Memory Lymphocyte Clusters in Genital Immunity: Role of Tissue-Resident Memory T Cells (T_{RM})

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Contents

Abstract Development of front-line defenses in genital tissues is important to inhibit viral/bacterial replication and to eliminate sexually transmitted diseases. In this chapter, we discuss the cellular composition, location, and function of memory lymphocyte clusters deployed in mucosal tissues and compare them with those in secondary lymphoid organs and tertiary lymphoid structures.

Abbreviations

Ab	Antibodies
APC	Antigen-presenting cells
BALT	Bronchus-associated lymphoid tissues

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Blimp1	B lymphocyte-induced maturation protein-1
CLA	Cutaneous lymphocyte-associated antigen
DC	Dendritic cells
DLN	Draining lymph nodes
FABP	Fatty-acid-binding proteins
FcRn	Neonatal Fc receptor
FDC	Follicular dendritic cells
FRT	Female reproductive tract
HBV	Human hepatitis B virus
HEV	High endothelial venules
HPV	Human papilloma virus
HSV	Herpes simplex virus
ICAM-1	Intercellular adhesion molecule-1
IFITM3	Interferon-induced transmembrane protein 3
Ig	Immunoglobulin
KLF	Kruppel-like factors
LA	Lactic acid
LC	Langerhans cells
LN	Lymph nodes
LT	Lymphotoxin
LTβR	LT β receptor
LTi	Lymphoid tissue inducer cells
LTo	Lymphoid tissue organizer cells
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MLC	Memory lymphocyte clusters
NK	Natural killer
pIgR	Polymeric Ig receptor
RANK	Receptor activator of nuclear factor κB
PNAd	Peripheral node addressin
S1PR1	Sphingosine-1-phosphate receptor 1
SLO	Secondary lymphoid organs
STD	Sexually transmitted diseases
T_{CM}	Central memory T cells
TCR	T cell receptor
TD	Thoracic duct
T_E	Effector T cells
T_{EM}	Effector memory T cells
TF	Transcription factor
TGF-β	Transforming growth factor-beta
Th	T helper cells
TK	Thymidine kinase
TLS	Tertiary lymphoid structures
T_M	Memory T cells
TNF	Tumor necrosis factor

Treg	Regulatory T cells
T_{RM}	Tissue-resident memory T cells
VCAM-1	Vascular cell adhesion molecule-1
VEDC	Vaginal epithelial DC
VEGFC	Vascular endothelial growth factor C
WT	Wild-type

1 Introduction

Over the past three decades, various types of lymphoid aggregates have been found in the skin, mucosal tissues, and central nervous system. In contrast to secondary lymphoid organs, lymphoid aggregates in peripheral tissues are not preprogramed in early postnatal life. Instead, chronic inflammation, viral/bacterial infection, the onset of autoimmune diseases, and tumorigenesis can trigger the genesis of lymphoid aggregates containing lymphocytes and stromal cells. These include memory lymphocyte clusters (MLC) and tertiary lymphoid structures (TLS) such as inducible Bronchus-associated lymphoid tissues (iBALT).

In this chapter, we will compare and contrast the cellular composition, distribution, and function of MLC with that of TLS formation and secondary lymphoid organs (SLO) containing lymph nodes (LN). Given that tissue-resident memory T cells (T_{RM}) accumulate in MLC, we will discuss current knowledge regarding the function of T_{RM} retained in peripheral tissues, including in MLCs, and consider the implications of these cells for the development of next-generation medical treatments.

2 Tertiary Lymphoid Structures and Memory Lymphocyte Cluster-like Structures

SLO, including the white pulp of the spleen, LN, the appendix, and mucosal-associated lymphoid tissues are required to initiate the generation of the antigen-specific immunity mediated by T and B cells (van de Pavert and Mebius 2010). In this respect, SLO have all the functionality to maximize the adaptive immune response in terms of the localization of immune cells (Schulz et al. 2016). Stromal cells, antigen-presenting cells (APC) including dendritic cells (DC), follicular DC (FDC), macrophages, and B cells and naïve T cells are strategically deployed in SLO (Fig. 1a) (Randall et al. 2008). In addition, SLO are genetically programmed to be generated in predetermined places within the body (Drayton et al. 2006). During the development of SLO, CXCL13 produced by resident mesenchymal cells initially tempts the precursors of $CD45^+$ $CD3^-$ $CD4^+$ $CXCR5^+$

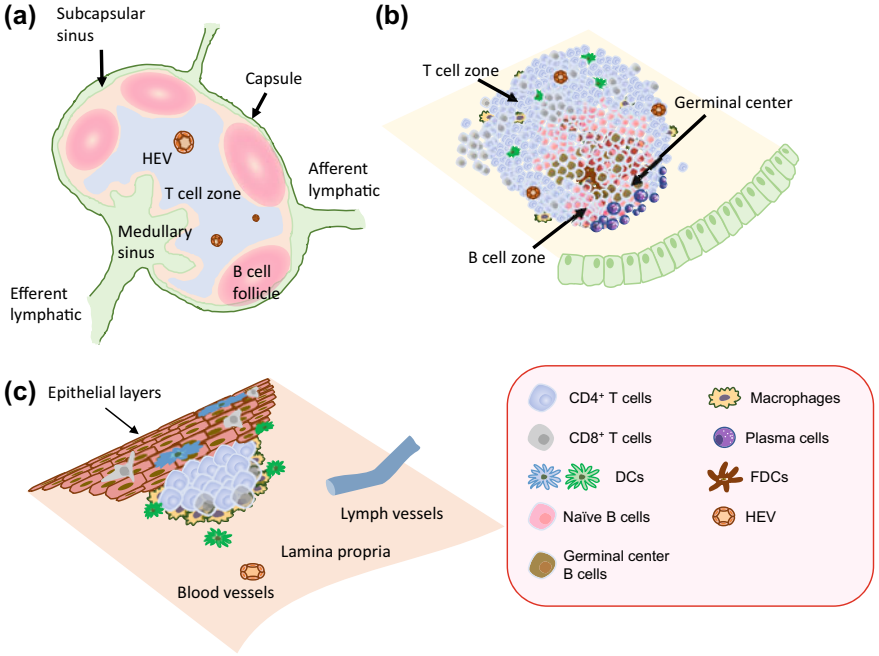

Fig. 1 Schematic representation of LN, TLS, and MLC. **a** LN comprise organized cell populations, including immune cells (T cells in T cell zones and B cells in B cell zones) and stromal cells covered by a fibrous capsule and an underlying subcapsular sinus. In addition, LN contain lymphatic vasculature (afferent and efferent lymphatics) and HEV. **b** The majority of TLS are not encapsulated. Instead, TLS form lymphoid aggregates consisting of T cell zones containing naïve T cells, T_{CM}, DC, macrophages, HEV, and fibroblastic reticular cells and a B cell zone with a germinal center, plasma cells, and FDC. **c** MLCs are formed beneath mucosal epithelial layers with the clusters of $CD4^+$ T_{RM}, $CD8^+$ T_{RM} and MHC class II^+ APC

lymphoid tissue inducer cells (LTi), also known as type 3 innate lymphoid cells bearing RAR-related orphan receptor gamma t to the lymph node anlagen from neighboring blood vessels. Thereafter, the accumulation of receptor activator of nuclear factor κB $(RANK)^+$ LTi facilitates the expression of lymphotoxin (LT) α1β2 and tumor necrosis factor (TNF)-related activation-induced cytokine so that LTi can activate LT β receptor $(LT\beta R)^+$ stromal cells to differentiate into lymphoid tissue organizer cells (LTo) (Chang and Turley 2015). As a consequence of LTβR and RANK activation, LTo attract other hematopoietic cells, mainly lymphocytes through the expression of CCL19 and CCL21 as well as further recruitment of LTi for the development of LN tissue progenitors. Simultaneously, LTo express adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) to adhere to infiltrating immune cells (Denton et al. 2019). Thereafter, LTo give rise to various LN stromal subsets, including FDC and marginal reticular cells (Barone et al. 2016). At this time, high

endothelial venules (HEV) express MAdCAM-1 but not peripheral node addressin (PNAd). During the maturation of LN, HEV become $PNAd^+$ and down-regulate MAdCAM-1 in the majority of peripheral LN, although in some LN (mesenteric, sacral, cervical), HEV express both PNAd and MAdCAM-1 (Ager 2017; Bistrup et al. 2004; Soderberg et al. 2004).

In some cases, acute inflammation within peripheral tissues results in prolonged production of CXCL13 and IL-7 from stromal cells to attract LTi, which produce IL-17 and LT $\alpha 1\beta 2$ along with the production of TNF-alpha (TNFα) and LTα. In response to the recruitment of LTi to the site of inflammation, the inflammatory response of T helper cell 17 (Th17) cells, B cells, and macrophages is suggested to initiate the generation of TLS in various pathological contexts (Guedj et al. 2014; Lochner et al. 2011; Peters et al. 2011). At the site of inflammation, the TLS initiator cells, including LTi and Th17 cells, interact with $LT\beta R^+$ local stromal cells through LT$\alpha 1\beta 2$ secretion so that stromal cells produce vascular endothelial growth factor C (VEGFC) to promote HEV development in peripheral tissues (Furtado et al. 2007). Likewise, stromal cells also secrete CCL19, CCL21, CXCL12, and CXCL13 and upregulate ICAM-1, VCAM-1, and MAdCAM-1 to recruit lymphocytes and promote TLS structural organization, including a T cell zone with clusters of $TCR\beta^+$ cells and a follicular $CD20^+$ B cell zone (Fig. 1b) (Barone et al. 2016). FDC differentiation from local fibroblasts is especially critical for the development of B cells in TLS (Sautes-Fridman et al. 2019). In these areas, the majority of lymphocytes are $CD62L^+CD44^-$ naïve T cells, naïve B cells, and $CD62L^+CD44^+$ central memory T cells (T_{CM}).

Draining LNs (DLN) are connected with peripheral tissues through afferent lymphatics, providing entry sites for invading pathogens (Fig. 1a). There is no doubt that SLO are indispensable for the induction of robust antigen-specific immunity, while, in general, it takes 5–7 days to generate antigen-specific effector T cells (T_E) that are poised to migrate to the site of infection (Permanyer et al. 2018). Meanwhile, pathogens invading through mucosal tissues are able to replicate and spread rapidly to various organs. In this regard, tertiary lymphoid structures (TLS) in peripheral tissues are capable of immediately challenging invading pathogens. It is well known that TLS-like structures appear in peripheral tissues following local inflammation, viral or bacterial infection, tumor progression or the onset of autoimmune diseases (Corsiero et al. 2019; Fleige et al. 2014; Fridman et al. 2017; Kabashima et al. 2019; Lucchesi et al. 2014; Moyron-Quiroz et al. 2004). Compared with LN, the majority of TLS lack an organized clustering of immune and stromal cells encapsulated by coated layers composed of a fibrous capsule and a subcapsular sinus (Fig. 1b). In most cases, TLS represent a non-encapsulated aggregation of immune and stromal cells confined in an organ or peripheral tissues (Alsughayyir et al. 2017).

In peripheral tissues, MLC-like structures are found in the female reproductive tract (FRT), skin, ocular tissue, and intestine in humans and rodents (Fig. 1c) (Collins et al. 2016; Iijima et al. 2008a; Morrison and Morrison 2000; Reacher et al. 1991; Zhu et al. 2009). In contrast to typical TLS formation, $PNAd^+$ HEV, $CD35^+$ cells, and B cell areas are lacking within these MLC, indicating that naïve T cells,

naïve B cells and $CD62^+$ $CD44^+$ T_{CM} are largely absent. Instead, the center of all known MLC consists of $CD11b^+$ and/or CX_3CR1^+ APC (Fig. 1c). In addition, $CD4^+$ T_{RM} mainly form clusters with APC in close proximity to the mucosal epithelium, whereas $CD8^+$ T_{RM} are scattered in the cluster. The majority of $CD103^+$ $CD8^+$ T_{RM} are retained in the epidermis or mucosal epithelium, which is required for signaling via the transforming growth factor-beta (TGF-β) cascade. In contrast, $CD8^+$ T_{RM} in lymphoid clusters located in the intestine express CD103 at low levels; therefore TGF-β signaling pathway is likely to be dispensable for the maintenance of $CD103^-$ $CD8^+$ T_{RM} in the intestinal lamina propria (Bergsbaken and Bevan 2015). Instead of TGF-β-mediated retention control, antigenic stimulation appears to be involved in the residency of $CD103^-$ $CD8^+$ T_{RM}. In the vaginal lamina propria and skin dermis, both IFN-γ from $CD4^+$ T_{RM} and CCL5 from APC are required for the integrity of MLC formation (Collins et al. 2016; Iijima and Iwasaki 2014). However, the mechanisms of MLC development remain unknown, although LTα-mediated pathways are not likely to be involved in the retention of virus-specific T_{RM} in genital tissues following immunization with attenuated HSV (Roth et al. 2013).

3 Protective Immunity in Genital Mucosa

Both the female and male genital tracts are essential for fertilization and pregnancy. Given that the secretion of sex hormones is tightly regulated during the menstrual cycle, immune responses in the genital microenvironment are uniquely affected by hormone control. At the timing of conception, the trophoblast cells of the embryo adhere to the uterine lining and then invade into the maternal uterine decidua (Schatz et al. 2016). Following trophoblast differentiation to form a mature placenta, the immune response at the maternal-fetal interface establishes tolerance over time (Munoz-Suano et al. 2011). Natural killer (NK) cells and APC, including DC and macrophages in the decidua in close proximity to invading trophoblasts and paternally-derived alloantigens, are detected in the developing placental and fetal tissues (Houser et al. 2011). Their interaction triggers a sequence of immune responses that are initiated prior to conception and persist through gestation and delivery of the newborn. Among innate immune cells, $CD56^{hi}$ $CD16^{lo}$ NK cells, which are unique to the uterus, accumulate in the decidua during the progestational phase of the menstrual cycle after implantation is initiated (Koopman et al. 2003). The uterine NK cells are essential for placental development through the provision of growth factors and facilitation of adaptations to the uterine vasculature to help trophoblast invasion (Beaman et al. 2014). In terms of APC, decidual macrophages promote a tolerant microenvironment in healthy individuals through the expression of PD-1 and M2-like characteristics, whereas PD-1 is down-regulated on decidual macrophages that possess the M1 phenotype in patients of recurrent miscarriage (Shimada et al. 2018; Zhang et al. 2018). DC that accumulate in the uterus participate in decidual angiogenesis by secreting soluble fms-like tyrosine kinase-1 and

TGF-β1, which is independent of immunological tolerance (Plaks et al. 2008). Thereafter, Collins MK et al. (2009) demonstrated that DC enriched in the decidua fail to migrate into DLN following exposure to fetal/placental antigens, indicating that the decidual DC play a critical role in blocking immune rejection of the fetus (Collins et al. 2009). In addition to innate immune cells, the adaptive immune response based on regulatory T cells (Treg) and effector/memory T cells is also important for pregnancy tolerance. In particular, to allow reproduction, Treg are necessary to control inflammation in decidual tissues for embryo implantation and progression of gestation (Robertson et al. 2018).

In the human uterine endometrium and cervix, lymphoid clusters containing $CD8^+$ T cells and B cells develop in the stratum basalis of the endometrium during the proliferative phase of the menstrual cycle (Johansson et al. 1999; Wira et al. 2014; Yeaman et al. 2001). Given that the clusters are not found at menses or during gestation, their formation is likely to be under the control of the menstrual cycle (Yeaman et al. 1997). $CD20^+$ B cells are located at the center of lymphoid clusters and are surrounded by $CD45RO^+$ $CD8^+$ T cells and macrophages. Remarkably, these $CD45RO^+$ $CD8^+$ T cells express high levels of CD69 (Yeaman et al. 2001), suggesting that these memory $CD8^+$ T cells are a tissue-resident population. Similarly, structured lymphoid clusters are found in rats and mice and they enlarge to form mural structures by day 10.5 of gestation. As for the development of secondary lymphoid organs, LTα and/or LTβ play essential roles in initiating their organization. LTα is secreted from endometrial stromal cells and transformed decidual cells in human pregnancy (Vince et al. 1992). LTα and LTβ are detected in placental cells, fetal trophoblasts, and decidual macrophages (Phillips et al. 2001). In contrast, the formation of lymphoid clusters in the uterine endometrium does not appear to be affected by the expression of LTα or LTβ, indicating that the clusters are typical tertiary lymphoid structures (Kather et al. 2003; Moyron-Quiroz et al. 2004). Given that the size and maintenance of these lymphoid clusters are influenced by sex hormones, their formation in the uterus indicates involvement from pregnancy to childbirth, although their exact function remains unknown.

For genital tissues to perform their reproductive functions without problems, a local immune response must protect against causative infectious agents of sexually transmitted diseases (STD) (Iwasaki 2010). To this end, genitourinary tracts must deploy highly robust and effective first-line of defenses against viral, bacterial, fungal, and parasitic pathogens, while maintaining tolerance to allo-immune responses against spermatozoa and the semi-allogeneic fetus (Wira et al. 2014).

In contrast to other mucosal tissues, the immune defense system in the FRT is affected by its distinctive features of anatomy, hormonal control, mucus, microbiota, lymphatics, and cellular composition (Deruaz and Luster 2015; Iwasaki 2016; Wira et al. 2015). In terms of vaginal microbiota, the FRT is dominated by *Lactovacillus* species, which may play a role in protection against HIV-1 transmission (Chen et al. 2017). In addition, reduced colonization of vaginal lactobacilli is closely associated with the onset of opportunistic diseases and an increased risk of HIV-1 infection (Quinn and Overbaugh 2005). As a potential mechanism of

protection mediated by *Lactovacillus* species, vaginal *Lactobacillus* spp. is known to secrete antimicrobial factors, such as bacteriocins and organic acid metabolites, mainly lactic acid (LA). Protonated LA levels in cervicovaginal fluid inactivate HIV-1 replication; while the exact mechanism of this remains unclear, and it is not likely to be mediated by lactate anions or pH (Tyssen et al. 2018). Microbiota, including vaginal lactobacilli, has distinct types of immunoregulatory properties that have an impact on unique characteristics and functions of resident immune cells (Song et al. 2018).

To drive the robust protective immunity mediated by antigen-specific T and B cells, tissue-resident DC have highly potent antigen-presenting capacity to initiate the antigen-specific immune response (Iwasaki 2007). Therefore, the phenotypic and functional signatures of DC in genital tissues determine the quality of immune protection. The phenotypic features of DC in genital tissues are quite distinct from those of DC in other tissues (Iijima et al. 2008b; Parr et al. 1991). In particular, vaginal epithelial DC (VEDC) consist of several distinct populations in mice (Iijima et al. 2007) and humans (Ballweber et al. 2011; Duluc et al. 2013), whereas Langerhans cells (LC) ($CD207^{hi}$ $CD326^{+}$ $CD11c^{+}$ $MHCII^{+}$ DC) are predominantly located in skin epidermis (Merad et al. 2002). Likewise, dendritic cell populations in ocular and oral epithelia can be segregated into at least two distinct populations based on the expression of CD11b and CD103 ($F4/80^{-}$) (Capucha et al. 2015; Hattori et al. 2011). These findings suggested the fate of dendritic cell differentiation depending on whether stratified squamous epithelia is cornified or not. In fact, in adult skin epidermis, LC are not replenished by circulating BM-derived precursors (Hovav 2018). Instead, Langerhans cell precursors enter the epidermis during embryonic development and then self-renew there (Chorro et al. 2009; Hoeffel et al. 2012). During early embryogenesis, yolk sac macrophages give rise to LC. In addition, LC are likely to arise from liver monocytes (Hoeffel et al. 2012). In contrast, VEDC develop from circulating BM-derived precursors, including monocytes, common dendritic cell progenitors, and pre-DC (Capucha et al. 2015; Iijima et al. 2007). As another hallmark of DC in epithelia, skin, oral, and ocular DC expresses high levels of CD207, whereas VEDC express CD207 at low levels (Capucha et al. 2015; Duluc et al. 2013), suggesting that CD207 expression in vaginal tissues is influenced by unknown genital-specific factors. These findings indicate that the final differentiation of genital DC is determined by the properties of genital tissues. As for the function of DC in genital tissues, submucosal DC in the vaginal lamina propria but not VEDC are required for the generation of Th1 cells in DLN (Zhao et al. 2003), whereas VEDC appear to be involved in the differentiation of Th17 cells in DLN (Anipindi et al. 2016; Hervouet et al. 2010). To control the migration of these DC, CTLA4 expressed on $FoxP3^{+}$ Treg is involved in the recruitment of vaginal DC into DLN to initiate antigen-specific T cell responses (Lund et al. 2008; Soerens et al. 2016). In addition, $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC in DLN present viral antigens to $CD8^{+}$ T cells to differentiate into IFN-γ producing cells, whereas the origin of DC in vaginal tissues remains elusive (Lee et al. 2009). After migratory DC leave genital tissues, rapid recruitment of $Ly6C^{hi}$ monocytes from the blood occurs in vaginal tissues (Iijima et al. 2007). Thereafter, $Ly6C^{hi}$ monocytes

capture viral antigens and upregulate MHC class II and costimulatory molecules (Iijima et al. 2011). Furthermore, the monocyte-derived APC are able to present antigens to effector Th1 cells that subsequently enter into vaginal tissues. In addition, the reactivated Th1 cells are critical for inhibiting viral replication in the vaginal epithelium (Iijima et al. 2011). With respect to the function of other dendritic cell subsets, topical application of aminoglycoside antibiotics (neomycin) to vaginal tissues triggers the recruitment of $CD103^+$ $XCR1^+$ DC, which is responsible for a significant upregulation of interferon-stimulated genes in vaginal tissues. This contributes to the protection against vaginal HSV-2 and Zika virus infection in a microbiota-independent manner (Gopinath et al. 2018).

More than 30 pathogens cause STD. With regard to bacterial infection, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum,* and *Trichomonas vaginalis* lead to reproductive failure and possible death if untreated. In terms of virus infection, human papillomavirus (HPV) infection causes cervical cancer. In addition, HIV and genital herpes cause chronic infection and are difficult to completely cure. Thus, safe and effective vaccines to prevent sexually transmitted infections are urgently needed. However, vaccines are currently only available for two of pathogens causing STD (HPV and hepatitis B virus (HBV)), despite many of vaccine trials having been conducted. Protection against HPV infection by the prophylactic vaccine is thought to be largely mediated by antibodies (Ab); however, the control of pathogens that replicate intracellularly is likely to require cellular immunity, requiring the development of therapeutic vaccines. To block invading pathogens binding with host cells, including epithelial cells, Ab must be secreted from the mucosal lamina propria into the lumen. In FRT, the upper genital tract is classified as a type I mucosal surface, which consists of simple columnar epithelium. On type I mucosal epithelial cells, both neonatal Fc receptor (FcRn), which plays a prominent role in the transport of immunoglobulin G (IgG) into the lumen, and polymeric Ig receptor (pIgR), which binds dimeric IgA to transport it into the lumen, are expressed. However, the lower genital tissues, including the ectocervix and vagina, are classified as type II mucosal surfaces and are covered by stratified squamous epithelial cells. On type II mucosal epithelia, FcRn but not pIgR is expressed, indicating that IgG but not IgA is the main effector molecule inhibiting the attachment of infectious agents to epithelial cells in the lower reproductive tract. For IgG to exert a protective function against invading pathogens, Igs in the FRT must be adequately delivered to the mucosal lumen. In addition, Ig levels in the lumen of genital tissues are influenced by sex hormones. (Wira et al. 2015). In fact, IgG levels in secretions from uterine tissues are much higher than those in the fallopian tube during the periovulatory phase (Safaeian et al. 2009a). Furthermore, following HPV vaccine immunization, transition to the mid-cycle during ovulatory cycles causes a dramatic decrease in IgG levels that react to HPV virus-like particles in cervical secretions (Nardelli-Haefliger et al. 2003). Collectively, unique immune defense systems that adapt to the estrous cycle are strategically deployed in genital tissues.

It is currently unclear whether hormonal control affects the efficacy of HPV vaccines, but licensed prophylactic vaccines against HPV infection are highly

effective in preventing the infection (Joura et al. 2015). In contrast, no vaccine trials for genital herpes have succeeded in blocking HSV-2 infection in vaginal tissues. Following HPV vaccines or HSV vaccine candidate immunization, high titers of antigen-specific Igs are maintained in peripheral blood for over years and long-term antibody responses have also been observed (Belshe et al. 2012; Slifka and Amanna 2019). Contrary to serum antibody responses, IgA and IgG levels in cervical tissues are not associated with levels in blood (Safaeian et al. 2009b). In addition, Ig levels in genital tissues are markedly lower than those in peripheral blood, indicating that passive transfer of IgA and IgG thorough pIgR and FcRn into the lumen of genital tissues does not completely explain the mechanism of Ig transport. To establish HPV infection, the virus must reach epithelial basal cells with stem cell-like properties at the bottom of an epithelium. Following wound or epithelial trauma, HPV viral particles are able to access to the epithelial stem cells (Egawa et al. 2015). HSV infection, however, does not need epithelial trauma to access epithelial basal cells because it directly infects vaginal epithelial cells through viral entry receptors, including nectin-1 (Linehan et al. 2004). Recently, Oh JE et al. (2019) clearly demonstrated that the levels of antigen-specific Ab are significantly increased in the murine vaginal lumen following epithelial barrier breach (Oh et al. 2019), indicating that following epithelial trauma, leakage of HPV-specific Ab into the lumen blocks the establishment of HPV infection in individuals who received a prophylactic HPV vaccine. Furthermore, the mouse model evidence explains why the robust antibody responses induced by a prophylactic for HSV-2 vaccine are entirely ineffective at preventing the infection.

4 Generation and Function of T_{RM} in Peripheral Tissues

Following immunization or exposure of vaginal tissues to a pathogen, DC localized in the vaginal epithelium and lamina propria capture the antigen and migrate into DLN to generate antigen-specific T_E (Deruaz and Luster 2015; Iwasaki 2010). Once T_E migrate from DLN to the infection site, including the mucosa, they have the intrinsic capability to combat a replicating pathogen by means of cytotoxic activity and cytokine production. However, after the clearance of an invading pathogen or a few weeks after immunization, T_E mostly lead to apoptosis during a contraction phase (Badovinac et al. 2002). This process is likely to be preprogrammed to cease an undesirable inflammatory response and tissue damage mediated by T_E. However, a small number of T_E differentiate into long-lived memory T cells (T_M). T_M are able to rapidly exert a powerful effector function based on cytokine production and cytotoxic activity following reinfection with the same pathogen. This hallmark of T_M substantially contributes to combat against a secondary encounter with the same pathogen. Therefore, the generation of T_M following primary infection with a pathogen or immunization with a vaccine is an important component in achieving long-term protection and developing effective therapeutics. Two distinct types of T_M are generated: central memory T cells (T_{CM}) and effector memory T cells (T_{EM})

(Sallusto et al. 1999). T_{CM} express LN homing receptors, including CCR7 and CD62L, so that T_M can access to LN, which consist of a network of $CCL19^+$ stromal cells and $PNAd^+$ endothelial cells. Given their phenotypic features, T_{CM} are able to circulate between the blood and LN by entering the efferent lymph and returning to the circulation via the thoracic duct (TD) (Sigmundsdottir and Butcher 2008). In contrast, T_{EM} barely express CCR7 and CD62L. Instead, T_{EM} express tissue-homing receptors including α4β7 and CCR9 (for intestine), α1β1 (VLA-1), CXCR3 and CXCR6 (for lung tissues), CXCR3 and CCR5 (for genital tissues), α4β1 (VLA-4), CXCR3 and CCR6 (for neuronal tissues) and E-selectin ligands, P-selectin ligands, CCR4, cutaneous lymphocyte-associated antigen (CLA) and CCR8 (for skin tissues) (Bromley et al. 2008; McCully et al. 2012). Accordingly, it is conceivable that almost all T_{EM} continuously circulate between the blood and peripheral tissues. To elucidate the mechanism by which T_{EM} move from tissues, Bromley SK et al. (2005) and Debes GF et al. (2005) demonstrated that CCR7 expressed on T cells is involved in their exit from peripheral tissues, indicating that CCR7 might be upregulated on T_{EM} (Bromley et al. 2005; Debes et al. 2005). However, in various experimental settings, including pathogen infection, asthma and autoimmune diseases, $CD44^{hi}$ $CD62L^-$ T_E in peripheral tissues are $CCR7^{low}$ and direct evidence of continuous circulation was nearly lacking (Jameson and Masopust 2018; Masopust and Soerens 2019). Similarly, sphingosine-1-phosphate receptor 1 (S1PR1) guides naïve $CD4^+$ T cells but not activated T cells toward afferent lymphatic vessels (Ledgerwood et al. 2008). These data indicate that S1PR1 rather than CCR7 signaling might regulate the movement of T cells to the afferent lymphatics from tissues. However, the detailed mechanism by which T cells enter the lymphatic system remains to be fully elucidated. As such, recent findings of $CD8^+$ T_M subsets based on the expression of CX_3CR1 provide a more feasible classification of T_M to extend the traditional concept regarding T_{CM} and T_{EM}. Gerlach C et al. identified three distinct populations of murine T_M: CX_3CR1^{lo} $CXCR3^+$ $CD8^+$ T_{CM}, CX_3CR1^{int} $CXCR3^+$ $CD8^+$ $T_{M,}$ and CX_3CR1^{hi} $CXCR3^-$ T_{EM} (Gerlach et al. 2016). Both CX_3CR1^{lo} $CXCR3^+$ $CD8^+$ T_{CM} and CX_3CR1^{int} $CXCR3^+$ $CD8^+$ T_M are $CD62L^+$ $CD27^+$; therefore, that they can access LN. In contrast, surprisingly, CX_3CR1^{hi} $CXCR3^-$ T_{EM} are not found in peripheral tissues. Instead, CX_3CR1^{int} $CXCR3^+$ $CD8^+$ T_M are able to survey peripheral tissues. Furthermore, CX_3CR1^{int} $CXCR3^+$ $CD8^+$ T_M are responsive to CCL19 and are detected in TD lymph regardless of CD62L expression, indicating that this population is a genuine continuously circulating T_M (Gerlach et al. 2016). In human blood, CX_3CR1^+ $CD62L^-$ and $CX_3CR1^+CD62L^+$ $CD8^+$ T_M are also characterized (Bottcher et al. 2015). Both have the capability to secrete granzyme B and perforin, which are identical to T_{EM}, while CX_3CR1^- $CD62L^+$ $CD8^+$ T_M are able to produce a high level of IL-2 which are analogous to T_{CM}. As for continuously circulating T_M, $CD69^-$ $CD8^+$ T_M, which resemble CX_3CR1^{int} $CXCR3^+$ $CD8^+$ T_M, recirculate between skin tissues and blood under the control of TGF-β activation through keratinocytes expressing $\alpha_v\beta6$ and $\alpha_v\beta8$ (Hirai et al. 2019). In peripheral tissues, approximately 10%-20% of T_M are CX_3CR1^{int} $CXCR3^+$ $CD8^+$ T_M, and the

remaining population represents CX_3CR1^- $CD69^+$ $CD62L^-$ $CD103^{+/-}$ $CD8^+$ T_M, which are distinct from T_{EM} and T_{CM} (Gerlach et al. 2016).

Over the past decade, many groups have tried to clarify whether T_M detected in the peripheral tissues are continuously circulating cells or tissue-resident populations (Clark et al. 2012; Masopust et al. 2001). To answer this question experimentally, a parabiosis technique involving the sharing of blood circulation between two mice confirmed that a distinct subset of T_M representing the $CD44^+$ $CD62L^-$ $CD69^+$ $CD103^{+/-}$ population are T_{RM} that are largely disconnected from blood circulation (Iijima and Iwasaki 2014; Jiang et al. 2012; Klonowski et al. 2004; Schenkel et al. 2013; Teijaro et al. 2011). Furthermore, using photoconversion with the parabiosis technique, Park et al. (2018a) elegantly demonstrated that a minority of non-photoconverted circulating $CD4^+$ T_M, $CCR7^+$ $CD62L^-$ $CD69^-$ cells is completely segregated from $CD4^+$ T_{RM} in the skin dermis following *Candida albicans* infection (Park et al. 2018a), which is consistent with a similar population of previously reported circulating CX_3CR1^{int} $CXCR3^+$ T_M (Bromley et al. 2005; Debes et al. 2005; Gerlach et al. 2016; Hirai et al. 2019).

In human tissues, based on phenotypical and functional features of T_{RM}, T_M with a similar phenotype to mouse T_{RM} have been observed (Clark 2015). In a study of patients with genital herpes, HSV-specific CD8αα memory T cells were identified at the dermal-epidermal junction for a long time following resolution of HSV reactivation (Zhu et al. 2013). Furthermore, following alemtuzumab treatment (an anti-CD52 antibody), Watanabe R et al. (2015) elegantly demonstrated that a large number of T cells with an effector function analogous to that of T_{RM} remained in the skin of leukemic cutaneous T cell lymphoma patients despite complete depletion of all circulating T cells (Watanabe et al. 2015). These data directly confirmed the existence of long-lived T_{RM} in human peripheral tissues. In contrast to long-term resident T_{RM}, a minority population of $CD103^+$ CLA^+ $CD69^+$ $CD4^+$ T_{RM} down-regulates CD69 and then transits from the skin to the blood circulation (Klicznik et al. 2019). In particular, $CD103^+$ CLA^+ $CD4^+$ T_{RM} have the capability to produce IL-22 and IL-13 but not IL-17, IFN-γ, IL-4 and GM-CSF, indicating that control of retention is largely mediated by cytokine production from $CD4^+$ T_{RM}.

T_{RM} are mainly found to be seeded in non-lymphoid tissues, particularly at barrier tissues such as mucosal tissues and skin. As tissue-resident T cell populations, $CD8^+$ T_{RM} and $CD4^+$ T_{RM} are found in a variety of peripheral tissues, while their location, differentiation process, phenotypical, and functional features differ as described below.

4.1 The Role of $CD8^+$ T_{RM} in Barrier Tissues

Naïve $CD8^+$ T cells have the capacity to migrate the blood circulation, lymphoid tissues (LNs, spleen and Peyer's patches), and return to the blood via the efferent lymph. In contrast, $CD8^+$ T_M ($CD44^{hi}$, $CD62L^{lo}$) are distributed in peripheral tissues, especially, in the epithelia but not in lamina propria or dermis, long after the

clearance of a pathogen (Masopust et al. 2001). Based on their phenotypic characteristics, the majority of $CD8^+$ T_{RM} in skin epithelium, vaginal epithelium, lung tissues (airway, lung interstitium, and upper respiratory tract), brain, salivary grand, liver, and kidney express high levels of CD103 and CD69 (Masopust and Soerens 2019). In particular, CD103 is required for $CD8^+$ T_{RM} retention in skin epidermis (Mackay et al. 2013), and is likely to be involved in the survival of $CD8^+$ T_{RM} in brain tissue following virus infection (Wakim et al. 2010). CD103 appears to interact with E-cadherin expressed on epithelial cells; therefore, CD103 would be essential for the retention of $CD8^+$ T_{RM} in squamous epithelia (type II mucosa) and monolayer epithelium (type I mucosa) but not in brain tissues. To differentiate into $CD8^+$ T_{RM}, T_{RM} precursors in blood must enter peripheral tissues following some sort of stimulus. Topical application of CXCR3 ligands or nonspecific inflammatory stimuli were able to tempt T_{RM} precursors from the blood into peripheral tissues (Mackay et al. 2012; Shin and Iwasaki 2012); therefore, both $CXCR3^{hi}$ $CX3CR1^-$ T_{CM} and $CXCR3^{hi}$ $CX3CR1^{int}$ T_M have the potential to differentiate into T_{RM}. Following transit into peripheral tissues, TGF-β signaling can control the expression of CD103, which is indispensable for the maintenance of $CD8^+$ T_{RM} in skin epidermis and gut tissues (Casey et al. 2012; Mackay et al. 2013; Sheridan et al. 2014; Skon et al. 2013; Zhang and Bevan 2013). Furthermore, Mohammed J et al. (2016) demonstrated that retention of $CD8^+$ T_{RM} requires $\alpha_v\beta6$ and $\alpha_v\beta8$ expressed on keratinocytes through latent TGF-β activation in the epidermis (Mohammed et al. 2016). In the intestine, $\alpha_v\beta6$ controls the residence of $CD8^+$ T_{RM}, indicating that $\alpha_v\beta6$ plus $\alpha_v\beta8$ or $\alpha_v\beta6$ are necessary for $CD8^+$ T_{RM} to reside in type II mucosa or type I mucosa, respectively.

With regard to factors for $CD8^+$ T_{RM} survival, the expression of Bcl2 is significantly increased in $CD8^+$ T_{RM} in skin epidermis (Park et al. 2018b) and intestine (Bergsbaken and Bevan 2015) indicating long-term persistence in tissue. Of note, Wakim LM et al. (2013) demonstrated that $CD8^+$ T_{RM} maintain a high level of interferon-induced transmembrane protein 3 (IFITM3) expression to survive in lung parenchyma following influenza infection (Wakim et al. 2013). In skin epidermis, IL-15 produced by radio-resistant cells is also essential for the long-term survival of $CD8^+$ T_{RM} (Adachi et al. 2015; Mackay et al. 2015). Furthermore, fatty-acid-binding proteins 4 and 5 (FABP4 and FABP5) and P2RX7 play pivotal roles in the maintenance and function of long-lived $CD8^+$ T_{RM} mediated by regulating oxidative metabolism through exogenous free fatty acid intake (Pan et al. 2017) or by promoting mitochondrial homeostasis (Borges da Silva et al. 2018). However, it is unclear whether Bcl2, IFITM3, IL-15, FABP4, FABP5, and P2RX7 share commonalities to control the survival of $CD8^+$ T_{RM} in mucosal tissues and skin.

In addition to $CD103^+$ $CD8^+$ T_{RM}, long-lived $CD103^-$ $CD8^+$ T_{RM} have been found in intestine (Bergsbaken and Bevan 2015), brain (Steinbach et al. 2016), liver (Mackay et al. 2016), and secondary lymphoid organs (Beura et al. 2018; Schenkel et al. 2014b). Although $CD103^+$ $CD8^+$ T_{RM} need keratinocyte-mediated TGF-β activation for their retention, $CD103^-$ $CD8^+$ T_{RM} appear to require interaction with $CD4^+$ T cells and CX_3CR1^+ APC, which form clusters in the lamina propria of the intestine (Bergsbaken and Bevan 2015), suggesting that the recognition of cognate

antigen is involved in the retention. This is in contrast to CD103$^+$ CD8$^+$ T_{RM}, which maintain the residency in a local antigen-independent manner.

As is the case for CD103, upregulation of CD69 in CD8$^+$ T_{RM} has also been reported in various tissues. At the beginning of an immune response, CD69 is an early activation marker that is triggered by type-I interferon signaling in DLN. Upon the upregulation of CD69 on activated T cells, receptor sphingosine 1-phosphate receptor 1 (S1PR1) is down-regulated so that these T cells are retained in DLN and differentiate into T_E (Matloubian et al. 2004). Once T_E move to the blood circulation, the level of CD69 expression is low, whereas, following tissue-resident memory T cell differentiation in tissues, CD69 is re-expressed on T_{RM}. Skon et al. (2013) elegantly elucidated that repression of S1PR1 and the transcriptional factor, KLF2 (Kruppel-like factors 2), which controls expression of S1PR1, is required for the establishment of CD8$^+$ T_{RM} (Skon et al. 2013), indicating that downregulation of S1PR1 expression is also critical for CD8$^+$ T_{RM} generation. However, in various types of experimental setting, including influenza infection, chronic virus infection and protein immunization, the requirement of CD69 for the maintenance of CD8$^+$ T_{RM} varies according to the type of peripheral tissue (Beura et al. 2018). This indicates that other factors also control the expression of KLF2 and S1PR1 for retention (Walsh et al. 2019).

KLF2 downregulation has been extensively recognized as a hallmark of T_{RM}. Recently, homolog of B lymphocyte-induced maturation protein-1 (Blimp1) in T cells (Hobit) was identified to be remarkably upregulated in skin-resident CD8$^+$ T_{RM}, NKT cells and liver-resident NK cells (Mackay et al. 2016). Both Hobit and Blimp-1 control the expression of KLF2, S1PR1, CCR7, CD69, and cytotoxic molecules including granzyme B and TRAIL. In contrast to skin epidermis, Blimp-1 rather than Hobit is required for the establishment of CD8$^+$ T_{RM} in lung tissues (Behr et al. 2019). Furthermore, in human, the level of Hobit expression in T_M in blood is higher than that in T_{RM} in brain (Smolders et al. 2018). With regard to other transcription factors related to the control of CD8$^+$ T_{RM}, RUNX3 also regulates multiple targets that influence the retention of CD8$^+$ T_{RM} (Milner et al. 2017). Hence, CD8$^+$ T_{RM} development might be regulated by an intricate network of transcription factor expression that is influenced by each tissue microenvironment.

An intriguing feature of T_{RM} is their ability to exert a rapid effector function following re-encounter with the same pathogen at the site of infection. The presence of CD8$^+$ T_{RM} facilitates the clearance of invading pathogens, including viruses, bacteria, and fungi. Rapid secretion of cytokines (IFN-γ and TNF-α) and cytotoxic molecules, including granzyme B and perforin, directly blocks the replication of previously encountered pathogen in infected cells. T_{RM} therefore contribute substantially to prevent the spread of infectious agents (Mackay et al. 2013). Furthermore, immediate production of IFN-γ and TNF-α reinforces innate immunity, including DC maturation and NK cell activation, and triggers robust production of chemokines and rapid recruitment of leukocytes from blood circulation (Schenkel et al. 2014a; Schenkel et al. 2013). Collectively, CD8$^+$ T_{RM} are able to orchestrate immediate robust protective immunity as a front-line of defense.

The topical application of chemokines including CXCL9, surfactants including nonoxynol-9 or local antigen expression, recruits circulating $CD8^+$ T cells to mucosal tissues, leading to their retention as $CD8^+$ T_{RM} in the epithelium and lamina propria (Cuburu et al. 2019; Mackay et al. 2012; Shin and Iwasaki 2012; Takamura et al. 2016). These findings have important implications for the future development of effective mucosal vaccines.

4.2 The Role of $CD4^+$ T_{RM} in Peripheral Tissues

Over the last few decades, the existence of $CD4^+$ T cells in addition to $CD8^+$ T cells in local tissues has been observed (Reinhardt et al. 2001). At the same time, tissue-tropic T_{EM}-like cells circulate in blood until they are recruited to site of inflammation, while non-inflamed local tissues contain very few T cells. T cells that had migrated into tissues during infections were thought to either exit the tissue or undergo apoptosis after clearance of the infection. However, $CD4^+$ T cells and $CD8^+$ T cells that accumulate at the site of virus infection or protein immunization have been observed (Masopust et al. 2001; Reinhardt et al. 2001). In addition, activated $CD4^+$ T cells ($CD25^+$ and $CD69^+$) persist in lung tissues for several months following virus infection (Hogan et al. 2001). Furthermore, $CD4^+$ T cells that enter into peripheral tissues barely proliferated (Reinhardt et al. 2003), indicating that $CD4^+$ T cells that settle within peripheral tissues have a unique system to control their retention (Schenkel and Masopust 2014).

Similar to $CD8^+$ T_{RM}, $CD4^+$ T_{RM} localize to non-lymphoid tissues, such as skin and mucosal tissues of the lung, small intestine, and FRT for a long period of time (Collins et al. 2016; Glennie et al. 2015; Iijima and Iwasaki 2014; Iijima et al. 2008a; Stary et al. 2015; Steinfelder et al. 2017; Teijaro et al. 2011; Turner et al. 2018; Wilk et al. 2017). As is the case of $CD8^+$ T_{RM}, the majority of $CD4^+$ T_{RM} express high levels of CD69, but CD103 expression varies according to tissue. Contrary to $CD8^+$ T_{RM} that are retained in skin epidermis or mucosal epithelia, the majority of $CD4^+$ T_{RM} are distributed in the skin dermis or mucosal lamina propria, suggesting that the retention mechanism of $CD4^+$ T_{RM} is distinct from that of $CD8^+$ T_{RM}. TGF-β signaling contributes to the maintenance of $CD8^+$ T_{RM} in the epithelial layer, whereas, for IFN-γ^+ $CD4^+$ T_{RM}, the formation of clusters with other resident immune cells, including $CD8^+$ T cells and APC such as macrophages and DC, is required for the retention in the lamina propria of the FRT, lung, and skin dermis (Acosta-Ramirez et al. 2016; Collins et al. 2016; Iijima and Iwasaki 2014). In contrast, IL-17^+ $CD4^+$ T_{RM} do not form clusters in skin dermis following *Candida albicans* infection but these cells are retained in the superficial dermis (Park et al. 2018a). Following skin HSV infection, the localization of IFN-γ^+ $CD4^+$ T_{RM} is, however, confined to lymphocyte clusters formed around hair follicles (Collins et al. 2016). Therefore, each $CD4^+$ T_{RM} subset is suggested to have its own retention-control system in peripheral tissues.

Substantial advances have been made in understanding the differentiation of $CD8^+$ T_{RM}; however, the detailed molecular mechanism of $CD4^+$ T_{RM} development remains relatively elusive. Given the existence of functionally distinct effector Th subsets, such as Th1, Th17, and Th2, identification of $CD4^+$ T_{RM} precursors is much more complicated. Furthermore, both $CD4^+$ T_E and $CD4^+$ T_M exhibit substantial plasticity and easily convert to different linages (Hegazy et al. 2010). Similarly, in $CD8^+$ memory T cell development, $KLRG1^{hi}$ $CD127^{lo}$ cells were identified as a terminal effector population, while the $KLRG1^{lo}$ $CD127^{hi}$ subset contains memory precursor cells with the potential to differentiate into long-lived $CD8^+$ T_M (Joshi et al. 2007). In contrast, Marshall HD et al. (2011) clearly demonstrated that the expression of CD127, the IL-7 receptor α chain, does not characterize memory precursors from $CD4^+$ T_E (Marshall et al. 2011). Instead, $PSGL1^{hi}$ $Ly6C^{lo}$ T-bet^{int} $CD4^+$ T_E become long-lived T_M, such as $CCR7^{hi}$ $CD62L^{hi}$ T_{CM}. Interestingly, the gene expression level of CX_3CR1 in the $PSGL1^{hi}$ $Ly6C^{lo}$ T-bet^{int} population is significantly lower than that in the $PSGL1^{hi}$ $Ly6C^{hi}$ T-bet^{hi} population, which resembles T_{EM}-like cells, indicating that the definition of $CD4^+$ T_M subsets can also be classified by CX_3CR1 expression. It still remains unclear whether the precursor for $CD4^+$ T_{RM} originates from T_{CM}, T_{EM}-like cells or another type of memory T cell. A recent finding clearly demonstrated that effector Th17 cells reached in lung tissues differentiate into $CD4^+$ T_{RM} that have the capability to produce IFN-γ but not IL-17 (Amezcua Vesely et al. 2019), although it remains unknown whether all $CD4^+$ T_{RM} originate from effector Th17 cells.

For long-term survival of $CD4^+$ T_{RM} in peripheral tissues, $CD4^+$ T_{RM} must receive survival signals within the local microenvironment during their generation and differentiation. The common gamma-chain cytokines, including IL-2, IL-7 and IL-15 play pivotal roles in the generation of $CD4^+$ T_M. Among them, IL-2 receptor signaling is necessary for the generation of $CD4^+$ T_{RM} in lung tissues following the induction of allergic asthma or lymphocytic choriomeningitis virus infection (Hondowicz et al. 2016, 2018). The generation of $CD4^+$ T_{RM} through an IL-2-independent pathway has also been reported following lung influenza infection. Besides, IL-2-independent $CD4^+$ T_{RM} require IL-15 for T cell activation, whereas IL-15 was redundant for the maintenance of $CD4^+$ T_{RM} (Strutt et al. 2018). The maintenance of long-lived $CD4^+$ T_M, however, requires IL-7 signaling because IL-7R is highly expressed on naïve $CD4^+$ T cells and $CD4^+$ T_M (Seddon et al. 2003). In a skin model of contact hypersensitivity, $CD4^+$ T_M in skin tissues fail to be retained following ablation of IL-7 from epidermal keratinocytes, indicating that IL-7 signaling in the local microenvironment is essential for the entry or survival of $CD4^+$ T_M (Adachi et al. 2015). Likewise, following lung *Klebsiella pneumonia* infection, $CD4^+$ T_{RM} derived from Th17 cells express high levels of CD127 so that the cells can be maintained in lung tissue through IL-7-mediated signaling (Amezcua Vesely et al. 2019). In contrast, the expression of CD127 on $CD4^+$ T_{RM} is comparable to that on circulating $CD4^+$ T_{EM} in Peyer's patches (Ugur et al. 2014). Similarly, the expression of CD127 on $CD4^+$ T_{RM} was significantly lower

than that on circulating $CD4^+$ T_{EM} in the FRT (Beura et al. 2018). Furthermore, a parabiosis study demonstrated that the level of CD122 expression on $CD4^+$ T_{RM} is almost identical to that on circulating $CD4^+$ T_{EM} in the FRT (Beura et al. 2018), suggesting that IL-15 signaling is also dispensable for the retention of $CD4^+$ T_{RM} in genital tissues. Thus, the requirement of IL-7 and IL-15 for the survival of $CD4^+$ T_{RM} appears to be dependent on the properties of peripheral tissues. Collectively, the inflammatory status and immune responses in tissue microenvironments are more likely to shape the retention and function of $CD4^+$ T_{RM} compared with $CD8^+$ T_{RM}.

For this reason, the topical application of any product is not able to generate MLC formation containing $CD4^+$ T_{RM}. Currently, charge-switching synthetic adjuvant particles containing UV-inactivated *Chlamydia trachomatis* is the only topically applied artificial compound that has generated $CD4^+$ T_{RM} in the uterine mucosa (Stary et al. 2015). This finding has profound implications for the development of other vaccines, although it remains unknown whether the retention of $CD4^+$ T_{RM} themselves is sufficient to inhibit the spreading of invading pathogens.

5 Generation and Maintenance of MLC in Genital Tissues

For almost two decades, lymphocyte aggregates, memory lymphocyte clusters (MLC), have been reported in genital tissues following HSV-2 or *Chlamydia trachomatis* infection; however, the exact function of MLC remained elusive until fairly recently (Gillgrass et al. 2005; Morrison and Morrison 2000). In contrast to TLS, $CD62L^{hi}$ $CD44^{lo}$ naïve T cells are not found within the clusters of $CD4^+$ T cells, $CD8^+$ T cells and APC. Similarly, the expression of PNAd, CD31, lymphatic vessel endothelial hyaluronan receptor 1 and CD21/CD35 as the basal components of TLS is not detected within MLC (Fig. 1c) (Table 1), indicating that MLC are not the sites for induction of antigen-specific effector T cells from naïve T cells (Iijima and Iwasaki 2014, 2015). Furthermore, the expression of CCL19, CCL21, CXCL12, and CXCL13 is not found in MLC. Instead, CXCL9 and CCL5 were detected after MLC formed in vaginal tissues (Iijima and Iwasaki 2014) (Table 1). No MLC-like structure has been demonstrated to be affected by sex hormones, except for lymphoid aggregates centered around $CD20^+$ B cells in the uterine endometrium.

At present, MLC are mainly observed in the lamina propria beneath vaginal epithelial layers, which is the entry site of the HSV-2 virus. However, *Chlamydia*-induced MLC are found in vaginal tissues, cervix, and fallopian tubes. Thus, the mechanism of MLC generation and maintenance following HSV-2 infection and *Chlamydia trachomatis* infection is shown separately as follows.

Table 1 Components, immune cells, and fibroblastic cells in secondary lymphoid organs, tertiary lymphoid structures, or memory lymphocyte clusters

	Encapsulated	T cell zone	B cell zone	HEV	Naive T and B cells	T_{CM}	T_{RM}	FDC	Chemokines and cytokines
SLO	+	+	+	+	+	+	+	+	LT, IL-17, CCL19, CCL21, CXCL13
TLS	−	+	+	+	+	+	N/D	+	IL-17, CCL19, CCL21, CXCL13
MLC	−	+	−	−	−	−	+	−	CCL5, CXCL9

N/D not determined
SLO secondary lymphoid organs; *TLS* tertiary lymphoid structures; *MLC* memory lymphocyte clusters; *HEV* high endothelial venules; T_{CM} central memory T cells; T_{RM} tissue-resident memory T cells; *FDC* follicular dendritic cells; *LT* lymphotoxin; *CCL* CC-chemokine ligand; *CXCL* CXC-chemokine ligand

5.1 MLC Formation Following HSV-2 Infection

HSV-2 is one of the most prevalent sexually transmitted pathogens with more than 400 million people infected worldwide. HSV-2 is a double-stranded DNA virus that belongs to the *Herpesviridae* family and is a major cause of genital herpes, symptoms of which include recurrent local inflammation along with severe pain in genital tissues leading to reduced quality of life (Schiffer et al. 2018). Following massive HSV-2 replication in the genital epithelium, the virus enters peripheral nerve terminals and then travels to dorsal root ganglia to replicate and/or lead to latency (Diefenbach et al. 2008). During latent infection in neuronal tissues, viral particles and viral DNA are rarely detected in vaginal and neuronal tissues. Instead, latency associated transcripts are abundantly localized in neuronal cell bodies but not genital tissues. Despite the lack of HSV-2 replication in vaginal tissues, lymphoid aggregates have been observed in human and mice (Gillgrass et al. 2005; Iijima et al. 2008a; Kiviat et al. 1990; Zhu et al. 2009), although their significance remained unclear until recently. To prevent HSV-2 infection in genital tissues and to treat HSV-2-mediated diseases, a large number of prophylactic vaccine trials have been performed; however, none of these trials has succeeded in generating a genital herpes vaccine (Awasthi et al. 2014; Belshe et al. 2012; Corey et al. 1999; Stanberry et al. 2002). In a murine model of genital herpes infection, intravaginal immunization with live attenuated HSV-2 was highly effective at inducing protective immune responses and the protection against HSV-2 (McDermott et al. 1984; Parr et al. 1994). Furthermore, at least four weeks after thymidine kinase negative (TK^-) HSV-2 intravaginal immunization, both T cells and IFN-γR signaling were required to inhibit virus replication in murine vaginal tissues following wild-type (WT) HSV-2 intravaginal challenge (Milligan and Bernstein 1997;

Milligan et al. 1998; Parr and Parr 1998, 1999). Likewise, in a guinea pig model of vaginal HSV-2 infection, $CD4^+$ T cells were required to the control HSV recurrence (Bourne et al. 2019). In contrast, intravaginal immunization with TK^- HSV-2 in B cell-deficient mice protected against HSV-2 secondary challenge, whereas intranasal immunization with attenuated virus in B cell-deficient mice allowed WT HSV-2 to invade neuronal tissues to replicate following intravaginal challenge (Iijima and Iwasaki 2016; Milligan et al. 2004). Furthermore, HSV-2-specific Ab required circulating $CD4^+$ T_M cells to achieve protection in neuronal tissues against WT HSV-2 intravaginal challenge (Iijima and Iwasaki 2016; Morrison et al. 2001). These findings suggested that tissue-resident immunity in vaginal tissues is critical for rapid viral clearance to block viral spreading into neuronal tissues. Certainly, HSV-specific $CD4^+$ T cells are found to accumulate in lamina propria of vaginal tissues four weeks after intravaginal immunization with TK^- HSV-2 (Iijima et al. 2008a). Furthermore, $CD4^+$ T cells form clusters beneath the vaginal epithelium (Roth et al. 2013). To inhibit HSV-2 replication in vaginal tissues, MHC class II^+ APC, including $CD11c^+$ DC and $CD20^+$ B cells are required for the IFN-γ-mediated but not cytotoxic function of $CD4^+$ T_M following HSV-2 secondary challenge (Iijima et al. 2008a). To dissect the mechanism for the maintenance of $CD4^+$ T_M in vaginal tissues following TK^- HSV-2 immunization, immunized C57BL/6 mice were conjoined with immunized congenic C57BL/6 mice to examine whether vaginal $CD4^+$ T_M constitute a tissue-resident population or a continuously circulating population. Two to seven weeks after surgery, HSV-2-specific $CD4^+$ T_M were predominantly a host-derived and not a blood-derived population (Iijima and Iwasaki 2014), indicating that these $CD4^+$ T_M are identified as T_{RM}. Furthermore, HSV-2-specific $CD4^+$ T_M bearing TCRVβ1 accumulate within MLC, although viral antigen sequence recognized by TCRVβ1 remains unclear. Regarding phenotypic features of $CD4^+$ T_{RM} in MLC, CD44, CD69, and CD49d are highly expressed, while CD103 and CD62L are rarely detected in HSV-2-specific $CD4^+$ T_{RM}. Remarkably, the expression of KLF2, KLF13, CCL5, and S1PR1 is barely observed in $CD4^+$ T_{RM} in MLC. In contrast, $CD4^+$ T_{RM} in MLC express T-bet, Bcl-xL and Bcl-2, indicating that IFN-γ-producing $CD4^+$ T_{RM} in MLC are a long-lived population (Iijima and Iwasaki 2014) (unpublished data). To maintain the formation of MLC, $CD11b^+$ macrophages within MLC are required and these cells secrete CCL5. Although viral genomic DNA or RNA transcripts have not been detected in vaginal tissues of TK^- HSV-2 immunized mice, constitutive low level secretion of IFN-γ from $CD4^+$ T_{RM} is observed in vaginal tissues (Iijima and Iwasaki 2014), indicating that $CD11b^+$ macrophages that stimulate $CD4^+$ T_{RM} to secrete IFN-γ produce CCL5 as a feedback loop mechanism. One of the mechanisms regarding MLC maintenance has, therefore, been elucidated but adhesion molecules and transcription factors that define $CD4^+$ T_{RM} and APC within MLC remain unknown. In the lamina propria of vaginal tissues, collagen type III, collagen type IV or desmin but not collagen type I is broadly distributed as major constituents of basement membranes, connective tissues and filaments (Fig. 2). In addition, PDPN/$gp38^+$ fibroblastic reticular cells and ER-$TR7^+$ fibroblasts are not found within MLC (Fig. 2). Future studies may

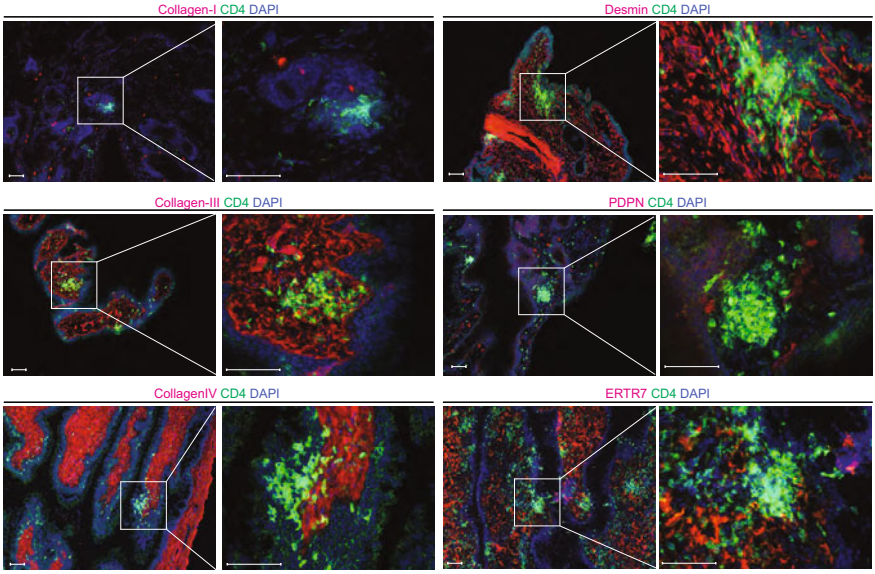

Fig. 2 Expression of collagen I, collagen III, collagen IV, desmin, PDPN or ER-TR7 in the lamina propria of vaginal tissues following immunization with attenuated HSV-2. C57BL/6 mice were immunized intravaginally with attenuated HSV-2. Five weeks later, frozen sections of vaginal tissue were stained with antibodies against collagen I, collagen III, collagen IV, desmin, PDPN/gp38 or ER-TR7 (red) and CD4 (green). Nuclei were stained by 4′,6′-diamidino-2-phenylindole (DAPI) (blue). Images were captured using a 10x or 40x objective lens. Scale bars indicate 100 μm

elucidate in more detail the interaction between $CD4^+$ T_{RM} and the network of fibroblastic reticular cells in genital tissues.

5.2 *MLC Formation Following* Chlamydia *Infection*

Chlamydia trachomatis infection of the genital mucosa causes an STD that is prevalent worldwide. Following invasion into the columnar epithelial cell lining of the endocervix and endometrial epithelium of the fallopian tubes, *Chlamydia trachomatis* triggers persistent inflammation leading to cause urethritis, proctitis, cervicitis, endometritis, and salpingitis (Poston and Darville 2018). More than six distinct types of curative antimicrobial drugs are available; however, there is no effective vaccine to prevent *Chlamydia* infection because of several strategies to evade the host immune response. Protective immunity against *Chlamydia* infection develops in DLN and genital tissues (Johnson and Brunham 2016); however, there is currently no critical target to exploit for development of an effective vaccine. Several groups has demonstrated that mice deficient in CD4, MHC class II, IL-12 or IFN-γ have increased susceptibility to genital *Chlamydia* infection (Morrison et al.

1995; Morrison et al. 2000; Perry et al. 1997; Wang et al. 1999), indicating that IFN-γ producing $CD4^+$ T cells are required for the inhibition of *Chlamydia* replication. Similarly to genital HSV-2 infection, lymphoid aggregates containing $CD4^+$ T cells beneath the epithelium have been observed after genital *Chlamydia* infection (Kiviat et al. 1990; Morrison and Morrison 2000). Interestingly, in children with active trachoma caused by ocular *Chlamydia* infection, lymphoid follicles also develop in the conjunctiva, in which the majority of immune cells are B cells not T cells (el-Asrar et al. 1989). In contrast, in adults infected with *Chlamydia trachomatis*, along with conjunctival scarring, lymphoid aggregates were observed in conjunctiva and a large number of $CD4^+$ T cells but not B cells formed clusters (Reacher et al. 1991), indicating that age-related factors might affect the composition of immune cells in lymphoid aggregates. It remains unknown whether age-related factors also influence the generation of MLC following genital *Chlamydia* infection. In a murine model of genital *Chlamydia* infection, $CD11b^+$ cells in addition to $CD4^+$ cells are the predominant cell populations within MLC-like structures in uterine tissues, which are analogous to MLC in the conjunctiva of adults (Morrison and Morrison 2000). Although the existence of MLC following *Chlamydia* infection has been confirmed by several groups (Johnson and Brunham 2016), it remains totally unknown whether $CD4^+$ T_M within MLC are a tissue-resident population or if they maintain an effector function for some time after *Chlamydia* infection. Stary G et al. (2015) elegantly demonstrated that intrauterine and intranasal immunization with charge-switching synthetic adjuvant particles containing UV-inactivated *Chlamydia trachomatis* succeeded in protecting against a genital *Chlamydia* challenge by generating $CD4^+$ T_{RM} in the uterine mucosa (Stary et al. 2015). Interestingly, immunization with UV-inactivated *Chlamydia* through the intrauterine route failed to achieve the protection against reinfection with *Chlamydia* because of massive accumulation of $FoxP3^+$ regulatory T cells in the uterine mucosa through the activation of tolerogenic $CD103^+$ DC. This study sheds light on the future development of an effective mucosal vaccine against *Chlamydia* infection based on the generation of protective T_{RM} in mucosal tissues.

6 Functional Features of MLC Against Sexually Transmitted Pathogens

There is currently no way to block the infection of genital epithelia by sexually transmitted pathogens, except for two viruses, HPV and HBV. In the case of HSV-2 infection, HSV gD initially binds to nectin-1 expressed on vaginal epithelial cells (Linehan et al. 2004). This interaction results in a conformational change in HSV gD so that the gH/gL heterodimer with gD induces a conformational change in HSV gB. Finally, HSV is able to fuse with nectin-1^+ epithelia through HSV gB to deliver the virion capsids containing HSV DNA (Eisenberg et al. 2012). These

glycoproteins are potential targets for prophylactic and therapeutic vaccines to induce durable memory responses of T cells and B cells. In a mouse model of genital herpes, intravaginal immunization with TK^- HSV-2 establishes complete protection against vaginal HSV-2 challenge (Parr et al. 1994). In contrast, although large numbers of T cells from TK^- HSV-2 immunized mice, which include HSV-2-specific T_E from DLN, were adoptively transferred, the recipients were only partially protected against genital herpes infection (McDermott et al. 1989), indicating that the generation of genital immunity following intravaginal immunization with TK^- HSV-2 is required for the establishment of complete protection. Subsequently, intravaginal immunization with TK^- HSV-2 was found to maximize the generation of a tissue-resident population in vaginal tissues, including MLC formation for the retention of HSV-2-specific $CD4^+$ T_{RM} (Iijima and Iwasaki 2014). To directly elucidate the requirement of tissue-resident immunity, TK^- HSV-2 immunized mice were conjoined with naïve mice to share blood circulation. In naïve pairs, HSV-2-specific cell populations, including memory T and B cells and Ab, were observed in blood, while T_{RM} were not established in vaginal tissues. Following WT HSV-2 challenge of the naïve pair, viral clearance was significantly delayed compared with challenge of the immune pair, indicating that T_{RM}, especially $CD4^+$ T_{RM}, are required for rapid viral clearance (Iijima and Iwasaki 2014).

The majority of $CD4^+$ T_{RM} are localized in the lamina propria of mucosal tissues or dermal tissues of the skin (Collins et al. 2016; Iijima and Iwasaki 2014), indicating that $CD4^+$ T_{RM} also play a prominent role in the front-line of defense against invading pathogens. Following re-encounter with the same pathogen, $CD4^+$ T_{RM} secrete high levels of IFN-γ within 12 h (Glennie et al. 2015; Iijima and Iwasaki 2014; Oh et al. 2019), suggesting that local antigen-presenting cells present viral antigens to $CD4^+$ T_{RM} instead of migrating into DLN. In addition, $CD4^+$ T_{RM} also trigger accumulation of monocytes, NK cells, and B cells from blood circulation and induce local dendritic cell activation through IFN-γ secretion and cytotoxic activity (Beura et al. 2019). Furthermore, disruption of MLC formation by $CD11b^+$ cell depletion reduced the level of CXCL9 expression in vaginal tissues following WT HSV-2 challenge, causing failure of memory B cells to migrate into vaginal tissues to secrete HSV-2-specific IgGs (Oh et al. 2019). This indicates that MLC formation is a platform for the maintenance of T_{RM} to rapidly exert their effector functions upon reencountering of invading pathogens.

7 Summary and Outlook

The generation of a robust immune response in peripheral tissues has a substantial impact on the protection against invading pathogens, the onset of autoimmune diseases, and tumor progression. On the one hand, SLO including LN are preprogrammed encapsulated organs that organize both innate and adaptive immune responses to maximize the initiation of antigen-specific immunity. On the other hand, TLS formation represents a non-encapsulated congregation of lymphocytes

and stromal cells in peripheral tissues. In both SLO and TLS, stromal cell-derived HEV develop to recruit $CD62L^+$ naïve B and T cells and T_{CM} so that these lymphocytes can undergo transition from blood circulation to B cell zones or T cell zones (Fig. 1a and b) (Table 1). In contrast, MLC lack HEV and B cell areas, and naïve T and B cells do not reside in MLC (Fig. 1c). Instead, MLC consist of $CD11b^+$ APC with $CD44^+$ $CD62L^-$ $CD69^+$ $CD4^+$ T_{RM} and $CD8^+$ T_{RM} beneath mucosal epithelial layers. SLO and TLS are, therefore, suggested to be inductive sites for antigen-specific immune responses upon encountering newly delivered antigens, while MLC appear to be specialized in the maintenance of tissue-resident memory populations in peripheral tissues to initiate rapid immune response at the mucosal surface. Currently, the molecular mechanisms underlying the generation of MLC are poorly understood. The cellular composition and distribution of MLC are obviously distinct from those of SLO and TLS (Table 1). In association with the deployment of immune cells in MLC, the findings regarding the neogenesis of iBALT formation within or outside of B cell areas following repetitive inhalation of heat-killed *P. aeruginosa* is significantly informative (Fleige et al. 2014). This study demonstrated that BALT formation mainly lacks B cell areas containing $CXCL12^+$ stromal cells in IL-17A and F deficient mice following inoculation of heat-inactivated *P. aeruginosa*, the formation consisting entirely of $CD3^+$ T cells. Consequently, the BALT formation appears to resemble MLC formation, although it remains unclear whether the B cell-deficient BALT contain $PNAd^+$ HEV and naïve T cells.

The following questions concerning MLC formation remain to be answered.

1. What type of cells initiates MLC formation in genital tissue?
2. What cytokines or chemokines are responsible for the generation of MLC?
3. Which integrins and adhesion molecules expressed on T_{RM} are involved in the interaction with stromal cells?
4. How do $CD4^+$ T_{RM} in the lamina propria attach to epithelial layers?
5. How is the effector function of T_{RM} in MLC maintained?
6. What type of APC is responsible for reactivation of T_{RM} in MLC?
7. How do antigen-captured APC reactivate T_{RM} in MLC to exert effector function?
8. How do reactivated T_{RM} in MLC migrate to the site of viral/bacterial replication?

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