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# Review

# Mucosal effector memory T cells: the other side of the coin

**H. Cheroutre* and L. Madakamutil**

The La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, California 92121 (USA), Fax: +1 858 678 4595, e-mail: hilde@liai.org

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**Abstract.** Immunological memory allows for rapid and effective protective immunity to previously encountered pathogens. New insights in understanding specific memory differentiation and function have now indicated that in addition to providing enhanced immunity, an important purpose of immunological memory is to provide immediate protection at all sites of the body, including non-lymphoid tissues. Effector memory CD8 T cells have the capacity to reside long-term at epithelial surfaces, where they allow for rapid containment of the invading pathogens at the local entry site and prevent systemic spreading and excessive immune responses. The accumulation of tissue-specific memory T cell subsets, together with cross-reactivity of these antigen-experienced T cells even to unrelated pathogens, provides flexibility and expansion of their specificity repertoire that over time greatly surpasses that of the declining naïve T cell populations. This review will discuss new insights into T cell memory. We will focus in particular on the generation and function of effector memory CD8 T cells at the intestinal mucosa, which represents one of the largest entry sites for pathogens.

**Key words.** Memory T cells; intestinal mucosal T cells; T cell differentiation; T cell priming; T cell homing; mucosal memory; systemic memory; protective immunity.

## Introduction

The adaptive immune system has the unique ability to generate immunological memory that provides the individual with long-term enhanced immunity to previously encountered pathogens. Protective immunity to re-occurring infections is provided by a combination of high-affinity neutralizing antibodies secreted by plasma cells and memory B cells together with eliminating effector functions by memory T cells. These memory lymphocytes can be defined as antigen-experienced B and T cells which clonally expanded and differentiated from primary responder cells and persist as 'memory cells' long after resolution of the initial infection. From this basic definition, one could argue that immunological memory is of little biological significance, because memory cells only emerge after a successful primary immune response. In effect, immune memory results when an immune-competent host effectively defeats the pathogen and survives the infection – without the requirement of an active immune memory. Although such arguments have some merit, they do not take into account important variable parameters, including the availability of primary effector cells at the initial site of the infection or the dose and route of the challenge. Furthermore, neutralizing antibodies might play major roles in providing systemic protective immunity, while memory T cells might play critical roles in mediating immune protection at epithelial surfaces and solid peripheral tissues not readily available to secreted antibodies.

In recent years, much new information has become available that underscores the many facets of

* Corresponding author.

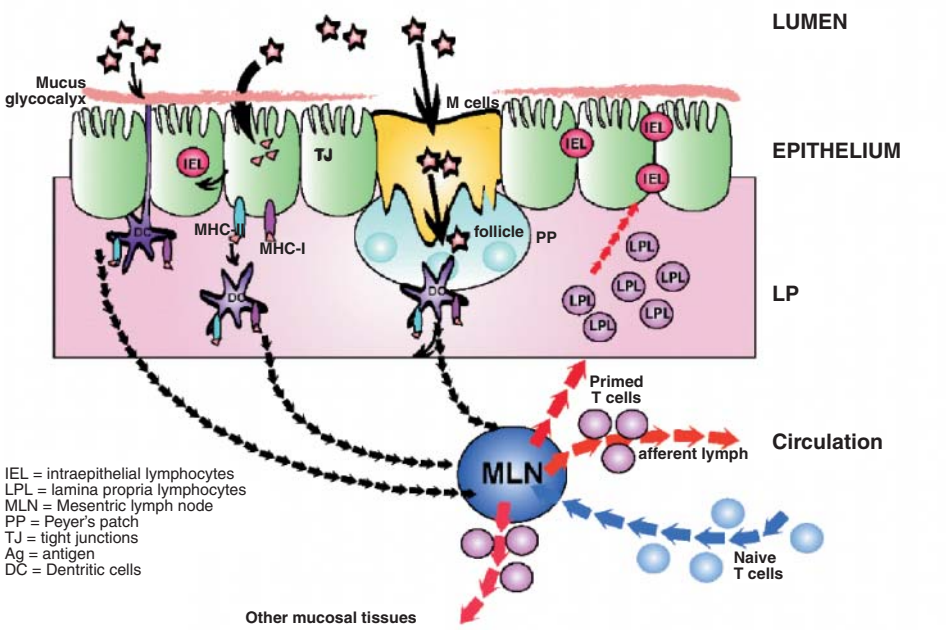


Figure 1. Lymphocytes at the gut epithelium as sentinels. Gut epithelium can be broadly classified according to immune function as immune effector and initiation sites. The effector compartment consists of the scattered LP lymphocytes and the terminally differentiated IELs seen embedded in the base of the epithelial cell layer. The initiation compartment is made of the organized lymphoid tissues, including the Peyer's patches and the mesenteric lymph nodes where naïve T cells are primed.

immunological memory and the importance of this advanced system for providing protection at all levels, ranging from self-antigen-based natural memory and maternal passive immunity for the newborn to providing long-lasting effective and immediate protection at lymphoid and non-lymphoid tissues and limiting excessive or potentially dangerous immune responses.

Although it is generally accepted that the antigen-stimulated generation of memory B cells is crucial for the effectiveness of systemic immunity against naturally occurring infections, unique and specific contributions of memory T cells are perhaps less appreciated [1]. Especially the unique capacity of certain subsets of memory T cells to provide rapid and effective but highly regulated protection at privileged sites and mucosal epithelial surfaces. Gut intraepithelial lymphocytes (IELs) are a good example of this mechanism (fig. 1); they are almost exclusively T cells, making the intestine a major T cell organ. The majority of the IELs in the small intestine in most species are CD8 T cells, which express an antigen-experienced phenotype, and immune memory provides a unifying hypothesis for understanding the phenotypic and functional characteristics of these mucosal T cells [2].

This review will focus on the complexity and diversity of T cell memory and in particular on the specific differentiation of mucosal effector memory CD8 T cells. Such differentiation endows these T cells with intrinsic functional ability to allow for the most effective immune protection at the immediate interface with the outside world and the major natural entry ports for most pathogens.

## Conventional T cell memory differentiation

Naïve T cells are mainly excluded from non-lymphoid peripheral tissues such as the gut epithelium [3–5], while they continuously circulate through secondary lymphoid tissues via the blood and lymph. They enter lymph nodes (LNs) via the high endothelial venules guided by their high levels of expression of lymph node homing receptors such as CD62L and the chemokine receptor CCR7 [6–8]. They can leave the LNs via the lymphatic vessels to re-enter the blood stream via the thoracic duct [9]. Recognition of their specific antigen, presented in the context of major histocompatibility complex (MHC) on professional antigen-presenting cells (APCs) or dendritic cells (DCs), triggers the naïve T cell to clonally expand and transform into effector cells. Costimulation by CD28 and CD40L expressed on the activated T cells, and B7-1/B7-2 and CD40, upregulated on mature DCs, further enhances the quality of the activation signal and the productive priming of the T cell [10–11]. During this primary effector phase, the activated T cell undergoes dramatic changes in terms of phenotype and functional ability as well as survival potential. Effector cells display enhanced and differential expression of cell adhesion and chemokine receptors which endow these effector cells with specific homing capacity preferentially to the site of the initial infection [3, 12–18], while they downregulate expression of the lymphoid homing receptors, CD62L and CCR7 [19–20]. CD8 T cells display enhanced expression of interferon γ (IFN-γ), and acquire cell contact-dependent effector functions with the formation

of granules containing perforin and granzymes necessary to destroy the infected tissue [21]. Following the peak of the immune response, when the acute infection has been resolved, most of the effector cells undergo apoptosis [22, 23], while a small fraction of them survive and further differentiate into long-lived memory T cells. Similarly to the primary effector cells, some of these memory T cells have the capacity to reside in non-lymphoid peripheral tissue, where they can persist long-term and provide immediate and enhanced immune protection upon re-encounter of the pathogen. This scenario not only allows for more effective immune protection but also for immune protection at peripheral sites, where naïve T cells are excluded. The presence of these effector memory T cells at the immediate entry site of pathogens also limits the chance of systemic spreading and immune pathology caused by excessive immune responses. Memory T cells further differ from naïve T cells in that they do not absolutely require stimulation by DCs in the context of appropriate costimulatory molecules. 'Non-professional' APCs, such as infected intestinal enterocytes, which normally do not express B7 *in vivo* [24, 25], might directly serve as APCs to restimulate these resident mucosal effector memory T cells or IELs (fig. 1).

## Primary effector T cells and memory precursor cells

It is not understood why some primary effector cells differentiate to memory T cells while most of them undergo apoptosis. Several studies now provide evidence that the initial differentiation of memory T cell precursors occurs during the priming phase [26–32]. Activated T cells initially upregulate anti-apoptotic factors (Bcl-$x_L$, Bcl-2), followed by a sharp decrease in expression, consistent with their resulting cell death [33]. Sustained expression of these survival factors is associated with resistance to cell death and depends on the strength of stimulation [34]. Concordantly, cytokine responsiveness of T cells, such as interleukin (IL)-15 responsiveness is regulated by activation and correlates with signal strength [34, 35]. Interestingly the enhanced expression of Bcl-$x_L$ and IL-15 responsiveness are two characteristics of memory T cells suggesting that the quality of signal strength during primary activation might contribute to the initial differentiation of memory precursor cells [34, 36]. Although survival cytokines, including IL-15 and IL-7, are known to play important roles in the generation and maintenance of memory CD8 T cells, a direct role of IL-7R or IL-15R signaling for the initial differentiation of memory T cell precursors has not been demonstrated [37, 38]. Nevertheless, several studies have indicated that the expression of high levels of IL-7R on a subset of primary CD8 effector cells is consistent with the capacity of these cells to survive and differentiate to memory T cells [26, 27]. Recently, we showed that the expression of a CD8 isoform, CD8αα, on primary activated CD8αβ T cells also marks the subset of CD8 memory precursor cells [28]. CD8αα is normally not detected on conventional T cells in the spleen or LNs, while it is constitutively expressed on the majority of T cells residing within the epithelium of the small intestine in mice [39]. Like CD8αβ, CD8αα also interacts with the conserved α3 domain of classical MHC class I molecules, but CD8αα interacts much more strongly with a non-classical class I molecule, the thymic leukemia antigen (TL) [39, 40]. Similarly to CD8αα, TL is not expressed in the periphery, but it is abundantly and constitutively expressed on the epithelial cells of the small intestine in immediate proximity to the CD8αα-expressing IELs [41]. Using *in vitro* stimulation, we showed that interaction of CD8αα on T cells with TL on the APCs promotes enhanced survival of the activated T cells while inhibiting their proliferation [28]. Because of the preferential binding of TL to CD8αα compared with CD8αβ, TL tetramers allow for specific detection of CD8αα expression even in the presence of CD8αβ expression [28, 39]. Using polyclonal activation *in vitro* or a lymphocytic choriomeningitis virus (LCMV) infection *in vivo*, we were able to show that a fraction of *in vitro* or *in vivo* activated CD8αβ$^+$ T cells transiently induced CD8αα [28]. Consistent with a memory precursor phenotype, these CD8αα$^+$ effector cells also contained high levels of Bcl-$x_L$ and showed enhanced expression of IL-7Rα and IL-15Rβ on their cell surface [28]. Adoptive transfer of sorted CD8αα$^+$ CD8αβ$^+$ TCRαβ$^+$ effector cells further demonstrated that these CD8αα$^+$ effector cells survived long-term *in vivo* and readily differentiated to mature memory T cells. Moreover, using mice deficient for a region of the CD8α-enhancer (E8I–/– mice), which fail to induce CD8αα on polyclonally activated or LCMV-specific effector cells, we further showed that the absence of efficient CD8αα induction on primary effector cells severely impairs the generation of long-lasting memory CD8 T cells *in vivo* [28]. These observations suggested an important role for transient CD8αα expression on memory precursor cells during primary activation. Consistent with activation-induced expression, CD8αα is rapidly reinduced on memory T cells upon secondary stimulation [28] or upon cytokine stimulation with IL-15 but not with IL-7 [unpublished results]. Interestingly, the majority of the intestinal mucosal CD8 memory T cells constitutively express CD8αα, suggesting that the microenvironment of the intestine might specifically promote the reinduction and persistent expression of CD8αα on primary effectors and memory T cells. In this context it should also be noted that although a human homologue for TL has not been identified, CD8αα expression has been detected on activated human

$CD8\alpha\beta^+$ T cells [42, 43]. A recent study further demonstrated that in humans, $CD8\alpha^+\beta^{low}$ cells are exclusively composed of memory T cells and that they represent direct descendants of activated $CD8\alpha^{high}\beta^{high}$ effector cells [44].

CD4 help and CD40L-CD40 interaction might also play important roles in the differentiation and maintenance of CD8 memory T cells [29–31, 36, 45–50]. It is well established that CD4 T cell help can regulate pathogen-specific CD8 memory responses. The quality of protective immunity gradually declines after initial priming in the absence of CD4 help, and helpless CD8 memory T cells respond very poorly upon restimulation [29, 31, 32]. The dependence on CD4 help for CD8 memory differentiation is important during the primary response; it is not required for secondary responses. Not only does CD4 help play a role during initial differentiation of CD8 memory precursor cells, but a recent study showed that CD4 T cell help is important for the survival and maintenance of already established CD8 memory T cells [49]. Work with adoptive transfer of *Listeria monocytogenes* or LCMV-specific CD8 memory T cells to normal mice or to CD4-deficient animals showed that CD8 memory T cells gradually decrease in MHC class II-deficient recipient mice, irrespective of whether the initial priming occurred in the presence or absence of CD4 help. In this case it was suggested that CD4 help plays a more important role in maintaining CD8 memory T cells than in initial memory programming [49]. Consistent with this hypothesis, in response to pathogens, $IL\text{-}7R^{high}$ [49] and $CD8\alpha\alpha^+$ [unpublished results] primary effector cells are present regardless of the presence or absence of CD4 help during the initial priming. It is thus possible that in the absence of sufficient danger or activation signals, CD4 help is required for the efficient differentiation of functional CD8 memory precursor T cells, and that once generated, CD8 memory T cells remain dependent to some degree on continuous CD4 help for their long-term survival and maintenance in situ.

CD40-CD40L interactions have been shown to be involved in certain steps of help [51] and CD4 T cell help-dependent priming of $CD8^+$ T cells could be prevented by blocking the CD40 interaction [52–54]. It is believed that antigen presentation by DCs to CD4 T cells induces upregulation of CD40L, which then interacts with CD40 on the DCs, allowing the DCs to actively prime the CD8 T cells with processed MHC class I restricted antigens [52]. CD8 T cells also induce CD40L, and a direct role for CD40L expressed by the CD8 T cells during cross-priming has been demonstrated. In addition, CD40 was reported to be induced on the CD8 T cells themselves, and one study showed that CD40 expressed by H-Y-specific CD8 effector cells played an important role during initial priming for the effective differentiation of functional H-Y-specific CD8 memory T cells [30]. A role for CD40 expressed by CD8 effector T cells was not observed, however, using infection with influenza [55], LCMV or *Lysteria monocytogenes* [56], suggesting that CD40 signaling directly to the CD8 T cell might play crucial roles under less optimal conditions or in the absence of sufficient danger or activation signals which also seem to require CD4 help.

It is evident from this that many factors control the initial differentiation of memory precursor cells, and that depending on the antigenic stimulus, the quality and quantity of the activation signals, the microenvironment of the initial priming and the overall condition of the organism, specific differentiation events might or might not occur that determine the fate of the responder cells and ultimately the immunity status of the individual.

## Central and effector memory T cells

Memory differentiation is certainly not a uniform process leading to a uniform memory T cell population, and memory T cell subsets can be distinguished based on phenotypic, functional and specific homing characteristics. Memory T cells can be divided into effector memory T cells ($T_{EM}$'s ) and central memory T cells ($T_{CM}$'s ) (fig. 2). Similar to primary effector CD8 T cells, $T_{EM}$'s downregulate lymphoid homing receptors such as CD62L and CCR7, thus gaining the capacity to migrate to inflamed peripheral tissue, and they display immediate effector function. By contrast, like naïve T cells, $T_{CM}$'s remain $CD62L^+$ and $CCR7^+$, and they preferentially home to the T cell areas of secondary lymphoid tissues [15, 57–59]. They are further characterized by vigorous homeostatic and antigen-driven proliferation, and although they display little or no direct effector function, $T_{CM}$'s readily proliferate and differentiate into effector cells upon secondary stimulation [15, 57–59]. The expression of CCR7 and CD62L is not a completely accurate marker for $T_{CM}$'s, and some $T_{EM}$'s express CCR7 and lack CD62L [60] while others lack CCR7 but express detectable levels of CD62L [57]. Furthermore, neither CCR7 nor CD62L expression correlates consistently with the functional characteristics of $T_{CM}$'s [61, 62]. The ratio of $T_{EM}$'s to $T_{CM}$'s varies for CD4 and CD8 T cells, and while CD4 memory T cells are predominantly $T_{CM}$'s, $CD8\alpha\beta$ memory T cells contain a higher proportion of $T_{EM}$'s [59]. Within tissues, $T_{EM}$'s are greatly enriched among mucosal tissues, including the intestine and lung, while $T_{CM}$'s reside predominantly in secondary lymphoid tissue (fig. 2). The combined expression of chemokine receptors and adhesion molecules, such as CCR4 and CLA or CCR9 and $\alpha_4\beta_7$, further subdivide $T_{EM}$'s into tissue-specific subsets [5, 63], such as skin-homing [13]

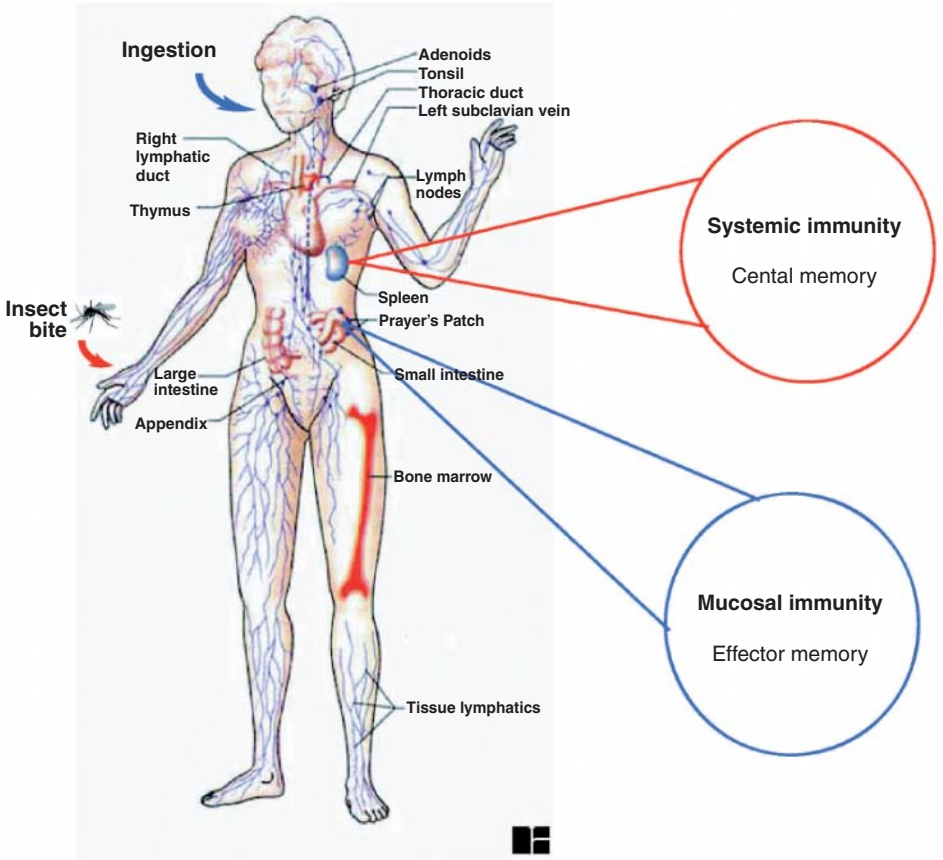


Figure 2. Various routes of infection and various subsets of memory T cells. There are multiple routes to infection. Many food-borne microorganisms are entero-invasive pathogens which have developed the means to efficiently adhere to and/or invade enterocytes and subsequently disseminate and cause systemic infections. Thus the majority of pathogens, including respiratory, gastrointestinal and sexually transmitted agents, initiate infection at mucosal surfaces. Other routes, including, animal bites, insect bites and blood transfusions or tissue transplants can transmit the pathogen directly to the blood stream, causing systemic infection. The immune system can respond to the various pathogenic invasions by providing systemic immunity to blood-borne pathogens or mucosal immunity to pathogens that gain entry through various mucosal surfaces.

or intestine-homing $T_{EM}$'s [64], respectively. While antigen stimulation can drive the proliferation of these memory T cell subsets, they also slowly turn over in the absence of T-cell-receptor (TCR) triggering [65], but driven by cytokines, including IL-7 and IL-15 [66–70]. Recent studies have also shown that this TCR-independent cytokine-mediated signaling can readily drive differentiation of $T_{CM}$'s into fully activated effector T cells [71, 72]. Using the CD127/IL-7Rα and CD62L as early markers characteristic of CD8 memory precursor cells, a recent study showed an early phenotypic and functional segregation of the two memory T cells subsets. And while $T_{CM}$'s ($CD127^{high}$ and $CD62L^{high}$) are effectively generated in the absence of CD4 help, $T_{EM}$ ($CD127^{high}$ and $CD62L^{low}$) differentiation depended on CD4 help and CD40-CD40L interactions [26]. The identification of different memory precursor subsets moves away from, but does not completely contradict, the initial 'progressive differentiation model', in which it was proposed that $T_{EM}$'s provide the first line of defense at peripheral tissues and epithelium, while $T_{CM}$'s form a reservoir that could readily replenish the $T_{EM}$ pool if needed [57, 59]. The linear relationship between the memory subsets was further challenged by the results obtained from adoptive transfer studies in mice of LCMV-specific memory T cells. It was shown that $T_{CM}$'s and $T_{EM}$'s are both capable of immediate killing of target cells in an *in vivo* cytotoxic assay [61]. Furthermore, over time $T_{EM}$'s reinduced high levels of CD62L, suggesting that in the absence of antigen, $T_{EM}$'s convert back to $T_{CM}$'s, making $T_{CM}$'s the true *in vivo* effector cells that provide long-term protection [61]. It is possible, however, that adoptive transfer and a change of their initial microenvironment might reprogram the transferred cells, providing them with differentiation patterns that do not normally occur under physiological conditions. Furthermore, and in contrast to the results from systemic LCMV infection, $T_{EM}$'s and not $T_{CM}$'s were shown to be the protective cell type, providing long-term immunity against a pulmonary infection with Sendai virus, which preferentially infects epithelial cells in the lung [73]. In support of separate lineage precursors leading to the generation of $T_{EM}$'s and $T_{CM}$'s, it was shown in a TCR repertoire analysis of influenza-specific CD8 $T_{CM}$'s ($CD62L^{high}$) and $T_{EM}$'s ($CD62L^{low/neg.}$), that the TCR repertoire of each subset is rather stable, with little or no evidence for conversion between the two subsets over time [74]. Additional data indicating expression of specific cell surface markers [59] and functional differences [75] that further characterize discrepancies

between these subsets support the hypothesis that they might represent distinct sublineages. In a very elegant system using adoptive cell transfer and parabiotic mice, it was recently shown that in fact three distinct memory T cell pools can be distinguished based on the migration ability of CD8 memory T cells [76]. There are memory T cells resident in tertiary tissues, including the lung and liver, and blood-borne memory T cells which have a broad migratory ability and the capacity to redistribute between lymphoid and non-lymphoid tissues, while other memory T cell subsets, including the intestinal mucosal CD8 memory T cells, are terminally differentiated $T_{EM}$'s which display a strict and rather permanent tissue-specific tropism and unique functional characteristics, making them a well-defined separate subset of memory T cells [76].

## Mucosal immune memory

The majority of pathogens, including respiratory, gastrointestinal and sexually transmitted agents, initiate infection at mucosal surfaces. Complex innate and mechanical mucosal defense mechanisms have evolved to limit the penetration of microorganisms and macromolecules. These mucosal barriers together with maternal passive immunity provide the initial protection for the newborn, and these barriers remain the first line of defense throughout the life of the organism. Bacterial and viral pathogens have developed complex invasive mechanisms, however, allowing them to cross these passive barriers and invade the organism. For example, many food-borne microorganisms, including *Listeria monocytogenes*, *Yersinia, Shigella, Salmonella, Toxoplasma gondii* and rotavirus are entero-invasive pathogens that have developed means to efficiently adhere to and/or invade enterocytes. These agents cause local inflammation and destruction of the intestinal mucosa or go on to cause more regional infections involving the intestinal draining LNs, the mesenteric lymph nodes (MLNs) and subsequently to disseminate and cause systemic infections (fig. 2). The resulting need for constitutive and active immunity to rapidly and effectively destroy invading pathogens and infected epithelial cells underscores the importance of mucosal T and B cell immune memory.

Although mucosal surfaces are largely protected by secretory immunoglobulin (Ig) A, T-cell-mediated immunity also plays a critical role in mucosal protection against pathogens, especially within the intestinal mucosa, where CD8 effector memory T cells form the majority of immune cells within the intestinal epithelium [2, 77]. Whereas splenic antibacterial or antiviral CD8 memory T cells do not display immediate cytolytic activity, mucosal CD8 memory T cells mediate direct lytic activity, surpassing the strength of primary effector cells [78]. The realization that these memory CD8 T cells have the ability to reside long-term at tertiary mucosal surfaces and provide immediate and enhanced protection at the most likely entry site of reinvading pathogens has significantly advanced our understanding of the selective forces driving immune memory and more specifically mucosal effector memory.

## Mucosal effector memory CD8 T cells at the intestinal frontline

Intestinal mucosal $CD8^+$ $TCR\alpha\beta^+$ T cells are a heterogeneous population of T cells in mice, and although they have the activated phenotype in common, the differentiation pathway they took to acquire the memory phenotype, the nature of the antigens they recognize and the effector functions they display are all very different. The CD8 $TCR\alpha\beta^+$ memory T cells of the intestine can be divided into two major subsets based on the differentiation process that led to their appearance. The more conventional $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ IELs can functionally be characterized as typical antigen-induced $T_{EM}$ cells [2, 79]. This population of conventional memory T cells gradually increases with age as more and more antigen-experienced T cells migrate and accumulate in the gut mucosa as long-lived memory T cells.

Concomitantly, at birth or at a young age, very few of these conventional $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ memory T cells can be identified within the intestinal epithelium or any other site. Nevertheless, unique antigen-experienced CD8 $TCR\alpha\beta^+$ T cells, which typically express $CD8\alpha\alpha$ but not $CD8\alpha\beta$, are already present in the small intestine epithelium of newborns [2, 77]. In young mice, $CD8\alpha\alpha^+$ IELs are the dominant mucosal T cell population [80], after which they are gradually taken over by the conventional $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ memory T cells. Similar to mice, the $\alpha\beta$ $TCR^+$ $CD8\alpha\alpha^+$ IEL numbers in humans gradually decline with development. They are prevalent in the human fetal intestine as early as from 12–14 weeks of gestation [81], but they are rather rare in adults.

## Mucosal $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ natural memory T cells

Mucosal $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ T cells are almost exclusively confined to the epithelium. They are rarely detected in the lamina propria (LP), blood, lymphatics or the thoracic duct [79], and never as naïve T cells in circulation or residing in secondary lymphoid tissues. They display an oligoclonal TCR repertoire that does not overlap with that of the $CD8\alpha\beta^+TCR\alpha\beta^+$ mucosal memory T cells [82], indicating that these IEL subtypes have differentiated along diverse pathways. Additionally, autoreactive TCRs

accumulate among the TCRs expressed by the CD8αα$^+$ TCRαβ$^+$ T cells [83–85], while they are normally deleted from the TCR repertoire of conventional T cells during negative selection in the thymus. This, together with their early appearance in neonatal mice [86] and in human fetal intestine [87], before any significant exposure to exogenous non-self antigens, supports the hypothesis that the CD8αα$^+$ TCRαβ$^+$ IELs are self-antigen-specific T cells. In addition, these T cells isolated at fetal or newborn stages already express an antigen-experienced memory phenotype [87], indicating that a distinct process of memory differentiation and different types of antigens are responsible for the generation of these self-reactive natural memory T lymphocytes.

The sequential appearance of self-antigen-specific natural memory T cells followed by non-self-antigen-specific conventional memory IELs might indicate that these T cell subsets mediate qualitatively and/or quantitatively different functions at various stages of development. It is possible that the self-antigen-specific CD8αα TCRαβ$^+$ memory T cells, which appear early on when initial colonization of the gut by commensal bacteria should be permissive, allow for immune protection by direct recognition of self-antigens induced on damaged or stressed epithelial cells. Transformed cells also induce self-antigens, and the close proximity of these self-antigen-specific memory T cells with the rapidly turning over epithelium might provide a continuous survey to eliminate transformed and malignant enterocytes.

The memory differentiation process that leads to the generation of these self-specific natural memory T cells is based on a unique selection process in the thymus, distinct from the antigen-driven memory differentiation of non-self-specific conventional CD8αβ$^+$ TCRαβ$^+$ memory T cells in the periphery. The generation of CD8αα$^+$ TCRαβ$^+$ natural memory IELs has been reviewed elsewhere and will not be further discussed here [2, 77].

## Mucosal CD8αβ$^+$ TCRαβ$^+$ conventional memory T cells

CD8αβ$^+$ TCRαβ$^+$ mucosal memory T cells have much in common with the conventional memory T cells in the periphery, yet they also display distinct characteristics in their appearance, function and conditions required for memory differentiation, which might imply a unique memory differentiation process for intestinal mucosal CD8 memory T cells.

In contrast to CD8αα$^+$TCRαβ$^+$ natural memory T cells, CD8αβ$^+$ and CD4$^+$ TCRαβ$^+$ mucosal memory T cells are not confined to the epithelial compartment, and they can be detected in the LP and circulating in the thoracic duct lymph [79]. In the epithelium of the small intestine, however, they frequently coexpress CD8αα, together with the classical TCR coreceptors CD4 [88] and CD8αβ [85]. Because LP memory T cells infrequently coexpress CD8αα, IELs may be further differentiated or further stimulated than the LP lymphocytes (LPLs) (fig. 1). This hypothesis is also supported by the observation that similar to restimulated memory T cells in the periphery [28], under inflammatory conditions CD8αα can be induced on T cells of the large intestine epithelium and in the LP [89].

Conventional TCRαβ$^+$ mucosal memory T cells express an oligoclonal TCR repertoire that differs among mice exposed to the same microenvironment [79], indicating that identical peptide antigens may stimulate diverse TCRs within individual mice. Based on the mature memory phenotype of these cells it was proposed that the oligoclonal repertoire is in part the result of repeated restimulation within the intestine leading to selection and focusing of TCR diversity [79].

The observation that the LP and IEL TCRαβ$^+$ memory T cells share some TCR-β clonotypes within the same mouse [79], suggests that they have differentiated along the same pathway. The presence of identical clones among T cell blasts circulating into the thoracic duct lymph further indicates that the mucosal memory T cells have differentiated from circulating naïve T cells [79].

## Intestinal mucosal primary effector T cells

The concept that primary effector cells actively migrate to non-lymphoid tissues, including the intestinal mucosa, was directly demonstrated in mice by following the specific migration of adoptively transferred conventional TCR transgenic T cells *in vivo* using peptide-MHC tetramers that react specifically with the transgenic TCRs [15, 18, 58, 78, 90–93]. In the absence of antigen stimulation, adoptively transferred naïve CD8αβ$^+$ T cells expressing a transgenic TCR (OT-I) specific for a chicken ovalbumin peptide (OVAp), SIINFEKL, presented by the class I molecule K$^b$, accumulate in the lymphoid tissues of the spleen and LNs but did not migrate to non-lymphoid tissues of the recipient mice. Upon immunization with whole ova (sOVA) *in vivo*, however, activated OT-I CD8$^+$ T cells readily migrated to tertiary tissues, including the LP and intestinal epithelium [90–92]. Because most of the influx of cells into the intestine occurred within a timeframe of 24 h, this provided supportive evidence that these mucosal T cells were effector T cells that had actively migrated from the periphery. Direct evidence for this was demonstrated in an elegant experiment using immunohistochemical analysis of whole-body sections to track labeled donor T cells in naïve and immunized recipient mice. While naïve

donor cells localized to lymphoid tissues only, antigen-reactive T cells redistributed and actively migrated to non-lymphoid tissues such as the intestine [58]. The T cells that migrate to the gut in response to antigen express a typical mucosal effector T cell phenotype, including expression of the mucosal integrins and downregulation of the lymph node homing receptor, CD62L. In the absence of innate stimuli, or adjuvant, sOVA-activated OT-I IEL and LPL displayed cytolytic effector functions, while their splenic counterparts did not, suggesting that the gut environment provides some kind of intrinsic stimulatory environment, perhaps provided in part by the intestinal microflora [91]. Under inflammatory conditions, using immunizations with OVA-expressing vesicular stomatitis virus (rVSV-OVA), all OT-I responder cells displayed lytic activity, including splenocytes. But the IEL response was more intense, demonstrating differences in the initial priming events between mucosal and systemic effector cells [92]. Moreover, specific differences in costimulatory requirements between systemic and mucosal priming were also demonstrated. Costimulation by CD28 and B7-1 was critical for sOVA- stimulated mucosal effector cells but not for activation of peripheral CD8 T cells, while co-stimulation was critical for all rVSV-OVA-stimulated CD8 T cells [92].

## Specific migration of activated primary effector T cells to the intestine

The interactions of integrins with mucosal addressins play a role in the specific migration of activated T cells to the intestine. Studies on mice deficient for the genes encoding integrin subunits indicate that LFA-1 [94], which interacts with intercellular adhesion molecules (ICAMs), and VLA-1, which binds to collagen [95], are important for the generation of normal IEL numbers. The $\beta_7$ integrins are also typically expressed by mucosal lymphocytes. $\beta_7$ can pair with two $\alpha$ subunits, $\alpha_4$ or $\alpha_E$, and while $\alpha_4\beta_7$ is typical expressed by LPL, $\alpha_E\beta_7$ is expressed predominantly by the IELs [2, 77]. The $\alpha_4\beta_7$ integrin binds to an Ig-like domain in the MAdCAM-1 addressin expressed by endothelium in Peyer's patches (PPs) and LP [96, 97], while $\alpha_E\beta_7$ binds to E-cadherin expressed by intestinal epithelial cells [98]. Mice deficient for $\beta_7$ integrins have a greatly reduced number of IELs [99]. Mice defective for the $\alpha_E$ subunit also have decreased IELs [100], although the decrease is less pronounced. Consistent with the importance of $\alpha_4\beta_7$ for gut-specific migration, the ability of adoptively transferred IELs to adhere to villus microvessels was inhibited by antibodies to $\beta_7$ integrin and MAdCAM-1.

DCs of the MLNs and PPs, which initially prime the naive T cells in mucosal lymphoid tissues, are known to play crucial roles during the instruction and differentiation processes that lead to efficient homing of activated T cells to the LP and epithelium (fig. 1) [17, 101, 102]. Productive stimulation by mucosal DCs uniquely upregulated the expression of the mucosal integrin $\alpha_4\beta_7$ on the T cells they activate. Specific $\alpha_4\beta_7$ integrin induction on the activated T cells, however, is not required under all circumstances for specific gut migration [103], and it was shown that $\beta7^{-/-}$ mice are able to clear an infection of gut-specific rotavirus as quickly as wild-type mice [104]. On the other hand, transferred $\alpha_4\beta_7^+$ memory CD8 T cells isolated from mice orally infected with rotavirus cleared the virus much more efficiently than $\alpha_4\beta_7^-$ memory CD8 cells [104], demonstrating that although it is not absolutely required, $\alpha_4\beta_7$ enhances specific antigen-induced trafficking to the gut.

Chemokines also play an important role in gut tropism of activated T cells, and similar to the $\alpha_4\beta_7$ integrin, the chemokine receptor CCR9 was shown to be selectively induced on effector T cells directed to the gut [16]. The ligand for this chemokine receptor, the thymus-expressed chemokine (TECK) or CCL25, is abundantly and selectively produced by epithelial cells of the small intestine but not by those of the large intestine in mice [105, 106]. Consistent with this, $CCR9^+$ IELs are found in the mouse small intestine but not in the large intestine [107]. Analysis of $CCR9^{-/-}$ mice [108, 109], however, indicated only modest decreases in IELs, with the knockout having the strongest effect of $\gamma\delta$ $TCR^+$ IELs. Treatment with CCR9-blocking antibodies likewise only partially decreased IEL numbers [16]. It is therefore possible that there is redundancy in these chemokine/chemokine-receptor systems. Imprinting by mucosal DCs is not an absolute requirement for the primed T cells to migrate to the gut, and T cells primed by spleen DCs which do not mediate active induction of gut-homing receptors still migrate to the intestine and other non-lymphoid tissues under some experimental conditions [unpublished results], emphasizing the complexity and perhaps redundancy of some of these organ-specific homing systems.

The molecular basis of the gut-homing imprint instructed by mucosal DCs is not fully understood, although recently it was shown that this might be partially due to their specific capacity to produce the vitamin A (retinol) metabolite retinoic acid (RA), which enhances the expression of retinoic acid-sensitive genes, including $\alpha_4\beta_7$ and CCR9 [110].

## Intestinal mucosal effector memory CD8 T cells

Although adoptively transferred OT-I CD8 T cells actively migrate to the intestine and differentiate into cytolytic effector cells in response to systemic

immunization with sOVA, long-lived memory CD8 T cells were not generated [92]. In sharp contrast, recipient mice immunized with rVSV-OVA generated systemic as well as mucosal memory [92]. The mucosal OT-I CD8 memory T cells displayed constitutive lytic activity characteristic of the functional phenotype of $T_{EM}$'s. Mucosal $T_{EM}$'s differentiated under specific conditions that distinguished them from other memory T cells. For example, while functional mucosal OT-I CTLs (cytotoxic T lymphocytes) were generated in $CD40^{-/-}$ animals, their numbers were drastically reduced compared with normal animals identifying a unique CD40-CD40L dependency for effective accumulation and maintenance of mucosal $T_{EM}$'s that was not observed for the spleen memory T cell pool [111]. The dichotomy between the two memory subsets originated from the initial priming, and it was shown that the accumulation of primary activated OT-I $CD8\alpha\beta^+$ mucosal T cells but not splenic OT-I $CD8\alpha\beta^+$ effectors was dependent on direct CD40L signals received by the activated OT-I CD8 T cells themselves [111]. A CD40 signal was not critical for their cytolytic differentiation, however, and the few mucosal memory $CD8\alpha\beta^+$ T cells of $CD40^{-/-}$ animals exhibited normal killing activity [111]. Mucosal memory cells display a significantly lower threshold for restimulation, and the secondary response of mucosal CD8 memory T cells in $CD40^{-/-}$ animals was equal to or even greater than that observed in the control animals [78]. CD4 help and MHC class II expression or secretion of the pro-inflammatory cytokine IL-12 by the mucosal DCs was also not required for the functional differentiation of the mucosal OT-I $CD8\alpha\beta^+$ CTLs [111], but such help was important for effective migration and accumulation of activated $CD8\alpha\beta^+$ effector T cells to the gut mucosa [93].

The mucosal $CD8\alpha\beta^+$ $T_{EM}$ cells resident to the epithelium and the LP can readily be restimulated by the numerous DCs present at that site (fig. 1). Although an important role for DCs in secondary stimulation of memory T cells was recently demonstrated [112], the lack of costimulatory requirements for recall responses by mucosal $CD8\alpha\beta^+$ $T_{EM}$'s allows these cells to be reactivated also by non-professional APCs such as the intestinal epithelial cells, thus providing a local protective immune system that can respond directly and highly effectively to infected or transformed epithelial cells (fig. 1). The generation of antigen-specific mucosal CTLs that exert potent antigen-specific cytotoxicity that exceeds the maximum cytotoxicity of their spleen counterparts has been reported in several other systems, including rotavirus [113, 114], reovirus [115–117], *Toxoplasma gondii* [118, 119], *Listeria monocytogenes* [15, 78, 93] and LCMV [120]. Long-term effective host protection by the mucosal $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ memory T cells has been directly demonstrated by adoptive transfer of these cells to recipient mice that were orally or systemically infected with pathogens [18, 73, 92, 119–121]. These studies indicated that mucosal memory could provide effective immune protection not only of mucosal tissues but systemically as well.

## Mucosal versus systemic immune memory

The ability of adoptively transferred mucosal memory T cells also to provide protection systemically is consistent with the observations that reactivated memory T cells can migrate to all tissues. This behavior is in sharp contrast to adoptively transferred resting memory T cells, including mucosal memory T cells, which rapidly migrate to every tissue except the brain and intestinal mucosa [76]. These observations suggest that the resting mucosal $T_{EM}$ pool is rather intrinsic to the intestine and that once established, intestinal mucosal T cells do not actively recirculate nor do blood-borne or tissue-specific memory T cells contribute much to the steady state pool of mucosal memory T cells of the intestine. Such a unique distribution of tissue-specific memory T cell pools could indicate that different memory differentiation processes can lead to unique memory T cell subsets. The existence of a separate memory differentiation pathway that leads to bona fide intestinal mucosal effector memory T cells is consistent with their distinct functional characteristics and the unique requirements for their initial differentiation. Intestinal mucosal $T_{EM}$'s also display some phenotypic characteristics, including the expression of typical homing and chemokine receptors, but also the constitutive expression of CD69 [76] and $CD8\alpha\alpha$, while expression of IL-7R$\alpha$ is low or not detectable [unpublished observations]. Similarly to CD69, $CD8\alpha\alpha$ is induced upon antigen-mediated activation of $CD8\alpha\beta$ T cells [28]. However, expression of CD69 and $CD8\alpha\alpha$ is transient on the peripheral T cells and is rapidly lost after stimulation [28, 76]. Work with parabiotic mice showed that some memory CD8 T cells reinduce CD69 upon entering the intestinal mucosa [76], and $CD8\alpha\alpha$ is likewise readily induced on T cells migrating to the intestine [unpublished results]. Although it is not known which factors promote the constitutive expression of CD69 and $CD8\alpha\alpha$ on mucosal $T_{EM}$ cells, it is possible that the inflammatory milieu of the antigen-rich intestine is a promoting factor. Furthermore it is possible that the reinduction of these molecules is cytokine driven, and in that context we were able to show that antigen-experienced but not naïve T cells readily induce $CD8\alpha\alpha$ in response to IL-15 [unpublished results].

The presence of intestinal mucosal memory T cells could imply that selective differentiation pathways exist that give rise to unique subsets of memory T cells. In that context it was indeed demonstrated that mice immunized with rotavirus – a pathogen largely limited to the villus

enterocytes of the small intestine – generate $\alpha_4\beta_7^+$ and $\alpha_4\beta_7^-$ memory T cells [121]. Adoptive transfer of $\alpha_4\beta_7^+$ CD8 memory T cells isolated from the spleen of rotavirus-immunized donor mice was highly efficient in clearing rotavirus in chronically infected recipient mice, while the $\alpha_4\beta_7^-$ memory CD8 T cells were less efficient or ineffective depending on the cell number transferred [121]. Similar results were obtained for $\alpha_4\beta_7^+$ memory CD4 T cells [122]. These observations also indicate that intestinal mucosal priming not only leads to the generation of memory T cells residing permanently in the gut but also contributes to the recirculating memory T cell pool and to memory T cell pools at other mucosal sites. Conversely, gut-homing memory T cells are not exclusively generated upon priming by mucosal DCs, and spleen DCs are also able to prime T cells with homing ability to the gut. Nevertheless, it was shown that although the generation of short-term CTL memory is independent of the initial priming site and is maintained in all tissues, long-term CTL memory becomes more tissue specific over time and is dependent on the route of the initial immunization [123, 124]. This mechanism would thus indicate that maintenance of specific T cell memory against pathogens depends in part on the presence of long-lived tissue-specific memory T cells. Additionally, memory T cells might be maintained as a result of constant stimulation by persistent antigen or by cross-reactivity with related or even unrelated antigens. The phenotypic transformation of mucosal memory T cells initially supplied by blood-borne or recirculating memory T cells from other tertiary tissue would also imply that these memory T cells undergo selective modifications upon entering mucosal peripheral tissues.

## Conclusion

Why immunological memory? Perhaps one of the main goals of T cell memory is to generate compartmentalized long-term immune protection. The naïve immune system residing within secondary lymphoid tissues leaves tertiary non-lymphoid tissues largely unguarded. The ability of memory T cells to home and reside long-term in non-lymphoid tissues, preferentially to the tissue where the initial exposure to the pathogen first occurred, provides the immune system with a mechanism for targeting long-term immune protection to the site where the chance for re-entry of that particular pathogen is most likely (fig. 2). Additionally, the capacity of memory T cells to initially migrate to all tissues, regardless of where the pathogen was first encountered, allows the immune system to guard all potential entry ports, at least short-term, at a time when it is probable that the pathogen is still present. Eventually, memory T cell pools compartmentalize and reside long-term at the original site where the pathogen first invaded and related tissues (fig. 2). The gradual accumulation of memory T cells upon various antigen exposures generates an expanding pool of memory T cells with diverse specificities, at the same time that the pool of recent thymic emigrants and naïve T cells declines. The repertoire diversity of the memory pool is further expanded by the ability of these antigen-experienced T cells to participate in immune responses to related or even unrelated pathogens through the recognition of cross-reactive antigens [125–128].

Memory differentiation thus presents a dynamic mechanism for the adaptive immune system to guard and survey all tissues of the body for infected or transformed cells, and to provide immediate and highly effective long-term protection against a wide variety of pathogens. Recently it was proposed that the effector cells and effector memory T cells form a separate branch of the adaptive immune system defined as effector lymphoid tissue, or ELT, that is distinct from the primary and secondary lymphoid tissue [129]. It was further proposed that the ultimate goal of the immune system is to gradually build up the ELT to establish immune protection at all sites [129]. The presence of highly effective immune cells poised to act at mucosal tissues throughout the body makes teleological sense, as the majority of pathogens invade the body through mucosal barriers. Not only does the intestinal epithelium form the largest interface with the outside world, but the high turnover of continuously dividing epithelial cells also imposes an imminent and persistent risk of cell transformation. It is therefore perhaps not a coincidence that the intestinal mucosa forms one of the most expanded and complex branches of the immune system, dominated by the largest and most diverse pool of effector memory T cells.

The novel findings that DCs and the local milieu provide specific imprints to differentiating naïve T cells is of utmost importance to incorporate into vaccine protocols to specifically enhance mucosal versus systemic priming. So doing will make it possible to direct long-term memory to specific tissues depending on the pathogen or the transformed cell targets. There is accordingly a need to identify and incorporate tissue-specific adjuvants and mechanisms that will specifically program the DCs either systemically or at local mucosal tissues. Such mechanisms will arm the body and, in particular, the peripheral non-lymphoid mucosal tissues with effector memory T cells as sentinels for invading pathogens or malignant cell growth.

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