular labelling of CD8\(^+\) and CD4\(^+\) T cells using i.v. injection of fluorochrome-coupled antibodies [78, 87]. While more than 97% of Maxi cells were stained intravascularly (iv\(^+\)) in the lung and spleen, more than 80% of Maxi cells in the SG were spared from in vivo administered antibodies (iv\(^-\)) (Figure 5.1E, upper panel). The majority of SG isolated iv\(^-\) Maxi cells expressed CD103 (Figure 5.1E, lower panel). Similar patterns were observed for MCMV-specific CD4\(^+\) T cells, with the majority of M25 cells being spared from intravascular labelling in the SG (Figure 5.1F, upper panel) coinciding
5.3 Results

with pronounced expression of the signature CD4^T_{RM} phenotype (Figure 5.1F, lower panel). These results were not due to insufficient antibody transfer to the SG as intravenous labelling of CD31^+ endothelial cells was equally effective in the SG and the lung (Figure 5.2).

Taken together, these data indicate that MCMV infection results in substantial formation of MCMV-specific SG-resident CD8^+ and CD4^+ T cell populations which express T_{RM} signatures and show restricted vascular accessibility.

Figure 5.1: CD8^+ and CD4^+ T_{RM} cells develop in the SG upon MCMV infection. Naive Maxi CD8^+ T cells and M25 CD4^+ T cells were adoptively transferred into naive recipients followed by i.v. infection with 5x10^6 pfu MCMV. (A,B) Representative flow cytometry contour plots of Maxi (A) and M25 (B) T_{RM} formation in the SG, lung, and spleen at 8 wpi are shown. The percentage of Maxi (C) and M25 (D) cells with a T_{RM} phenotype at 4, 8, and >20 wpi is shown. Data are shown as mean ± SEM of at least n=10 mice per time point pooled from at least 3 independent experiments. *p<0.05 (unpaired two-tailed t-test). (E,F) Mice were injected i.v. with anti-CD8-PE or anti-CD4-PerCP 3 minutes before sacrifice. Representative contour plots of intravascular labelling and T_{RM} marker expression of Maxi (E) and M25 (F) cells are shown.
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Figure 5.2: Intravascular staining of the SG is not compromised by limited SG vascularisation. (A,B) Intravascular staining of CD31+ endothelial cells with 5 µg anti-CD31-APC 3 minutes before sacrifice. (A) Contour plots of ex vivo stained CD31+ endothelial cells in SG and lung. (B) Histogram of CD31 staining intensity after i.v. staining comparing CD31+ cells from the lung and the SG. Results from 1 experiment with 1 mouse are shown.

Local Antigen is Required for CD4+ but not CD8+ T_{RM} Formation and Maintenance

The presence of both CD8+ and CD4+ T_{RM} cells within the same organ allowed us to directly compare the requirements for CD8+ and CD4+ T cell recruitment, retention, and T_{RM} phenotype induction. Previous studies indicate that the tissue of residence dictates whether local antigen is required for CD8+ T_{RM} formation (reviewed in [88]). We directly compared the impact of absence or presence of cognate antigen on T_{RM} formation in the SG by employing three experimental setups with varying local and systemic, i.e. splenic, antigen availability (Figure 5.3A). To create a situation providing cognate antigen in the spleen as well as locally in the SG, we adoptively transferred MCMV-specific TCR transgenic (TCRtg) M25 and Maxi T cells and subsequently infected mice with MCMV (Figure 5.3A, panel 1). To provide antigen in the spleen but not in the SG, LCMV-specific TCRtg Smarta CD4+ T cells and P14 CD8+ T cells were transferred with subsequent low dose LCMV-WE infection (Figure 5.3A, panel 2). To evaluate T_{RM} induction in absence of systemic and local SG antigen, we adoptively transferred purified, previously in vivo MCMV activated Maxi and M25 cells into naïve recipients (Figure 5.3A, panel 3). The ability of CD8+ and CD4+ TCRtg T cells to migrate to the SG and to establish a T_{RM} phenotype was analyzed at 4 and 8 weeks post transfer. CD4+ TCRtg T cells were detectable in the SG at 4 weeks post transfer regardless of whether systemic or local antigen was present (Figure 5.3B). At 8 weeks post transfer, CD4+ TCRtg T cells were strikingly reduced and the magnitude of reduction correlated positively with increased
deprivation of antigen, culminating in a complete loss of M25 cells in some mice when antigen was entirely absent (Figure 5.3B). In contrast, CD8+ TCRtg T cells were unaffected by antigen deprivation and cellularities were maintained even in the absence of local or systemic antigen (Figure 5.3C).

Figure 5.3: Local cognate antigen is required for CD4+ but not CD8+ T\textsubscript{RM} formation and maintenance. (A) Experimental setup for the generation of 3 different settings of antigen availability. M25 and Maxi cells were adoptively co-transferred into C57BL/6 recipients with subsequent MCMV infection (5x10^6 pfu) to provide antigen in the SG and spleen (1). Smarta and P14 cells were adoptively co-transferred into C57BL/6 mice with subsequent LCMV-WE infection (200 ffu) to provide antigen in the spleen but not in the SG (2). M25 and Maxi cells were adoptively co-transferred prior to MCMV infection. At 1 wpi, CD4+ and CD8+ splenocytes were isolated by MACS and transferred into naive recipients with no cognate antigen in the SG and spleen (3). Total numbers of CD4+ (B) and CD8+ (C) TCR transgenic (TCRtg) cells per organ are shown. CD4+ and CD8+ T\textsubscript{RM} formation at 4 weeks post cell transfer is shown in (D). Data are shown as mean ± SEM of at least n=10 mice pooled from 2 independent experiments. (E) Representative contour plots (left) and statistical analysis (right) of gp33-tet CD8+ T cells 8 wpi with 200 ffu LCMV-WE or 10^6 ffu LCMV Docile are shown as mean ± SEM of n=5 mice from 1 experiment. (B, C, D, E) ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (unpaired two-tailed t-test).
We concluded that while both CD8+ and CD4+ T cells are able to enter the SG independently of antigen, they have differential requirements to be retained within the SG over time.

Consistent with the differential antigen requirement in maintaining T cell numbers, restriction of systemic and local antigen availability negatively affected the extent of T_{RM} formation in CD4+ but not in CD8+ TCRtg T cells at 4 weeks post transfer (Figure 5.3D). Previously published data suggest that chronic antigen exposure prevents CD8+ T_{RM} differentiation in the gut [89]. To confirm this notion in the SG, we compared the phenotypes of LCMV-specific P14 T cells in the SG during chronic or acute LCMV infection. Indeed, CD8+ T cells were significantly impaired in CD103 expression during chronic compared to resolved LCMV infection (Figure 5.3E), indicating a negative impact of persistent antigen on CD8+ T_{RM} formation in the SG. Taken together, these data show that while both CD8+ and CD4+ T cells can populate the SG independently of cognate antigen, CD4+ T cells require local antigen for tissue maintenance and T_{RM} formation, whereas CD8+ T cells are maintained independently of local antigen.

**TGFβ is Required for Induction of CD103 but not CD69 Expression**

With cognate antigen not being required for CD8+ T_{RM} induction in the SG, we investigated the role of cytokines in this process. TGFβ has been shown to induce CD103 expression in CD8+ T cells in various experimental systems [89-91]. We therefore analyzed the endogenous MCMV M38_316-323-specific CD8+ T cell response in mice exhibiting a T cell specific deletion of the TGFβRII (Tgfbr2^{f/f} dLck-Cre mice, [92]). At 4 wpi, M38-specific CD8+ T cells in the SG were severely compromised in CD103 and CD69 co-expression in contrast to Cre- littermates (Figure 5.4A and Figure 5.4B), despite comparable virus titres (Figure 5.4C).
Figure 5.4: TGFβ is required for induction of CD103 but not CD69 expression. Tgfbr2\textsuperscript{-/-}dlck-Cre\textsuperscript{+} mice and Tgfbr2\textsuperscript{-/-}dlck-Cre\textsuperscript{-/-} littermates were infected i.v. with 5x10\textsuperscript{6} pfu MCMV and M38-specific cells were tracked by tetramer staining at 4 wpi. (A) Representative contour plots of M38-tet\textsuperscript{+} cells are shown. (B) Percentages of M38-tet\textsuperscript{+} cells expressing CD103 and CD69 are shown as mean + SEM of n=10 mice pooled from 2 independent experiments. (C) Viral titres in the SG at 4 wpi are shown as mean + SEM. (D) Total numbers of M38-specific CD8\textsuperscript{+} T cells are shown as mean + SEM of n=10 mice pooled from 2 independent experiments. (B – D) ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (unpaired two-tailed t-test). (E) Representative contour plots of total CD4\textsuperscript{+} T cells are shown at 4 wpi. (F) Percentages of CD4\textsuperscript{+} T cells expressing CD11a and CD69 at 4 wpi are shown as mean + SEM of n=10 mice pooled from 2 independent experiments.
While CD103 expression on CD8+ T cells was completely dependent on TGFβ, CD69 expression was not affected by defective TGFβ signalling. Tgfbr2<sup>fl/fl</sup> dLck-Cre mice showed a significant reduction of the M38-specific CD8+ T cell pool, which was most prominent in the SG, reaching a 5.7x reduction compared to Cre<sup>+</sup> controls at 4 wpi (Figure 5.4D). This reduction was not due to the absence of the CD103<sup>+</sup> fraction, as the CD69<sup>+</sup> and CD69<sup>-</sup>CD103<sup>-</sup> M38-specific CD8+ T cell populations were also strikingly reduced. Hence, besides being essential for CD103 induction in CD8+ T cells in the SG, TGFβ also promotes the overall size of the SG-resident CD8+ T cell population by additional mechanisms unrelated to CD103 induction. Consistent with the CD8+ T cells, CD4+ T cells were not restricted in their ability to express CD69 in the SG of Tgfbr2<sup>fl/fl</sup> dLck-Cre mice (Figure 5.4E), neither were they affected to express high levels of CD11a and the size of the endogenous CD4+ T<sub>RM</sub> population was unaffected by defective TGFβRII signalling (Figure 5.4F). These results were confirmed using mice expressing a T cell specific dominant-negative variant of the TGFβRII, in which T cells are substantially, yet not entirely impaired in TGFβ signalling [93], leading to profound but incomplete reduction of CD103 expression in M38-specific CD8+ T cells in the SG (Figure 5.5).

![Figure 5.5: dnTGFβRII mice are compromised in CD8<sup>+</sup> T<sub>RM</sub> formation in the SG. (A) Representative contour plots of T<sub>RM</sub> formation in M38-tet<sup>+</sup> cells in the SG at 4 and 8 wpi in dnTGFβRII and C57BL/6 mice. (B) Statistical analysis of CD103<sup>+</sup> CD69<sup>+</sup> expression within M38-tet<sup>+</sup> cells at 8 wpi shown as mean + SEM of at least n=3 mice from 1 experiment. **p<0.01; (unpaired two-tailed t-test).](image-url)
IL-15 was previously identified as an inducer of CD103 in CD8+ T<sub>RM</sub> cells in the skin [94]. By adoptively transferring <i>in vivo</i> activated Maxi cells into naïve IL-15<sup>−/−</sup> recipients, we could demonstrate that despite a minor reduction in the MFI of CD103 staining, CD103<sup>+</sup> CD69<sup>+</sup> co-expression was not compromised in the SG in absence of IL-15 (Figure 5.6A and Figure 5.6B). Apart from TCR stimulation, IFNα induces CD69 expression in T cells [95]. To investigate whether IFNα was required to induce and sustain CD69 expression in CD8<sup>+</sup> T<sub>RM</sub> cells in the SG, we adoptively co-transferred wild type (WT) P14 cells and P14 cells that lack the IFNα receptor (IFNAR<sup>−/−</sup> P14) followed by infection with recombinant Vaccinia virus expressing the glycoprotein of LCMV (VV-G2). We found that both, IFNAR<sup>−/−</sup> P14 as well as WT P14 were not compromised in CD8<sup>+</sup> T<sub>RM</sub> formation (Figure 5.6C), suggesting that IFNα is not required for CD69 expression in CD8<sup>+</sup> T cells in the SG.

**Figure 5.6: CD8<sup>+</sup> T<sub>RM</sub> formation is independent of IL-15 and IFNα. (A+B) WT Maxi cells were adoptively transferred into donor mice followed by MCMV infection (5x10<sup>6</sup> pfu), isolated from the spleens of donor mice at 1 wpi and transferred into IL-15<sup>−/−</sup> recipients or C57BL/6 recipients, respectively. (A) Representative contour plots of Maxi T<sub>RM</sub> formation in the SG at 4 weeks post transfer are shown. (B) Statistical analysis of T<sub>RM</sub> formation in Maxi cells in the SG is shown as mean ± SEM of n=5 mice from 1 experiment. ns = not significant (unpaired two-tailed t-test). (C) WT P14 (Ly5.1) and IFNAR<sup>−/−</sup> P14 (Thy1.1) T cells were co-transferred into C57BL/6 recipients one day prior to infection with 5x10<sup>6</sup> pfu VV-G2. Shown are representative contour plots of T<sub>RM</sub> formation of P14 cells in the SG at 4 wpi. Results are representative of 5 mice from 1 experiment.
Taken together, these data indicate that CD8+ T cells in the SG depend on TGFβ for CD103 expression, that TGFβ additionally exerts a CD103 independent role in the establishment of a MCMV-specific CD8+ T cell population in the SG and that IL-15 does not promote CD103 upregulation in the SG.

**CD103 Contributes to Retention of CD8+ T Cells in the SG**

Next, we investigated whether CD103 expression promotes retention of SG-resident CD8+ T_{RM} cells compared to their CD103- counterparts, as CD103 has been implicated to support T cell retention in epithelial tissues by tethering CD8+ T cells to epithelial cells via interaction with E-Cadherin [96]. We adoptively transferred Maxi cells which lack CD103 (CD103−/− Maxi) into C57BL/6 recipients and compared their abundance with WT Maxi in the SG following MCMV infection (Figure 5.7A). CD103−/− Maxi cells were equally well established and maintained in the SG and spleen as WT Maxi throughout the course of infection (Figure 5.7B). We previously showed that MCMV-derived antigen presentation in lymph nodes reactivates local CD8+ T_{CM} cells, which subsequently migrate to peripheral tissues to maintain a constant effector CD8+ T cell pool [59]. To exclude that continuous recruitment of new effector CD8+ T cells masks a dependence of CD103 for SG retention, we restricted reseeding of the SG from lymph nodes by abrogating lymph node egress of T cells via FTY720 treatment starting at 1 wpi (Figure 5.7C). Despite their ability to expand to equal levels in the blood at 1 wpi and to maintain their population in the spleen at 4 wpi, CD103−/− Maxi in the SG were markedly reduced compared to WT Maxi at 4 wpi (Figure 5.7D). FTY720 treatment also resulted in a reduction of CD103−/− Maxi cells in the spleen by 8 wpi, indicative of a global deficit of CD103−/− T cells at later time points. With the reduction of CD103−/− Maxi cells emerging earlier and being more profound in the SG compared to the spleen, we conclude that CD103 expression significantly contributes to maintenance of CD8+ T cells in the SG. The loss of CD103−/− CD8+ T cells in the SG could either be due to a survival deficit or due to selective egress of CD103−/− CD8+ T cells from the SG. To investigate if the
latter was the case, we adoptively transferred and tracked MCMV-specific CD8+ T cells that lack CCR7, a receptor required for egress from peripheral tissues [97, 98] as well as for T cell entry into lymph nodes [99]. Being incapable of tissue emigration, CCR7-/ CD8+ T cells are trapped in the respective organ. Furthermore, CCR7-/ CD8+ T cells are incapable of entering lymph nodes, thus their seeding to the periphery relies solely on the spleen.

Figure 5.7: CD103 contributes to retention of CD8+ T cells in the SG. (A) Experimental setup. CD103-/ Maxi and WT Maxi cells were transferred separately into C57BL/6 hosts with subsequent MCMV infection. (B) Total numbers of CD103-/ and WT Maxi cells are shown as mean + SEM of n=10 mice pooled from 2 independent experiments. (C) Experimental setup. CD103-/ Maxi and WT Maxi cells were separately transferred and recipients were infected as in (A). Mice were treated with 5 µg/ml FTY720 in drinking water starting 1 wpi. (D) Percentages of CD103-/ and WT Maxi in blood and total cell numbers in SG and spleen are shown as mean + SEM of n=10 mice pooled from 2 independent experiments. (E) Representative contour plots of CCR7-/- Mini and WT Mini cells in SGs of C57BL/6 recipients at 4 and 8 wpi are shown. (F) Statistical analyses of T RM markers at 4 and 8 wpi in CCR7-/- Mini and WT Mini cells are shown as mean + SEM of n=5 mice from 1 experiment. (G) MFI of Bcl-2 expression in CD103+ and CD103- Maxi cells in the SG at 8 wpi is shown as mean + SEM of n=5 mice representative of 3 independent experiments. (B, D, F, G) ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (unpaired two-tailed t-test).
CCR7−/− CD8+ T cells in the SG were strongly skewed towards a T\textsubscript{RM} phenotype, with CD103− cells being essentially absent by 8 wpi (Figure 5.7E and Figure 5.7F), indicating that the loss of CD103− cells is not a result of selective tissue emigration but rather of a survival deficit. In line with this hypothesis, CD103+ Maxi cells expressed significantly elevated levels of the anti-apoptotic molecule Bcl-2 compared to CD103− Maxi cells (Figure 5.7G).

**Differential Localisation of CD103+ CD8+ T Cells**

With CD103 acting as a molecular tether to E-Cadherin expressing epithelial cells [96], we reasoned that CD8+ T\textsubscript{RM} cells would be selectively sequestered to epithelial structures in the SG. To test this hypothesis, we analyzed sections of infected SG tissue by fluorescence microscopy. T cells located within the confined borders of EpCAM-labelled SG ducts and acini were classified as intraepithelial (Figure 5.8A). The percentage of CD103+ cells localising within epithelial ducts was significantly higher compared to CD103− cells (Figure 5.8B). The majority of CD103− CD8+ T cells and CD4+ T cells was localised extraepithelially, indicating a selective preference of CD103+ CD8+ T cells for intraepithelial localisation.

**Figure 5.8: Differential localisation of CD103+ CD8+ T cells compared to CD103− CD8+ and CD4+ T cells.**

(A) Sections of SG tissue at 4 wpi were stained for CD103 (orange), CD8/CD4 (green), EpCAM (red) and DAPI (blue). Scale bars: 20 µm. (B) Statistical analysis of CD4+ and CD8+ T cell localisation in relation to EpCAM+ ducts. Data are shown as mean ± SEM of n=4 mice from 3 independent experiments. In total, the location of 638 CD8+ T cells and 1'069 CD4+ T cells was determined. **p<0.01; ***p<0.001 (unpaired two-tailed t-test).
**CD8⁺ T Cells have Protective Capacity upon Local Infection**

During primary MCMV infection, the SG is uniquely resistant towards CD8⁺ T cell-mediated control of lytic replication and CD4⁺ T cells are required for viral control [22, 24]. Whether CD4⁺ T cells are also uniquely responsible to control viral reactivation or reinfection events is not known. To investigate the capacity of SG-resident CD8⁺ and CD4⁺ T cells to control localised reinfection, we employed a model of locally confined SG infection by introducing virus via focused intraglandular (i.g.) injection [100]. Infection of one of the two submandibular SG lobes with 10⁴ pfu MCMV remained confined to the injected lobe during the first 11 days post injection (Figure 5.9A), qualifying this method to study the control of localised infection. We selectively lodged in vivo activated Maxi or M25 cells in the SGs of naïve recipients for 5 weeks and determined if the presence of Maxi or M25 cells affected virus titres at 4 days post i.g. infection (Figure 5.9B). Surprisingly, more than 70% of mice harbouring T_{RM} Maxi cells could completely control i.g. administered virus, as opposed to mice that did not receive T cells via adoptive transfer or had received M25 cells (Figure 5.9C). These data suggest that despite their restricted potency to control virus after primary systemic infection, CD8⁺ T_{RM} cells in the SG can protect against local viral challenge.
Figure 5.9: CD8⁺ T cells have protective capacity upon local infection. (A) Virus kinetics after i.g. infection of one SG lobe with 10⁴ pfu MCMV (input). Data are shown as mean ± SEM of n=4 mice for each time point. LOD = limit of detection. (B) Experimental design to assess the protective capacity of Maxi and M25 cells. Maxi and M25 cells were adoptively transferred into C57BL/6 recipients followed by MCMV infection. At 1 wpi, CD8⁺ and CD4⁺ T cells were isolated from spleens by MACS purification and injected into naïve recipients. After 5 weeks, mice were i.g. challenged with 10⁴ pfu MCMV to both SG lobes and virus titres were determined 4 days later. (C) Percentage of controllers and non-controllers at 4 days after i.g. MCMV infection. Results are pooled data from at least 10 mice per group from 3 independent experiments. ns, not significant; *p<0.05 (Fisher’s exact test).

Cells Targeted by Local Infection Resist Complete Viral MHC I Downregulation

CMV-mediated MHC I downregulation in the SG substantially contributes to the inability of CD8⁺ T cells to control virus replication [24, 25]. To understand why T_RIM Maxi cells were able to control local infection, we characterised the cell types targeted early by i.g. injection. We visualised infected cells using an MCMV strain that recombinantly expresses GFP (MCMV-GFP). Cells infected by i.g. injection extended dendrites and were arranged in a network (Figure 5.10A and Figure 5.10B), reminiscent of the previously described macrophage network in the SG [31]. The morphology of the infected cells differed substantially from that of infected acinar glandular epithelial cells, the cell type of viral persistence following primary systemic infection (Figure 5.10C). Interestingly, infected acinar glandular epithelial cells were detectable at 2 weeks post i.g. challenge (Figure 5.10D), suggesting that the virus initially replicates in cells of non-epithelial origin before occupying its niche of persistence at later time points of infection.
5.3 Results

Figure 5.10: Cells targeted by local infection are refractory to complete CMV-mediated MHC I downregulation. (A, B) Sections of SG tissue at 4 days post i.g. infection with 5x10⁵ pfu MCMV-GFP representative of 3 mice. Scale bar: 50 µm (A) and 20 µm (B). (C) Section of SG tissue at 3 weeks post i.v. infection with 5x10⁶ pfu MCMV-GFP representative of 3 mice. Scale bar: 50 µm. (D) Section of SG tissue at 2 weeks post i.g. infection with 5x10⁵ pfu MCMV-GFP representative of 3 mice. Scale bar: 20 µm. (E-H) Flow cytometry analysis of SG isolated cells targeted by MCMV-GFP at 4 days post i.g. infection. Infected cells were distinguished from non-infected cells by GFP expression. Results are representative of 3 independent experiments. (E) Representative contour plot of GFP⁺ cells showing CD45 and CD11c expression. (F) Statistical analysis of cell types infected after i.g. MCMV-GFP infection. Shown are means ± SEM of n=3 mice from 1 experiment. (G) Representative histogram and contour plot of EpCAM, MHC I, and MHC II expression of non-hematopoietic (CD45⁻) GFP⁺ cells. The grey histogram shows the EpCAM FMO control staining. (H) Representative contour plots of MHC I and MHC II expression of hematopoietic (CD45⁺) GFP⁺ cells.

At 4 days post i.g. infection both hematopoietic and non-hematopoietic cells were infected and a fraction of the hematopoietic targets expressed CD11c, indicative of SG-resident APCs (Figure 5.10E and Figure 5.10F). Infected non-hematopoietic cells did not contain substantial contributions of EpCAM⁺ cells, confirming the notion that epithelial cells are not the prime target of locally introduced MCMV (Figure 5.10G). While MHC II expression was confined to a fraction of CD11c⁺ hematopoietic targets (Figure 5.10H), MHC I expression was abundant on the majority of infected cells, indicating that cells targeted early by i.g. infection largely resist virally mediated MHC I downregulation - at least up to 4 days post infection. We previously showed that complete viral MHC I downregulation in infected glandular epithelial cells renders CD8⁺ T cells unable to control lytic virus replication.
in these cells [24]. With the primary targets being of non-epithelial ontogeny, early local reinfection provides a window of opportunity for CD8+ T_{RM} cells to recognise infected cells and control virus replication despite the presence of viral immune evasion genes. These data indicate an important role for CD8+ T_{RM} cells to control early lytic virus replication in the SG following localised reinfection.

5.4 Discussion

CMV infection leads to protracted infection of the SG, fostering horizontal virus transmission in mice and humans. Our data identify the SG as a glandular barrier tissue with exquisite capacity to instruct and lodge tissue-resident memory CD8+ and CD4+ T cells following MCMV infection, suggesting that these cells might play pivotal roles in protection against localised reinfection, thereby curtailing CMV transmission episodes. While both MCMV-specific CD4+ and CD8+ T_{RM} cells were maintained long-term in the SG after control of lytic replication, the requirements for their local induction, their micro-anatomical localisation and their protective capacities in localised infection events differed substantially.

Previous studies indicate that local antigen requirement for induction of T_{RM} formation is largely dictated by the organ of residence. While antigen independent CD8+ T_{RM} induction is supported in the gut, the skin, the female reproductive tract, as well as in the SG upon LCMV infection [74, 89, 101], CD8+ T_{RM} cell formation in the brain requires local antigen [81]. We show here that local antigen is dispensable for T_{RM} induction in MCMV-specific CD8+ T cells in the SG. In contrast, CD4+ T_{RM} formation critically relied on the availability of local antigen, indicating that T cell subset-specific demands are surprisingly different and outweigh organ-specific effects in the T_{RM} differentiation program. Previous data indicate that lung-resident CD4+ T cells acquire T_{RM} signatures independently of local cognate antigen [78], suggesting that the necessity of antigen for CD4+ T_{RM} development depends on the tissue of residence.

Cytokines play a critical role in T_{RM} development with the list of candidates growing and their organ-specific relevance currently being unravelled. The expression of
CD103 and CD69 phenotypically characterises CD8⁺ T⁰ cells and TGFβ is a global instructive signal for CD103 expression. We confirm that this is also the case for MCMV-specific CD8⁺ T⁰ cells forming after MCMV infection in the SG. In line with our results, MCMV-specific T cells upregulated CD103 in response to TGFβ stimulation *in vitro* and MCMV infection did not increase levels of TGFβ protein in the SG, providing an explanation why the naïve SG supports CD103 upregulation equally well as the MCMV-infected SG (Smith et al. 2015, submitted manuscript). As IL-15 was recently shown to induce CD103 expression in HSV-specific CD8⁺ T cells in the skin [94], we assessed its role for CD8⁺ T⁰ induction in the SG but found no evidence for an involvement. The mechanisms leading to constitutive expression of CD69 on T⁰ cells remain unclear. The reduced CD69 expression in CD8⁺ T⁰ cells in the gut of Tgfbr2⁻/⁻ dLck-Cre mice suggested a role for TGFβ in CD69 upregulation [92]. Yet, our data show that neither MCMV-specific CD8⁺ nor CD4⁺ T⁰ cells in the SG required TGFβ signalling for CD69 expression. Type I interferons are also known to induce CD69 expression [95], but type I interferon receptor signalling was not required for the sustained expression of CD69 on MCMV-specific T⁰ cells in the SG.

The fact that T⁰ cells reside at peripheral sites and are maintained long after the primary infection is resolved has promoted the search for mechanisms of T⁰ cell survival and maintenance. CD103 plays a central role in CD8⁺ T⁰ cell maintenance through two mechanisms: CD103 promotes T cell survival by upregulation of the anti-apoptotic molecule Bcl-2 [81, 94] and exerts adhesive function by tethering T cells to E-Cadherin, which is mainly expressed on epithelial cells [96]. CD103⁺ CD8⁺ T⁰ cells in the SG appear to profit from both mechanisms, indicated by elevated levels of Bcl-2 as well as preferential localisation within epithelial ducts compared to CD103⁻ CD8⁺ T cells and CD4⁺ T cells, which were predominantly localised in the SG’s connective tissue. CD4⁺ T⁰ cells generally do not express CD103, excluding a function in CD4⁺ T⁰ maintenance. It remains to be determined whether CD4⁺ T⁰ retention is achieved by CD69 through its inhibitory effect on cell surface
expression of the sphingosine-1-phosphate receptor 1 (S1P1), which is required for T cell egress from organs [102, 103].

The fact that MCMV infection promotes the establishment of considerable populations of both CD4+ and CD8+ T cells specifically in the SG suggests that the strategic positioning of these MCMV-specific cells at relevant re-entry sites of the virus affords immediate local protection and hence curtails chances of horizontal transmission upon reinfection. During primary MCMV infection, CD4+ T cells are the only cell type capable of controlling lytic infection in the SG [22]. The inability of CD8+ T cells to control lytic MCMV replication in the SG upon primary infection is due to the exquisite ability of MCMV to profoundly downregulate MHC I expression on the main target cells of MCMV, the acinar glandular epithelial cells [24]. We therefore hypothesised that MCMV specific CD8+ T cells would also be incapable of controlling local reinfection events. Surprisingly, this was not the case and contrary to our expectations, CD8+ T cells were superior in providing local protection compared to CD4+ T cells. Detailed analysis of the cell types permissive for MCMV infection and replication at early time points in the SG revealed that MCMV targeted largely non-hematopoietic cells of non-epithelial origin and to a lesser extent hematopoietic cells including tissue macrophages. MCMV failed to achieve complete MHC I downregulation in these early SG target cells, thereby providing a window of opportunity for CD8+ T cells to detect viral antigen and control virus replication. Thus, the potential of CD8+ T cells to confer immediate protection against localised SG infection illustrates that the efforts of the SG in hosting a sizeable population of CD8+ T cells indeed have physiological advantages which are explained by the altered composition of MCMV-targeted cell types at early time points of localised infection.

So far CMV superinfection studies were mainly conducted using the subcutaneous route of reinfection, which is likely leading to a systemic viral challenge [104-107]. In these studies, systemic memory CD8+ T cells were shown to afford protection against lethal challenge in immunocompromised murine hosts [105, 106] or to provide protection against systemic reactivation of CMV in immunosuppressed...
organ transplant recipients [108-110]. Furthermore, expression of CMV-encoded immune evasion genes was linked to the inability of CMV-specific CD8$^+$ T cell immunity in restricting subcutaneous CMV superinfection [108]. Here we show that CMV-encoded immune evasion genes do not compromise the ability of SG-resident CD8$^+$ T$_{RM}$ cells in protecting against reinfection with CMV, owing to the early infection of cell types which are not overtly vulnerable to MHC I downregulation by MCMV.

In summary, we delineate the extraordinary capacity and the mechanisms by which the SG accommodates T$_{RM}$ memory cell development and maintenance in an epidemiologically relevant infection model and demonstrate the protective capacity of CD8$^+$ T$_{RM}$ cells upon localised MCMV infection of the SG, highlighting a yet unappreciated role of CD8$^+$ T cells in an organ that has so far been deemed resistant towards CD8$^+$ T cell-mediated control of lytic virus replication. The mechanisms by which SG CD8$^+$ T$_{RM}$ cells can mediate this immunity may involve direct cytotoxicity and effector cytokine production or the establishment of a broad, organ-wide antiviral state, such as shown for CD8$^+$ T$_{RM}$ cells in the female reproductive tract and the skin [111, 112].

5.5 Materials and Methods

Ethics Statement

This study was conducted in accordance to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocols were approved by the Cantonal Veterinary Office of the canton Zurich, Switzerland (permit number 109/2011, 110/2011, 127/2011).

Mice

All mouse strains were housed and bred in specific pathogen-free facilities: C57BL/6 mice, CCR7$^{-/-}$ mice [99], and CD11c-YFP reporter mice [34]. IL15$^{-/-}$ mice [113] were provided by Prof. O. Boyman (University Hospital of Zurich). DnTGFβRII mice [93]
were provided by Dr. J. Herkel (Universitaetsklinikum Hamburg-Eppendorf). Maxi transgenic (Ly5.1+) mice express a TCR specific for the MCMV peptide M38\textsubscript{316-323} \cite{59}. Mini transgenic (Ly5.1+) mice express the V\textbeta\textsubscript{10}J\textbeta\textsubscript{2} chain, while the TCR\alpha chain was of endogenous origin, leading to an increased percentage of naïve M38-specific CD8\textsuperscript{+} T cells from undetectable levels in C57BL/6 mice to 10% \cite{59}. M25 transgenic (Ly5.1+) mice express a TCR specific for the MCMV peptide M25\textsubscript{410-425} \cite{60}. P14 transgenic (Ly5.1+) mice express a TCR specific for the LCMV peptide gp\textsubscript{33-41} \cite{80}. Smarta transgenic (Ly5.1+) mice express a TCR specific for the LCMV peptide gp\textsubscript{61-80} \cite{114}. Tgfbr2\textsuperscript{+/−} dLck-Cre mice \cite{115} were a gift of Prof. M. Bevan (University of Washington).

**Viruses and Infections**

Recombinant MCMV deficient in m157 (MCMV-Δm157) was previously described \cite{116} and is referred to as MCMV in this study. The MCMV mutant expressing GFP under the m157 promoter was described in \cite{117}. MCMV strains were propagated on MEFs as described \cite{67}. Virus titres of organs were determined on M2-10B4 cells as described recently \cite{118}. For systemic infection, mice were infected intravenously with 5x10\textsuperscript{6} pfu MCMV-Δm157. Focused intraglandular infection was performed as previously described \cite{100}.

**Adoptive Transfer**

CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells were isolated from naïve Maxi, CD103\textsuperscript{−/−} Maxi, P14, M25, and Smarta (all Ly5.1+) or INFAR\textsuperscript{+/−} P14 (Thy1.1+) mice using magnetic anti-CD8 or anti-CD4 beads (Miltenyi Biotech). 10\textsuperscript{6} M25 cells, 10\textsuperscript{5} Mini cells or 10\textsuperscript{4} of all other transgenic cells were adoptively transferred into naïve recipient mice one day prior to infection. To study the migration and maintenance of in vivo activated T cells, splenocytes were isolated 1 wpi and 10\textsuperscript{6} T cells purified by MACS were transferred into naïve recipient mice. To study the maintenance of CD8\textsuperscript{+} T cells in the SG, mice received 5 μg/ml FTY720 (US Biological) in the drinking water, starting 1 wpi.
Lymphocyte Isolation, Intravascular, Intracellular, and Surface Staining

Lymphocytes were isolated from spleen and SG as previously described [69], with the modification that cell suspensions were not subjected to a percoll gradient. Staining for flow cytometry was performed on whole blood or single cell suspensions from the indicated organs as described in the supplemental experimental procedures. Data were acquired on a LSRII flow cytometer (BD Bioscience) and analyzed using Flowjo software (Treestar).

Immunofluorescence Microscopy

SGs were isolated from infected animals, fixed overnight in PBS containing 4% PFA at 4°C and incubated overnight in PBS containing 20% sucrose, followed by tissue embedding in O.C.T. compound (Sakura, Torrance, CA), snap-freezing in liquid N2 and storing at −80°C. Cryosections of 10 µm thickness were stained for immunofluorescence microscopy as described in the supplemental experimental procedures. Images were acquired with an inverted confocal microscope (Axiovert 200, Carl Zeiss, Inc., Zurich, Switzerland), equipped with an oil-phase contrast objective (Plan Neoflusr, Carl Zeiss, Inc., Zurich, Switzerland), an CSU-X1 spinning-disk confocal unit (Yokogawa) and a solid state laser unit with 4 laser lines (405, 488, 561, 647, Toptica). Data were analyzed with Volocity (Improvision). Images were subject to contrast enhancement.

Statistical Analysis

Statistical significance was determined as indicated either by two-tailed unpaired t-test or Fisher’s exact test using GraphPad Prism (La Jolla, CA, USA).
6 General Discussion

6.1 Introduction

Barrier tissues include all organs that are connected to the body surface, e.g. the skin, the mucosae of the gastrointestinal and respiratory tract, as well as exocrine glands. These tissues face unique challenges in the pursuit of immune integrity as they are exposed to a great variety of exogenous antigen in which indicators of pathogenic insult must be sensitively and specifically distinguished from the multitude of harmless commensal or environmental antigen. Moreover, many barrier tissues must not only defend themselves against pathogenic invasion, but are furthermore abused for pathogenic egress and certain viruses have co-evolved with their hosts for millions of years to specifically suppress immunity in these tissues and spread via mucosal secretions.

CMV has reached extraordinary prevalence by persisting in the SG and exploiting the saliva for horizontal transmission. Specifically in the SG, CMV achieves complete MHC I downregulation in saliva producing epithelial cells, thereby extinguishing the functionality of CD8$^+$ T cells, which are the principal effectors of CMV immunity in other tissues [22]. For a long time, it appeared that the SG is on the losing side of this arms race. Yet, the evidence presented in this thesis suggests that CD8$^+$ T cells in the SG can in fact exert local CMV control. They transform into long-lived T$^{RM}$ cells that are able to afford protection against local CMV infection and might even prevent viral reactivation from latency. In this chapter, we will set these new findings in context to the existing knowledge of T$^{RM}$ cells in barrier tissues and elaborate their distinctive features in the SG as a representative of exocrine glands.

6.2 Tissue-Resident Memory T Cells in Barrier Tissues

The importance of memory T cells in the protection against systemic secondary infection is undisputed. In the active debate over whether efficient protection at peripheral sites was achieved by circulating T$_{CM}$ or T$_{EM}$ cells, the discovery of non-recirculating, long-lived T$^{RM}$ cells has led to extensive adjustments of our
understanding of T cell memory. T\textsubscript{RM} cells are found at all epithelial barrier tissues and can afford immediate protection against reinfection. The T\textsubscript{RM} concept is now accepted as a universal frontline defence strategy that was long undiscovered as a result of technical shortcomings, which misinterpreted T\textsubscript{RM} cells as part of recirculating T\textsubscript{EM} cells.

**Defining Tissue-Resident Cells**

The discovery of T\textsubscript{RM} cells has created a new field of research that investigates their generation, their maintenance, and their functional contribution in the context of infection and autoimmunity. Addressing these questions firstly requires the precise identification of T\textsubscript{RM} cells and the discrimination from their circulating counterparts in the tissue of interest. This section provides an overview on the current approaches used to specify resident cells and discusses their advantages and pitfalls.

**Phenotypic Assessment**

Phenotypic analyses of T cells in various tissues have revealed a surprisingly uniform T\textsubscript{RM} signature that can be directly examined by flow cytometry. Yet, the enzymatic digestion and mechanical disintegration that is required to generate single cell suspensions from the tissue of interest is incomplete and therefore yields only partial lymphocyte extraction. Thus, flow cytometry might fail to detect small lymphocyte populations and bears the risk of underestimating the actual size of detectable populations. Moreover, organs consist of different anatomic compartments, such as epithelial structures, connective tissue, muscular tissue, as well as blood and lymphatic vessels, which differ in their resilience towards digestive enzymes and mechanical distress. The lymphocyte composition of a complex organ might be inaccurately reflected in its single cell suspension if the anatomic compartments are not equally well disintegrated. Epithelial tissues are particularly resistant to digestion due to their tight packing and their high
6.2 Tissue-Resident Memory T Cells in Barrier Tissues

abundance of tight junctions. Moreover, T\textsubscript{RM} cells are tightly tethered to epithelial cells [96] and might thus be trapped in cell aggregates, complicating or even invalidating their analysis in flow cytometry.

Apart from the risk of misinterpreting the composition of tissue lymphocytes, the analysis of single cell suspensions does not allow to identify blood-borne contaminations that were passing the vasculature at the time point of organ preparation. This problem is classically avoided by perfusing mice with saline before organ harvest. Yet, Andersen et al. have recently reported that perfusion fails to remove blood-borne lymphocytes and even disrupts tertiary lymphoid structures in lungs, thus introducing significant sources of error to the analysis [87]. This study suggests to refrain from perfusion and invites to include intravascular staining, i.e. the injection of a fluorescently labelled monoclonal antibody before organ preparation. This technique is based on the assumption that cells located in the blood are immediately accessible to intravenously administered antibodies, whereas cells located in the parenchyma are not accessible at all or are labelled with a significant time delay. Importantly, this protocol requires adoptions with respect to timing and antibody amounts for each organ, depending on its vascular accessibility and lymphocyte abundance.

Despite its caveats, flow cytometry remains an invaluable tool to dissect the variety of T cell subsets and assess their effector functions. In light of the aforementioned shortcomings, it is advisable to combine flow cytometry with intravascular labelling and imaging to add spatial information and to correct errors that are introduced by incomplete lymphocyte extraction.

\textit{Functional Assessment}

The T\textsubscript{RM} signature phenotype is a good indicator but not a sufficient criterion for tissue residence as demonstrated by the existence of CD103\textsuperscript{+} T\textsubscript{RM} cells in SLOs and the lung [119, 120]. The most indicative evidence of tissue residence is the lack of recirculation, which is assumed true if one of the two conditions applies: (1) the T cells in question cannot be detected in the circulation throughout a defined time
frame that starts after tissue residence is assumed established. (2) The size of the resident T cell population remains constant over time when lymphocyte resupply is absent.

The ability of T cells to egress a peripheral tissue can be addressed by transplanting the tissue in question under the kidney capsule of a naïve recipient. Tissue residence is assumed when transplant-derived T cells cannot be detected in the circulation of the recipient. This method was first described by Gebhardt et al. and enabled them to show that dorsal root ganglia harbour a population of HSV-specific CD8+ T_{RM} cells that protect against HSV reactivation [77]. Yet, it is not clear to which extent lymphocyte egress from the tissue in question is influenced by reimplanting it to a different anatomical location that does not provide the organ's physiological lymphatic infrastructure and blood vessel supply. Furthermore, this approach dictates a small transplant size. An elegant way to circumvent these shortcomings is parabiotic surgery – the vascular conjoining of a naïve recipient parabiont with a donor parabiont that harbours memory T cells. Donor-derived circulating T cells will redistribute within the naïve recipient, allowing to distinguish tissues that are accessible for blood-borne memory T cells from tissues with restricted access. Parabiosis revealed that LCMV-specific memory T cells repopulated many organs but not the female reproductive tract, the lamina propria, and the lung of a naïve parabiont. Nevertheless, CD8+ memory T cell populations in these tissues remained constant in the donor parabiont over time [75, 79, 121]. With these sites not being accessible for circulating memory T cells after a resolved infection, the authors concluded that the T cell populations found in these organs take up residence during the acute phase of infection and self-maintain thereafter. Gebhardt et al [86] confirmed the ability of T_{RM} cells to self-maintain without resupply from the circulation by injecting male HSV-specific gBT-I CD8+ T cells into female recipients, thus introducing a mismatch allotransplant, which is rejected by cytotoxic Y-antigen-specific CD8+ T cells in the female host. While circulating male T cells were eliminated within the first 2 weeks after transfer, a skin-resident population of male
T cells resisted rejection and persisted in the skin in absence of their circulating counterparts for 12 weeks post infection. Despite their elegance in theory, both, parabiotic surgery and male-to-female T cell transfer allow to distinguish circulating from resident populations only under the assumption that the tissue in question is not accessible for circulating T cells in steady state. Whether this holds true needs to be addressed before applying these methods for proof of tissue residence. The SG is accessible for activated CD8⁺ T cells even under homeostatic conditions. Hence, tissue-resident cells of male origin are not protected from the cytotoxic activity of Y-antigen-specific female CD8⁺ T cells, which will be activated in secondary lymphoid organs and are consecutively able to enter the SG irrespective of local presence of antigen. For the same reason, parabiotic surgery would result in the establishment of T_{RM} populations in the naïve parabiont, potentially with exceptionally high efficiency in the context of CMV infection, as low level viral reactivation in lymph nodes constantly activates circulating inflationary memory T cells [59]. Indeed, Smith et al. (2015, submitted manuscript) show that inflationary CD8⁺ T cells are able to form new T_{RM} cells at mucosal sites in steady state. Thus, both methods fail to precisely determine the existence of a T_{RM} population in the face of the constitutive ability of the SG to recruit activated effector cells and maintain them. Investigating T cell residence in the SG requires an approach that directly addresses the egress of cells located in the SG. One possibility to achieve this employs the local labelling of lymphocytes in the SG by injecting fluorescent cell staining dyes or antibodies into the Wharton’s duct, the main secretory duct of the SG that empties into the floor of the oral cavity [122]. Provided that the labelling agent successfully passes the epithelial barriers of the Wharton’s duct without simultaneous leakage into blood or lymph vessels, cells that egress the SG can specifically be identified by their positive staining. A second approach to demonstrate disequilibrium of a tissue-resident cell population with the circulation could be achieved by labelling cells with BrdU during the acute phase of infection and assessing the composition of CD8⁺ T cells in the SG after the infection is resolved. The continued proliferation of inflationary T cells after the
resolution of the acute infection will yield a growing population of BrdU\(^+\) CD8\(^+\) T cells, which will intermix with BrdU\(^+\) T cells in tissues, leading to dilution of circulating BrdU\(^+\) cells, while T\(_{RM}\) cells should maintain a high frequency of BrdU\(^+\) cells.

**CD8\(^+\) T\(_{RM}\) Cells in Mucosal Infection**

The discovery of memory T cell populations that reside in non-lymphoid tissue (NLT) was first made among CD8\(^+\) T cells. Based on the finding that CD8\(^+\) memory T cells isolated from NLT exhibited direct effector functions that were not evident in their splenic counterparts, Masopust et al. suggested that at least a subset of CD8\(^+\) T cells isolated from tissues is distinct from circulating CD8\(^+\) T\(_{EM}\) cells [123]. This hypothesis was followed by the final proof of the existence of resident memory CD8\(^+\) T cells that are seeded to various organs during the effector phase of the immune response, acquire the T\(_{RM}\) signature and are not in equilibrium with the circulation during the memory phase [78, 81, 124]. Being readily positioned at frontline sites of infection, T\(_{RM}\) cells have the unique ability to respond most immediately and prevent systemic infection.

The mechanisms by which T\(_{RM}\) cells contain localised infections are a field of active inquiry. T\(_{RM}\) cells isolated from the brain, skin, and intestinal mucosa exert enhanced effector functions, such as rapid production of IFN\(\gamma\) and target cell lysis [75, 76, 81, 123], demonstrating that T\(_{RM}\) cells are fully equipped to immediately control peripheral infection directly. Yet, the actions of T\(_{RM}\) cells stretch far beyond the classical antiviral effects of cytotoxic T cells: In a manner that is dependent on IFN\(\gamma\), TNF, and IL-2R, T\(_{RM}\) activation initiates broad local immune activation, including the maturation of dendritic cells, the activation of NK cells, as well as the recruitment of memory B and T cells to an extent that can generate near-sterilising immunity against antigen-unrelated pathogens [112, 119, 121].

The discovery of T\(_{RM}\) cells and their relevance in infection in the mouse model has prompted the search for equivalents of resident T cell populations in humans.
These efforts are highly limited by technical constraints, however, CD103+ CD8+ T cells exist in humans and appear to play a role in both protection against pathogens as well as immunopathology. CD103+ CD8+ T cells in epithelial and neuronal tissues have been associated with pathological conditions such as graft rejection, tumourigenesis, and autoimmunity. Specifically, psoriatic skin as well as fixed drug eruptions contain high frequencies of CD103+ CD8+ T cells that are tightly associated with recurring disease manifestation in identical anatomic sites, indicative of long-term persistence of CD8+ T cell subsets within the same region (reviewed in [88]). Additionally, influenza-specific CD103+ CD8+ T cells have been found in the alveolar epithelium of human lungs [125], EBV-specific CD103+ CD8+ T cells were found in human tonsils [126], and HSV-2 specific CD0103+ CD8+ T cells were identified in human vaginal skin [127].

**CD4+ T_{RM} Cells in Mucosal Infection**

In both mice and humans, memory CD4+ T cells outnumber memory CD8+ T cells in mucosal tissues [128, 129], implying an evolutionary benefit from accommodating not only CD8+ but also CD4+ T cells in tissues. Yet, the CD4+ T cell subset was long ignored in the field of T_{RM} research and our knowledge is still scarce. CD4+ T_{RM} T cells were first identified in the lungs of influenza-infected mice as a population of CD11a+ CD69+ CD4+ T cells that were not accessible for intravascular staining [78]. Later studies showed that CD4+ T_{RM} cells were also induced in the lung upon infection with LCMV, *M. tuberculosis*, and *N. brasiliensis* and in the HSV-infected female reproductive tract, as well as in the gut following *L. monocytogenes* infection (reviewed in [130]). CD4+ T_{RM} cells readily produce effector cytokines and were shown capable of completely protecting from lethal influenza lung infection [78], suggesting that CD4+ T_{RM} cells are as efficient as CD8+ T_{RM} cells in their ability to mount rapid recall responses and control localised infection. In HSV-2 infection, CD4+ T cells play a key role in protection, while CD8+ T cells appear dispensable [131] [132] and CD4+ T_{RM} cells were shown to efficiently protect in HSV-2 challenge.
CD4+ T_{RM} cells appear to exist in humans as well: Walrath et al. report tissue-retentive CD4+ T cells in the lungs of tuberculosis patients [133]. Purwar et al. describe a lung-resident population of CD4+ T_{RM} cells that are able to proliferate in response to stimulation with influenza antigen [134].

T_{RM} Generation and Maintenance

The protective benefit of both CD4+ as well as CD8+ T_{RM} cell subsets in different infectious models has encouraged efforts to specifically induce T_{RM} cells in the context of vaccination or therapeutic approaches. To this end, it is crucial to define and understand the different requirements for CD4+ and CD8+ T_{RM} generation and maintenance. In order to persist in a peripheral organ, T_{RM} cells must resist signals that induce tissue egress and instead be responsive to the specific survival signals provided by the tissue of residence.

Resisting Tissue Egress

Physical retention by integrins is an effective mechanism that prevents tissue egress in CD8+ T_{RM} cells. The integrin αE, also known as CD103, tethers T_{RM} cells to E-Cadherin, which is highly expressed on epithelial cells [96]. Imaging of CD8+ T_{RM} cells revealed that CD103+ T cells preferentially co-localise with epithelial layers and acquire a dendritic shape between tightly packed epithelial cells [135]. Tissue retention of CD103 deficient CD8+ T cells is strikingly impaired in a variety of peripheral tissues [81, 89, 94]. Yet, despite their expression of CD103, CD8+ T_{RM} cell maintenance in the small intestine is CD103 independent. Furthermore, CD103 is absent on the large majority of CD4+ T_{RM} cells, suggesting that other molecules additionally support tissue retention. The integrin VLA-1 interacts with collagen and laminin and its expression was found in CD8+ T_{RM} cells in the brain, gut, lung, and skin [77, 94, 120, 136], as well as on CD4+ T_{RM} cells [137].

Apart from mechanical retention, tissue egress is furthermore prevented by inhibiting signalling pathways that initiate tissue egress via efferent lymphatics.
Lymphocytes are guided to efferent lymphatics by a chemotactic gradient of sphingosine-1-phosphate (S1P), which is sensed by the S1P receptor 1 (S1PR1). CD69 is expressed on all T<sub>Rm</sub> cells and antagonises the action of the S1PR1 by interfering with its cell surface expression, thus desensitising the T<sub>Rm</sub> cell towards S1P-mediated egress stimuli. The transcription factor KLF-2 is required for S1PR1 expression on T cells, but is downregulated in T<sub>Rm</sub> cells [138]. Furthermore, T<sub>Rm</sub> cells downregulate the chemokine receptor CCR7 (reviewed in [139]), which is required for T cell exit from peripheral tissues [98].

Taken together, the retention of T<sub>Rm</sub> cells is mediated by the concerted action of integrins, which physically hold T<sub>Rm</sub> cells in place as well as the selective ignorance of chemokines that mediate tissue egress.

Integration of Local Survival Signals
Persistence within tissues does not only require T<sub>Rm</sub> cells to physically remain in their organ of residence, but they must furthermore be responsive to the locally provided survival signals. What these survival signals are and by which cell types they are produced, is largely unanswered. Several candidates have been identified, but their role appears to be dictated by the tissue environment. The cytokine IL-15 promotes CD8<sup>+</sup> T<sub>Rm</sub> survival in the skin, but not in SLOs [94, 119]. Our data indicate that IL-15 is not relevant for T<sub>Rm</sub> survival in the SG, either. As discussed before, TGF-β induces CD103 expression in CD8<sup>+</sup> T<sub>Rm</sub> cells. Besides its well-known role in tissue retention, CD103 promotes T cell survival by upregulation of the anti-apoptotic molecule Bcl-2. We furthermore observed that defective TGF-β signalling in T cells leads to a deficit in CD8<sup>+</sup> T cell retention in the SG, which is not selective for the CD103<sup>+</sup> subset, suggesting a CD103-independent role for TGF-β in the maintenance of T<sub>Rm</sub> cells. Further studies suggest a role for the aryl hydrocarbon receptor for T<sub>Rm</sub> cell survival in the skin [140]. Moreover, inflammatory stimuli, persistent antigenic stimulation, as well as a range of other cytokines, including IL-33, IL-2, and TNF have been suggested to be involved in T<sub>Rm</sub> cell homeostasis [89, 94].
It is unclear which cell types provide the necessary survival signals. In their capacity to produce cytokines and present antigenic peptides, APCs are potent modulators of immune responses in both lymphoid organs as well as peripheral tissues. In the recent years, the importance of tissue-resident macrophages in shaping the cytokine environment of peripheral organ has become strikingly evident. Current evidence corroborates the notion that APC networks might also contribute to $T_{RM}$ cell maintenance and survival. In the skin, CD8$^+$ $T_{RM}$ cells engage in close interactions with the network of Langerhans cells [140]. In the genital mucosa, protection from lethal HSV-2 infection required the presence of CD4$^+$ $T_{RM}$ cells, which critically depended on chemokines secreted by a local network of macrophages. Our data show that in the SG, a dense meshwork of macrophages tightly co-localises with both CD4$^+$ and CD8$^+$ T cells [31]. This network is present in steady state and is reminiscent of tissue-resident macrophages. Whether these macrophages play a role in the maintenance and function of $T_{RM}$ cells in the SG requires further investigation.

**Differential Requirements for CD8$^+$ and CD4$^+$ $T_{RM}$ Formation**

The impact of local cognate antigenic stimulation on the maintenance of CD8$^+$ as well as CD4$^+$ $T_{RM}$ cells seems to depend on the tissue of residence. In a broad variety of peripheral tissues including gut, lung, and skin, ongoing antigenic stimulation is not required to induce CD103 expression. Non-specific inflammation in the skin was sufficient to generate CD8$^+$ $T_{RM}$ cells [74], while lymphopenia-driven proliferation led to CD8$^+$ $T_{RM}$ formation in the gut [89]. However, CD103 expression on CD8$^+$ T cells in neuronal tissue appears to be strictly dependent on cognate antigen in a model of VSV brain infection [81, 89]. Teijaro et al. found that influenza-specific, lung-retentive CD4$^+$ $T_{RM}$ cells acquired $T_{RM}$ properties in a manner that is independent of antigen [78]. Our data indicate that SG-resident CD4$^+$ $T_{RM}$ cells rely on the supply of local antigen. Taken together, these data clearly indicate that the tissue of residence decides upon the antigen dependence of $T_{RM}$ formation and lodgement. Whether tissue-inherent factors outweigh subset-specific demands is
not sufficiently elucidated, as studies that directly compare CD8+ and CD4+ T<sub>Rm</sub> formation within the very same organ are scarce. Insightful data were obtained from intravaginal HSV-2 infection, which leads to the lodgement of both CD8+ and CD4+ T<sub>Rm</sub> populations. A vaccination strategy that involved a subcutaneous prime followed by an intravaginal chemokine pull only led to CD8+ T<sub>Rm</sub> but not CD4+ T<sub>Rm</sub> formation despite both cell subsets being recruited [141]. These results indicate that CD4+ T cells require additional factors provided by HSV-2 infection for long term maintenance. Our data corroborate the differing requirements that these two subsets demand from the same organ for T<sub>Rm</sub> generation: While CD4+ T<sub>Rm</sub> generation in the SG relies on local cognate antigen, CD8+ T<sub>Rm</sub> generation is even favoured in the absence of cognate antigen.

The mechanisms of T<sub>Rm</sub> induction and stasis are yet to be precisely defined. A continuous supply of microenvironment-specific factors seems crucial for survival and function of T<sub>Rm</sub> cells as indicated by impaired survival and recall responses of T<sub>Rm</sub> cells isolated from their organ of residence and reinjected into the circulation [142, 143].

**Peculiarities of SG-Resident Memory T Cell Populations**

Despite the large versatility of tissues that can accommodate T<sub>Rm</sub> cells, they are surprisingly homogenous in terms of their signature phenotype, their function, as well as their migratory behaviour. Parabiosis experiments have revealed that blood-borne memory T cell populations do not have access to mucosal and/or epithelial sites where tissue-resident T cell populations are found, suggesting that once the inflammatory environment of the acute phase of a viral infection has ceased, the T<sub>Rm</sub> population needs to self-maintain and cannot rely on the replenishment by circulating T cell pools [79]. The SG follows a different pattern, as it allows for the induction of a T<sub>Rm</sub> phenotype in any activated CD8+ T cell in a manner that is not only antigen- but also inflammation-independent. In striking contrast to the female reproductive tract or the gut, tissue entry to the SG is never restricted to circulating
CD8^+ T cell populations, even in steady state, allowing for constant replenishment of T\textsubscript{RM} cells. This phenomenon distinguishes the SG from all other T\textsubscript{RM}-inducing tissues and to this point, we can only speculate on the evolutionary benefit of equipping the SG with the strong potential to induce T\textsubscript{RM} cells of any specificity.

### 6.3 On the Evolutionary Purpose of Protecting Glandular Tissue

At first glance, it might not be apparent why to evolutionarily prioritise those individuals that put particular effort into protecting the SG, an organ that is not considered vital in its mere purpose of producing saliva. Yet, albeit not being essential to life, saliva provides a multitude of benefits that might yield small but decisive advantages in the course of evolution.

99% of the saliva consists of water. The residual 1% contains electrolytes and a multitude of proteins including mucosal glycoproteins, immunoglobulins, as well as digestive and antimicrobial enzymes. The digestive enzymes in saliva initiate breakdown of dietary compounds such as starches and fats. In fact, 30% of starch digestion takes place in the oral cavity and is supported by amylase. Lipases contained in saliva support fat digestion and are especially important in infants where the pancreas is not yet able to produce lipase. For the longest time in the existence of mammalian species, food was scarce and starvation has exerted profound evolutionary pressure, selecting those individuals that possess efficient digestive systems to fully utilise the energy contained in food. By producing saliva, the SG adds significant efficiency to the digestive system and a functional SG might have provided a striking selective advantage in periods of food shortage.

However, the functions of saliva extend way beyond its well-known role in digestion. In the oral cavity, saliva acts antivirally via Thrombospondin 1 \([144, 145]\) and exerts antibacterial functions via immunoglobulin A, which sterically hinders bacterial intrusion. Additionally, enzymes such as lysozyme, lactoferrin, and cystatins, which inhibit bacterial growth. Apart from antiviral and antibacterial defence mechanisms, saliva supports wound healing, which is mediated by regenerative growth factors and cytokines such as tissue factor, VEGF, TGF\beta, and
leptin. Experimental studies in mice demonstrate that epidermal growth factor contained in saliva significantly increased wound contraction and removal of the SG in mice slowed down wound healing [146]. In fact, wound licking is a highly conserved behavioural pattern across species and the saliva provides a valuable first aid kit for the injured, allowing for immediate disinfection, quicker wound healing, and recovery.

Whether other exocrine glands are equally well protected by T_{RM} cells requires further investigation. Yet, the existence of T_{RM} cells has been shown for mammary glands as well as lacrimal glands, and given their function, it appears evolutionarily favourable to protect these tissues, as well. Before the advent of breast milk substitutes, the mammary glands were essential to sustain the life of the offspring. Not only would a viral infection of the mammary gland threaten the offspring’s life through direct exposure to the virus itself, but it might also lead to malnutrition or starvation in case the milk producing tissue is destroyed as a consequence of the infection. Lacrimal glands produce a tear film that lubricates the eye and clears it of foreign particles. Tear fluids furthermore contain lysozyme that acts antibacterial, thereby protecting the eye from microbial infection. As a producer of tears, lacrimal glands therefore function to maintain an individual’s eye sight, which is essential in the escape from predators and the hunt for food. Overall, not all exocrine glands are critical to survival, but their proper function might constantly have added a selective advantage to those individuals whose immune system was specifically preparing for pathogenic attack of these glands.

So far, we can only speculate on why the SG is endowed with such great capacity to accommodate CD8^{+} T_{RM} cells of any specificity while simultaneously imposing severe restrictions on CD4^{+} T cell migration and T_{RM} formation. CD8^{+} T cells are the principal effectors in the immune response against many viruses. Regardless of the anatomic location in which a virus was initially encountered, the presence of CD8^{+} T_{RM} cells specific against the same virus in the SG is likely to be beneficial rather than harmful. Being equipped to directly detect and eliminate virally infected cells, CD8^{+} T cells are sufficient to control many viral infections even in the absence of
CD4+ T cells and the selective advantage of accommodating antiviral CD8+ T cells might have been decisive, while the evolutionary benefit of accommodating CD4+ T_{RM} cells might have been insignificant. In fact, the arbitrary accommodation of T_{RM} cells could even be harmful to an organ. The SG’s immunological tolerance is constantly challenged by harmless environmental antigens, which are taken up and presented by local APCs. T cells that were erroneously activated against such harmless antigen, i.e. at other sites of the gastrointestinal tract, can cause immunopathology in the SG when exposed to the same antigen. Hence, the enhanced protective capacity that goes along with the arbitrary accommodation of T_{RM} cells comes at the cost of increased risk of immunopathology. The severe restrictions that are imposed on CD4+ T_{RM} cells might therefore represent a precautionary measure to limit the damage of mistakenly primed CD4+ T cell responses. For CD8+ T cells, this measure of precaution is not required because SG-resident APCs entirely lack the ability to cross-present exogenous antigen in the context of MHC I molecules and therefore fail to activate CD8+ T cells that were primed elsewhere against harmless food antigen. With SG-resident APCs being well capable to present exogenous antigen in the context of MHC II while simultaneously being incapable to cross-present the same antigen in the context of MHC I, the accommodation of T_{RM} cells of various specificities only exposes the SG to a higher risk for immunopathology by CD4+ but not CD8+ T cells. Whether the complete lack of cross-presenting APCs in the SG was a decisive precondition or a consequence of the diametrical requirements for CD4+ and CD8+ T_{RM} cells in the SG is another matter of speculation.

6.4 Concluding Remarks

The concept of circulatory immune surveillance is massively challenged by a large body of elegant studies that demonstrate the existence of sessile populations of memory T cells. These tissue-resident T cells act as on-site guards of peripheral immunity and are superior to their circulating counterparts in containing localised reinfection of barrier tissues such as mucosae and skin. Their specific strength lies
within the ability to immediately purge infection when circulating cells are lagging behind due to their necessity of reactivation and redifferentiation in secondary lymphoid organs. The benefit of this circumstance is highlighted by the ability of CD8$^+$ T cells to contain CMV infection once they are readily positioned in the SG before CMVs immune evasion program achieves its maximal impact.

Given the extraordinary similarity of T$_{RM}$ phenotype and function regardless of their tissue of residence and the infectious or inflammatory circumstances under which they were generated, the T$_{RM}$ formation appears universally favourable. Yet, CD4$^+$ and CD8$^+$ T$_{RM}$ generation requires different factors that seem to depend on the tissue of lodgement. Understanding how T$_{RM}$ cells are recruited and maintained is critically required to unlock promising opportunities for therapeutic and prophylactic intervention strategies that utilise the unique benefits of T$_{RM}$ subsets.
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8 Curriculum Vitae

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Presentations

Short talk and poster presentation "Cytomegalovirus Infection Results in the Formation of CD8<sup>+</sup> and CD4<sup>+</sup> Tissue-Resident Memory T Cells in the Salivary Glands", Keystone symposium on Tissue-Resident Memory T cells, 2014, Snowbird, USA
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