Immunological Reviews

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The great balancing act: regulation and fate of antiviral T-cell interactions

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Acknowledgements
This study was supported by the National Institutes of Health (NIH) intramural program. The authors have no conflicts of interest to declare.

This article is part of a series of reviews covering Immunity to Viruses appearing in Volume 255 of *Immunological Reviews*.

Summary: The fate of T lymphocytes revolves around a continuous stream of interactions between the T-cell receptor (TCR) and peptide-major histocompatibility complex (MHC) molecules. Beginning in the thymus and continuing into the periphery, these interactions, refined by accessory molecules, direct the expansion, differentiation, and function of T-cell subsets. The cellular context of T-cell engagement with antigen-presenting cells, either in lymphoid or non-lymphoid tissues, plays an important role in determining how these cells respond to antigen encounters. $CD8^+$ T cells are essential for clearance of a lymphocytic choriomeningitis virus (LCMV) infection, but the virus can present a number of unique challenges that antiviral T cells must overcome. Peripheral LCMV infection can lead to rapid cytolytic clearance or chronic viral persistence; central nervous system infection can result in T-cell-dependent fatal meningitis or an asymptomatic carrier state amenable to immunotherapeutic clearance. These diverse outcomes all depend on interactions that require TCR engagement of cognate peptide-MHC complexes. In this review, we explore the diversity in antiviral T-cell behaviors resulting from TCR engagement, beginning with an overview of the immunological synapse and progressing to regulators of TCR signaling that shape the delicate balance between immunopathology and viral clearance.

Keywords: *T cells, cytotoxic T cells, viral infection, neuroimmunology, in vivo imaging, immunotherapies*

Introduction

Our lives are shaped by constant encounters with an extraordinarily diverse and staggering number of microorganisms in our environment. Most of these microorganisms pose little threat to vertebrates such as ourselves, and many enrich our lives tremendously, either directly (e.g. commensal gut microbiota) or indirectly (e.g. nitrogen fixation, cheese, beer). However, some microbes have evolved to prey upon vertebrate hosts, either by feeding on us or hijacking our cells to generate countless copies of themselves. In the evolutionary arms race between host and pathogen, vertebrates have evolved potent front line innate immune defenses that recognize conserved pathogen associated patterns as well as host-derived 'danger' signals (1).

Immunological Reviews 2013
Vol. 255: 110–124

Immunological Reviews
0105-2896

These innate immune mechanisms rely on germline-encoded receptors to recognize highly conserved and 'unchangeable' structural components that pathogens cannot easily mutate (2–4). Vertebrates have also evolved complementary adaptive immune mechanisms that are highly specific and establish immune memory such that future challenges of the same type will be abortive or blunted. Through an elegant series of stepwise DNA rearrangements, adaptive lymphocytes generate tremendous antigen receptor diversity using a limited number of germline-encoded antigen receptor genes, resulting in clonally diverse populations with wide-ranging specificities (5–7). The two classical adaptive immune cell types are B and T lymphocytes. B cells represent the humoral adaptive immune effector arm, secreting soluble antibody proteins directed against intact antigens to bind and neutralize pathogens (8). However, intracellular pathogens are often sequestered from antibody binding, and therefore must be eliminated using different strategies. T lymphocytes discriminate between host and pathogen via protein antigen fragments (peptides) presented in the context of cell surface-expressed major histocompatibility complex (MHC) molecules (9–12). T-cell antigen recognition is typified by T-cell receptor (TCR) binding to MHC-bound cognate peptide, and CD8 or CD4 coreceptor interaction with MHC class I and II, respectively. TCR and peptide-MHC (pMHC) engagement results in a cascade of intracellular signaling events that ultimately govern T-cell fate and function. It is these interactions that usually determine whether antiviral T-cell effector activity is pathogenic or non-pathogenic.

Stable versus dynamic T-cell interactions

The immunological synapse

Initial studies *in vitro* demonstrated the formation of stable interactions between T cells and antigen-presenting cells (APC) (13–15). These interactions were dependent on TCR recognition of cognate pMHC and resulted in a highly polarized surface of engagement. The junctional interface between an antigen-sensing T cell and APC is classically referred to as an immunological synapse. As the focal point for TCR signaling, this immunological synapse is thought to be an essential communication port. pMHC serves to nucleate synapse formation and establish an avenue for vectorial information to flow into T cells. Following pMHC engagement, an abundance of accessory and costimulatory molecules in and around the developing synapse allow APCs to ultimately authorize expansion, arming, and execution of T-cell effector functions. The priming and regulation of T-cell function is also heavily influenced by factors within the extracellular milieu; however, T-cell function is by necessity predicated on TCR signaling.

Work by Kupfer *et al.* (14, 15) illuminated the close juxtaposition and requirement for cell–cell contact between T cells and APCs (especially B cells) during T-helper responses *in vitro*. In 1998, Monks and Kupfer (16) provided seminal three-dimensional (3D) evidence for specific interaction domains on the T-cell surface during pMHC encounter. They showed that the central supramolecular activation cluster (cSMAC) is surrounded by a peripheral supramolecular activation cluster (pSMAC) to form an archetypal 'bullseye' pattern that represents the mature immunological synapse structure (16). Classically, the cSMAC is rich in clustering TCRs cross-junctionally engaging pMHC, whereas the pSMAC contains adhesion molecules such as leukocyte function-associated antigen-1 (LFA-1) that physically stabilizes long-term cell–cell interactions. Several additional cSMAC constituents have been identified, including CD2, CD4, CD8, CD28, PKC-θ, Lck, and Fyn (reviewed in 17, 18). The formation of the immune synapse was classically believed to be critical for directional and specific intercellular communication, such as CD40–CD40L interactions and delivery of effector molecules (e.g. lytic granule and cytokines) (19–21). Although the term immunological synapse was initially meant to describe a specific, highly dense region of TCR clustering with a peripheral adhesive ring, we now use the term more loosely to imply the contact interface through which cell–cell communication occurs between T cells and their cognate pMHC-bearing targets.

Using a planar bilayer system, Grakoui and Davis (22) observed initial TCR–pMHC engagement along with adhesion to intercellular adhesion molecule-1 (ICAM-1) at the T-cell periphery. This initial interaction phase was followed by the dynamic accumulation of pMHC-engaged TCR within a central cluster surrounded by a ring of bound ICAM-1. A broad range of TCR–pMHC affinities led to TCR clustering and Ca^{2+} signaling, indicating that cSMAC formation is a conserved feature of TCRs with different affinities. Lee *et al.* (23) provided similar evidence for cSMAC formation using a cell–cell instead of planar bilayer system; however, this group also noted that TCR signaling occurred at the periphery of the immunological synapse and actually preceded cSMAC formation, raising questions about the importance of mature synapse formation in T-cell activation.

In addition to TCR signaling, integrin signaling through LFA-1 can also occur during immunological synapse formation,

providing costimulation as well as a link between synapse formation and dramatic changes in T-cell cytoskeletal structure (24–26). In particular, the directional secretion of effector molecules relies on polarization of the microtubule organizing center (MTOC) to the immunological synapse (14). Interestingly, effector molecules are shuttled along the microtubule network toward the TCR–pMHC-driven synapse (13, 27). Many proteins are linked to MTOC polarization and the targeted release of effector molecules (reviewed in 28).

In vivo formation of SMACs

In vivo evidence of cSMAC formation has been difficult to acquire, particularly in priming interactions. This is partially a technical challenge in resolving protein microdomains within fixed or living tissues, but could also reflect the physiological infrequency of SMAC formation. By studying antiviral $CD8^+$ T cells in the lymphocytic choriomeningitis virus (LCMV)-infected brain, we demonstrated *in vivo* that cytotoxic T lymphocytes (CTLs) polarize signaling (TCR, Lck), adhesion (LFA-1), and effector (perforin) molecules toward the contact surface with virally infected target cells (29) (*Fig. 1*). In some instances, CTLs were able to form synapses with up to three separate targets simultaneously (*Fig. 1D*). Because these studies were performed on static tissue sections, we were unable to observe the temporal relationship between CTL-APC contact and the migratory behavior prior to arrest and polarization. Importantly, Barcia *et al.* (30) expanded upon our work by capturing 3D *in vivo* evidence of cSMAC and pSMAC formation along the contact interface of T cells and virally infected astrocytes in the brain. The formation of SMACs was specific to T cells engaging infected astrocytes and preceded T-cell-mediated clearance of these cells. Although these findings provide clear evidence that SMAC formation occurs *in vivo*, it remains unclear whether cSMAC generation is a functional necessity for cytolytic or helper T cells. In fact, Yang *et al.* recently concluded that mature immunological synapses did not appear to correlate with CTL-mediated clearance of brain tumors (31), suggesting that SMAC formation is not a requirement for delivery of CTL effector functions.

Serial T-cell interactions

Early studies utilizing bilayers and unsupported cells *in vitro* indicated that T cells rapidly halt their migration upon initial antigen encounter (32). However, it is still debated whether long-lived T-cell–APC interactions are required for priming and effector functions. Gunzer *et al.* (33) provided the first counterpoint to the stable immunological synapse paradigm by modeling *in vivo* tissue migration using a collagen matrix culture containing T cells and APCs. In this study, it was observed that T cells engaged in dynamic, short-lived interactions with cognate pMHC-bearing APCs instead of halting their migration and forming stable immune synapses (33). This observation led to the development of a serial encounter model in which a rapidly formed stable immunological synapse is not required after initial antigen encounter. Instead, a multitude of short-lived serial TCR–pMHC interactions occur, additively generating a cumulative activation signal (34). There is substantial evidence supporting the physiological relevance of serial antigen encounters during *in vivo* T-cell priming (35–37). There are also data showing that TCR–pMHC interactions can induce release of effector molecules in the absence of stable immunological synapse formation (38–40). Interestingly, a recent study demonstrated that nuclear localization of nuclear factor of activated T cells (NFAT) imprinted transient TCR signals and remained active for TCR tolerance genes; however, more sustained TCR signaling was required for interferon-γ (IFNγ) expression (41). These findings provide a mechanistic basis for why transient TCR signaling induces tolerance in naive T cells. Thus, it appears that prolonged TCR signaling, whether achieved serially or continuously, is required for T-cell priming and effector differentiation (42). Although serial TCR–pMHC encounters can eventually generate a cumulative stop signal resulting in T-cell arrest (36, 37), it remains unclear if the tight interactions observed after several hours of transient serial interactions are characterized by classic immunological synapse formation.

Dynamic interactions: 'kinapses'

The high antigen doses used in the initial characterization of the immunological synapse likely facilitated the development of a rapid, stable cell–cell interface (43). Increasing the frequency of TCR engagements *in vivo* markedly enhances cell–cell conjugate formation and migratory arrest, indicating a strong role for antigen dose in promoting rapid motility arrest (37). However, T cells operating *in vivo* often encounter priming and effector phase conditions in which cognate antigen is presented at a low level. This can occur when an APC is not infected or is simply presenting low levels of exogenously acquired antigen. In contrast, when T cells encounter infected target cells filled with antigen and densely covered in pMHC, the resultant engagement and

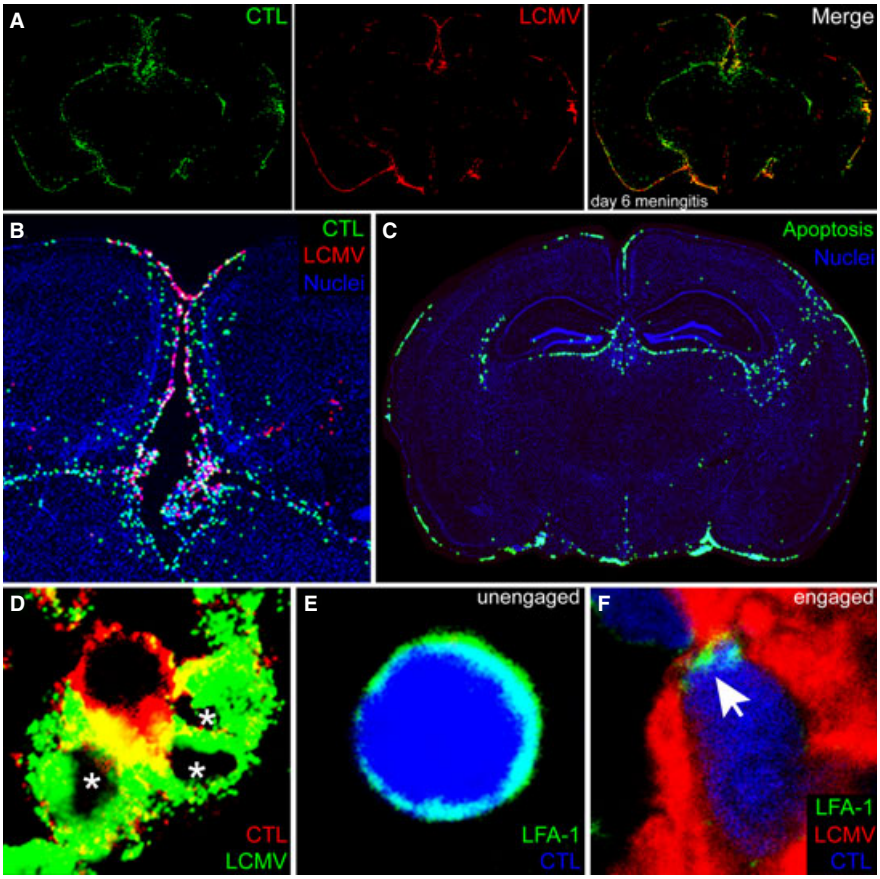

***Fig. 1.* Pathogenic CD8$^+$ T-cell interactions in the meninges during viral meningitis.** To study antiviral CTL interactions during the development of viral meningitis, naive B6 mice were seeded with 10^4 actin-GFP-tagged D^{b}GP$_{33-41}$-specific T-cell receptor-tg T cells (GFP$^+$ P14 cells) and then infected intracerebrally 1 day later with 10^3 PFU of LCMV Armstrong. Six-micron frozen brain sections were cut, stained, and analyzed by epifluorescence or confocal microscopy at day 6 post-infection. (A) A coronal brain reconstruction shows the meningeal distribution of GFP$^+$ P14 cells (green) and LCMV (red) in symptomatic mice at day 6. (B) An enlarged panel from the same coronal section shows P14 CTL (green), LCMV (red), and cell nuclei (blue). Note that virus and CTL localize almost exclusively to the meninges. (C) A pathologic consequence of CTL activity in the LCMV-infected meninges was revealed by performing TUNEL staining to label apoptotic cells (green). A marked increase in cell death was observed in the CTL-infiltrated meninges at day 6 post-infection. (D) Analysis of CD8$^+$ T-cell interactions revealed that a single CTL (red) can engage up to three different LCMV-infected targets (green; white asterisks) simultaneously. (E, F) The LFA-1 distribution (green) on unengaged (E) and engaged (F) P14 CTL (blue) was assessed in the LCMV-infected (red) meninges. Note that LFA-1 polarizes to contact interface of the engaged (white arrow) but not the unengaged cell. CTL, cytotoxic T lymphocyte; LCMV, lymphocytic choriomeningitis virus; GFP, green fluorescent protein; LFA-1, leukocyte function-associated antigen-1; GP, glycoprotein.

TCR signaling may be strong enough to favor formation of a stable, mature immunological synapse. Recently, Moreau *et al.* (44) reported clear associations among TCR affinity, signal strength, and the formation of stable immunological synapses. Stable synapses were associated with the strongest TCR–pMHC interactions, whereas dynamic interactions (referred to as kinapses) (45, 46) were observed *in vivo* with ligands of weak-to-moderate affinity. Importantly, kinapse interactions did result in T-cell activation and proliferation, albeit with a delay proportional to the pMHC signaling strength (44).

While stable cSMAC formation may occur *in vivo* under certain conditions (30), there is increasing evidence that many immune synapses can accommodate TCR signal integration with continued motility (47–50). These interactions have been termed 'kinapses' to reflect the importance of movement (or kinesis) (45, 46). A kinapse represents a region of cell–cell contact that serves to communicate information between the cells, but remains motile during signaling. Migrating T cells *in vitro* display motile synapses of TCR microclusters that move directionally along with the T cell, independent of cSMAC formation (51). Utilizing a transgenic mouse with green fluorescent protein (GFP)-tagged TCR, Friedman and colleagues (49) observed dynamic TCR trafficking *in vitro* and *in vivo* during naive T-cell priming. In these studies, the authors found little evidence of extended TCR clustering or classic cSMAC generation upon antigen encounter. Moreover, upon antigen encounter, TCR internal-

ization (which is evidence of TCR–pMHC signaling) also occurred rapidly without generation of cSMAC structure. Even more importantly, T cells were shown to cluster TCRs, flux calcium, and then internalize their TCR clusters all while maintaining motile surveillance of pMHC-presenting dendritic cells (DCs) (49). Similarly, Azar and colleagues, using a fluorescently tagged linker for activation of T-cells (LAT) protein, found little evidence for distinct areas of large scale TCR signaling clusters along the T-cell–DC contact zone *in vivo* (52). These authors also reported that activated effector T cells more frequently engage in kinapse-like interactions with APC when compared with naive T cells. These data indicate that T-cell activation state (naive, effector, memory) influences the type of cell–cell contact zone (e.g. mature synapse versus kinapse) that is formed (52). The formation of motile versus fixed synapses may also be linked to the APC itself, as interactions between T and B cells were shown to facilitate classical fixed synapse formation, where T-cell–DC interactions were more brief and serial (53). Collectively, the aforementioned studies provide a framework for the relationship between TCR signaling and the T-cell–APC interface. Strong, high affinity TCR–pMHC interactions are usually indicative of a T cell that has encountered a foreign peptide, which immediately authorizes the cell to activate. Following weaker TCR–pMHC interactions, T cells use serial engagements and record cumulative signaling events to gather more evidence before crossing over a threshold and committing to activation. This helps to ensure that the serially engaged peptide warrants T-cell activation.

Whether stable or dynamic, T-cell recognition of pMHC usually generates an interaction surface between cells. It is postulated that this interaction surface *in vivo* is highly dynamic, but does on occasion result in the formation of stable SMAC structures that facilitate prolonged signal integration. Numerous transient yet productive TCR–pMHC encounters also occur *in vivo* and can regulate T-cell priming and effector functions, both in lymphoid as well as in non-lymphoid tissues. In the following sections, we discuss how pMHC–TCR interactions modulated by accessory molecules can influence the fate of antiviral T-cell interactions.

Pathogenic and non-pathogenic consequences of T-cell interactions

Development of a fatal antiviral T-cell-mediated disease

B-cell production of neutralizing antibodies is the foundation for nearly all currently utilized vaccination strategies (54). Neutralizing antibodies are also critical to clearance of many viral infections; however, their very nature as extracellular soluble effector molecules often precludes their ability to clear intracellular viral reservoirs. Therefore, eradication of many viral infections requires coordinated effector activity by antiviral $CD8^+$ and $CD4^+$ T cells. LCMV virus is the prototypic model for T-cell-mediated viral clearance (55). LCMV is a non-cytopathic arenavirus that infects rodents and humans and provides an excellent system for understanding the mechanics of antiviral immune responses. Depending on the strain, dose, and infectious route, a wide array of responses to experimental LCMV infection can be elicited that include but are not limited to acute viral clearance, immune suppression, viral persistence, hepatitis, and fatal choriomeningitis. LCMV infection generates an enormous $CD8^+$ T-cell response dominated by well-documented pMHC specificities (56). The generation of LCMV glycoprotein (GP)-specific $CD8^+$ ($D^bGP_{33–41}$ specific; P14 mice) (57) and $CD4^+$ ($I\text{-}A^bGP_{61–80}$ specific; SMARTA mice) (58) TCR transgenic mice has provided transferable and traceable populations of virus-specific cells, allowing further characterization of antiviral T-cell responses.

LCMV has the capacity to induce a remarkably reproducible fatal meningitis (the disease for which the virus is named) 6 days following intracerebral inoculation into a murine host (reviewed in 59–63) (*Fig. 1*). LCMV is able to induce a similar disease in humans (64, 65). After intracerebral inoculation, LCMV gains access to systemic circulation and draining lymph nodes where it is available for naive T-cell priming. Professional APCs can be directly infected by LCMV, which may provide an abundance of pMHC for naive T-cell recognition (66, 67). Because strong TCR stimulation preferentially affects proliferation during priming (68), the abundance of pMHC presented by infected APCs may explain the massive burst in antiviral T cells, with up to 90% of circulating $CD8^+$ T cells becoming specific to LCMV (56, 69). LCMV-specific T cells traffic into many infected peripheral tissues, but their arrival into the central nervous system (CNS) precedes a cascade of cellular events that ultimately leads to death (*Fig. 1*).

A multistep adhesion cascade regulates the tethering and extravasation of circulating T cells into infected tissues. T-cell entry into the CNS relies on selectins, chemokines, and integrins (reviewed in 70). LCMV infection is associated with massive type I IFN (IFN-I) release (71), which plays an essential role in antiviral defense through induction of a myriad of antiviral proteins (72, 73) and by promoting adaptive immunity (74, 75). Our laboratory has shown that IFN-I also leads to widespread increases in MHC I through-

out the brain following LCMV infection, and this can be maintained indefinitely during states of persistent infection (76). Within the brains of mice persistently infected with LCMV, elevated MHC I expression was detected on endothelial cells and microglia. Endothelial cells have been reported to present antigen directly to T cells (77), and in one model, enhanced MHC I expression by CNS vascular endothelial cells facilitated the antigen-specific entry of $CD8^+$ T cells (78). It is known that TCR signaling events can link integrin and chemokine signaling to cytoskeletal changes required for motility (79, 80). Indeed, TCR signals can synergize with signals mediated by $\beta 1$ integrin (81), which is the very late antigen-4 complex that helps T cells gain access to the CNS. Thus, it is conceivable that pMHC-dependent immunological synapse or kinapse formation with vascular endothelial cells may sensitize T cells to transmigrate, thereby increasing the homing specificity. However, it is important to note that bystander T cells of an irrelevant TCR specificity are known to traffic into sites of infection, including the brain (82, 83). Therefore, the general role of TCR–pMHC interactions in the preferential recruitment of T cells to sites of infection remains unclear. That it does occur in certain scenarios (78) underscores the diversity of potential antigen-specific functions that TCR recognition can generate.

It is well described that T-cell entry into the LCMV-infected CNS is a harbinger of death (59). Fatal disease is absolutely dependent on LCMV-specific $CD8^+$ T cells, as shown by $CD8^+$ T-cell antibody depletion (84), genetic deletion (85), and peptide blocking (86) studies. Similarly, $CD8^+$ T cells specific to ovalbumin (OVA), an irrelevant antigen, are capable of entering the LCMV-infected CNS, but cannot cause fatal meningitis (82). $CD8^+$ TCR transgenic T cells specific to OVA (referred to as OT-I mice) (87) are unable to mount a LCMV-specific T-cell response and are completely resistant to LCMV meningitis (82). Following LCMV infection, these mice become asymptomatic, lifelong viral carriers. However, adoptive transfer of as few as 10^3 naive LCMV-specific $CD8^+$ P14 T cells into OT-I mice can fully restore lethal meningitis, illustrating the crucial role of CTL–pMHC interactions in mediating this disease (82). A single monoclonal population ($D^bGP_{33–41}$ specific) of virus-specific T cells operating in a repertoire of bystanders is sufficient to drive a fatal disorder.

To gain advanced real-time insights into this disease process and determine how virus-specific $CD8^+$ T cells induce a rapid onset fatal seizure disorder, we employed a technique referred to as intravital two-photon laser scanning microscopy (TPM) (63, 88–90) and imaged the brain through a surgically thinned skull window (91, 92). By imaging GFP-tagged LCMV-specific $CD8^+$ T cells in the living brain, we were able to define the real-time interactions of these cells during the development of LCMV meningitis (47, 63). In symptomatic mice, virus-specific CTLs invaded and interacted with the virally infected meninges. Interestingly, we observed that CTLs divided locally within the CNS environment (48). Up to 30% of CTLs were in active stages of cell cycle in the virus-infected meninges. This dynamic observation of virus-specific CTLs undergoing mitosis in the LCMV-infected meninges has expanded our general understanding of T-cell division programming. The traditional view of T-cell proliferation is that the proliferative burst, which occurs within lymphoid organs during priming is a hard-wired program (93) instituted by DCs (35). Contrary to this paradigm, we found that during LCMV meningitis, up to one third of antiviral CTLs depart lymphoid tissues and migrate through the blood while still in cell cycle (48). Peripherally cycling cells showed no overt differences in surface molecule expression or effector program from non-cycling cells in circulation or the CNS. These cycling cells likely represent a stochastic vestige of the proliferative program initiated in secondary lymphoid tissues during $CD8^+$ T-cell priming. Using TPM, we observed that the number of motile CTLs in the LCMV-infected meninges far outnumbered those that were stably arrested. We postulate that migrating CTLs integrate pMHC signals obtained from multiple infected target cells, which is consistent with the concept of serial signal integration. In the meninges, migrating CTLs often arrested briefly (approximately 10–15 min) to undergo mitosis before resuming their migration (48). Interaction with cognate pMHC was critical to advancing the CTL division program, but costimulatory molecules such as CD80 and CD86 were dispensable. These data extend upon the serial encounter model of cumulative TCR–pMHC signal integration in lymphoid tissues to include the summation of signals accumulated systemically. In other words, antiviral T cells likely have the capacity to record and integrate signals obtained from multiple tissues. Another advantage of advancing the T-cell division program serially is that antiviral T-cell numbers can be modulated locally at sites of viral infection. Based on the signals delivered, T-cell numbers can be easily increased to promote viral clearance or decreased to prevent immunopathology. Serial programming is more amenable to local control.

CTL interactions with pMHC drive the fatal consequences of LCMV meningitis (85); however, the exact effector

mechanism employed by T cells remained unclear. Mice deficient in perforin, granzyme B, tumor necrosis factor-α (TNFα), IFNγ receptor, Fas, and the degranulation pathway (Unc13d also known as Jinx) (94) are all susceptible to fatal meningitis (47). This was unexpected given the absolute requirement for CTLs in the disease process. Thus, we used TPM to determine how CTLs were linked to the development of fatal immunopathology. Time lapses revealed large numbers of antiviral CTL migrating through the meningeal space. In addition, increased CTL motility was observed following antibody-mediated disruption of TCR–pMHC interactions, which indicated that MHC I interactions regulate most if not all antiviral CTLs within the LCMV-infected meninges (47).

Blood brain barrier (BBB) breakdown (47, 95, 96) and convulsive seizures (97, 98) are two hallmarks of LCMV meningitis. At day 5 post-infection, CTLs begin to survey the meninges, but the BBB remains intact and mice are asymptomatic (47). However, at 6 day post-infection, CTL influx increases markedly and this coincides with a massive secondary recruitment of innate myelomonocytic cells (i.e. monocytes and neutrophils) that burst forth from meningeal vasculature, leading to vascular breakdown and seizure onset (47). This tidal wave of myelomonocytic recruitment into the CNS was precipitated in part by TCR–pMHC interactions driving meningeal CTL to release chemokines such as CCL3, 4, and 5. From a survival perspective, we postulate that direct chemokine release by CTLs is critical to the development of rapid onset fatal convulsive seizures. This does not, however, negate the importance of cell death induced by classical CTL effector pathways like lytic granule secretion onto virally infected target cells, which does occur in this model (29). Depletion of myelomonocytic cells eliminates rapid onset seizures (on day 6) and extends survival, but mice eventually succumb to a disease that is likely mediated by CTLs. Additional studies are required to prove this definitively. In general, these data revised our understanding of how antiviral T cells contribute to immunopathological diseases. It is well documented that CTLs cause immunopathology through release of cytotoxic effector molecules (99). Our studies demonstrate that they can also contribute to CNS disease by recruiting pathogenic innate immune cells.

Regulation of cytokine and chemokine secretion by TCR–pMHC is commonly associated with $CD4^+$ T-cell function; however, antiviral CTLs can produce large quantities of IFNγ, TNFα, and the aforementioned chemokines. Although it is not clear how CTL-derived chemokines trigger emigration of circulating cells, it is possible that endothelial pMHC complexes, in addition to enhancing transmigration (78), also elicit chemokine deposition within glycosaminoglycan networks on the luminal endothelial cell surface, which has been shown *in vivo* to facilitate interactions with circulating lymphocytes (100). It is expected that as the number of circulating antiviral CTLs increases, so too will the number of interactions between CTLs and brain endothelial cells. At some critical threshold, CTL-deposited chemokines lead to widespread integrin activation and transmigration by myelomonocytic cells. Further studies are required to determine how CTLs coordinate synchronous extravasation of myelomonocytic cells following CNS viral infection (47).

The original observation that cytokines were polarized (13) toward the immunological synapse led to the elegant model suggesting that TCR–pMHC interactions generate long-lived cell–cell interactions required for targeted delivery of effector molecules onto cells in need of them. Synaptically targeted delivery allows for strict communication between two 'authorized' partners, which spares the surrounding uninfected tissue from pathology and maintains the fundamental principles of antigen specificity within the adaptive immune system. However, although polarized delivery of lytic granules makes intuitive sense, and directional cytokine delivery by $CD4^+$ T cells during humoral T–B-cell interactions must be antigen specific, there are situations in which directional delivery simply cannot accomplish certain goals. In the case of $CD4^+$ T cells, relying solely on directional cytokine delivery precludes their ability to help cells that lack MHC II expression. As MHC II expression is far more restricted than MHC I, many cell types that could benefit from cytokine exposure would not be able to receive directional cytokine support. Importantly, Huse *et al.* (101) have shown that while some T-cell-derived cytokines (e.g. IFNγ, IL-2) are targeted to pMHC-expressing cells by an immunological synapse, others such as TNF and IL-4 are secreted multidirectionally (i.e. not only at the cell–cell interface). Moreover, the chemokines CCL3 and CCL5 also appear to be multidirectionally secreted (101). Whether this is the case for chemokine production by antiviral $CD8^+$ T cells remains unclear, but it seems reasonable to assume that meningeal TCR–pMHC interactions drive CTLs to produce a cloud of chemokines within the LCMV-infected meninges (47). In fact, even directional synapses have been shown to be 'leaky'. Barcia and colleagues (102) recently demonstrated that $CD8^+$ T cells within the virally infected CNS use directional (cSMAC-containing) immunological synapses to release IFNγ and lytic granules toward targets; however, cell contacts lacking cSMAC structure (referred to as non-Kupfer

interactions) were also shown to result in IFNγ secretion. These non-Kupfer interactions are still directional because they are regulated by TCR engagement. Using a clever *in vitro* technique to probe the true directionality of IFNγ release by $CD8^+$ T cells, this same group showed that while antigen-bearing target cells were directly exposed to IFNγ (evidenced by Stat1 relocalization), so were their non-antigen-bearing, unengaged neighbors (103). This observation implies that following formation of a TCR–pMHC-dependent contact interface, IFNγ can at least partially leak out into the surrounding extracellular area. This may be particularly relevant in immunoprivileged tissues where MHC I (and II) are not widely expressed. Within the virally infected CNS, many cells (especially neurons) express little to no MHC I (104, 105), making it difficult for T cells to directly engage them, even if they are infected. If nearby cells, such as microglia or DCs, present antigen to $CD8^+$ T cells, IFNγ production can exert antiviral effects regionally as opposed to only on the pMHC-bearing target cells.

$CD8^+$ T cells are idolized for their ability to specifically lyse pMHC-bearing cells. CTLs can mediate target cell lysis through the directional release of lytic granules or through interaction of CTL-presented FasL with target cell expressed Fas (99, 106). The life or death decision CTLs make based on TCR–pMHC contact is highly regulated, as little non-specific killing is observed (82). Interestingly, it is estimated that CTLs make the decision to kill after engagement of as few as 3 pMHC molecules, whereas 10 pMHC molecules are needed to establish the cSMAC (40). Although directional cytokine secretion requires greater TCR–pMHC signaling and favors formation of the cSMAC (i.e. a classical immunological synapse), very little antigen is needed to induce lytic granule secretion (27, 107). Thus, this pathway must be exquisitely regulated to avoid severe tissue injury.

Synaptic regulation of T-cell function

A number of factors and molecular queues modulate TCR–pMHC signaling. Interactions between B7 (primarily CD80/86) and CD28 superfamily members dominate the costimulatory landscape. T-cell expression of these factors, which include CD28, cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD-1), and inducible T-cell costimulator (ICOS), regulate T-cell functionality by dampening or enhancing TCR proximal signaling cascades (reviewed in 17). Activation by innate signaling pathways causes APCs to upregulate costimulatory molecule expression in preparation for T-cell priming interactions. Immature APCs, with low costimulatory molecule expression, can nevertheless present pMHC complexes to T cells. TCR–pMHC interactions on the surface of immature or tolerogenic DCs can result in T-cell tolerance induction. T-cell interactions with these immature DCs were shown to be shorter and less productive than those with mature APCs (108). Although these shorter interactions were deemed independent of CD80/86 influence, they nevertheless support an orchestrating role for APCs during T-cell priming interactions. In addition to upregulation of classic costimulatory molecules, APC maturation itself results in increased T-cell adhesiveness regardless of peptide presentation (109). The signals being exchanged in non-specific interactions remain unclear, but T cells themselves can engage in interactions with one another that facilitate differentiation and acquisition of effector functions (110, 111).

Naive T cells constitutively express CD28, and upon TCR engagement, it is recruited along with TCR to the signaling synapse. CD28 interactions with APC-expressed CD80/86 amplify TCR–pMHC signaling and permit T-cell activation by only a small number of triggered TCRs (112). Naive T cells are dependent on CD28 costimulation, especially when TCR–pMHC interactions are limiting (113–115). Although effector T cells appear less dependent on CD28 to elicit effector function, memory T cells benefit from a reactivating encounter with CD28 costimulation (116, 117). TCR and CD28 signaling elicits expression of the secondary costimulation marker ICOS by activated $CD4^+$ T cells (118). Therefore, ICOS ligation is important for activated rather than naive T cells and supporting continued $CD4^+$ T-cell expansion/differentiation (119, 120). ICOS ligation was also shown to augment the proliferative and cytokine responses of activated $CD8^+$ T cells *in vitro* (121), although ICOS blockade had no effect on antiviral CTL responses *in vivo* (122).

Upon TCR engagement, naive T cells upregulate CTLA-4 (123, 124), which subsequently traffics into the immunological synapse (125). Compared with CD28, CTLA-4 possesses a much higher affinity for CD80/86, and instead of enhancing TCR signals, CTLA-4 binding contributes to the dephosphorylation and suppression of proximal TCR signaling (reviewed in 126). This interference with TCR signaling allows CTLA-4 to override the TCR–pMHC 'stop signal' and further disrupt TCR–pMHC interactions as cells migrate away from their antigen (127, 128). Animals deficient in CTLA-4 succumb to a fatal multi-organ autoimmune reaction only weeks after birth, illustrating the crucial role in negatively regulating TCR signaling (129, 130). Although essential in preventing autoimmune activation of T cells,

CTLA-4 does not appear to critically regulate antiviral CTL function (131, 132).

Another T-cell-expressed negative regulator of TCR signaling is PD-1, an inhibitory molecule expressed on activated T cells that is especially pronounced in chronic infections (132). Although both PD-1 and CTLA-4 negatively regulate proximal TCR signals, they do so using distinct mechanisms (133). Studies have demonstrated that PD-1 and TCR co-cluster upon pMHC engagement and coalesce within the cSMAC in stable immunological synapses (134, 135). Interactions between PD-1 and one of its ligands, PD-L1, at membrane sites of TCR–pMHC interaction serve to promote dephosphorylation of proximal TCR-activating signals, which in turn blunts activation and effector functions. Dynamic *in vivo* studies of autoreactive $CD4^+$ T cells have indicated that PD-1:PD-L1 interactions disrupt TCR-based stop signals, resulting in increased motility and decreased interaction times between $CD4^+$ T cells and APCs (136). Overriding TCR-induced stop signals was proposed as an important mechanism to prevent the development autoimmune disease. However, our recent work with antiviral $CD8^+$ T cells in a model of chronic LCMV infection (using the clone 13 strain) (137) has shown that while PD-1 co-associates with TCR at the immunological synapse, PD-1:PD-L1 engagement promotes long-term stable arrest. Instead of reducing the contact time between antiviral $CD8^+$ T cells and APCs, PD-1 interactions stabilized cell contacts and immunological synapses, while remaining disruptive of proximal TCR signaling (135). In the LCMV clone 13 model of viral persistence, CTLs undergo active suppression to prevent severe immunopathology (138). We propose that motility paralysis imposed by the PD-1:PD-L1 pathway decreases T-cell scanning efficiency and facilitates continued engagement of regulatory pathways that negatively impact antiviral T-cell function. Importantly, PD-1 blockade resulted in a rapid recovery of $CD8^+$ T-cell motility, signaling, and expression of the antiviral cytokine, IFNγ. PD-1:PD-L1 management of TCR signaling quality was critical to maintaining a tolerable level of host–pathogen interplay, as PD-1 blockade resulted in rapid initiation of viral clearance followed by IFNγ-mediated death of the host (135). It is interesting that PD-1 blockade triggers the synthesis and/or release of IFNγ, which is a synaptically targeted cytokine (101). The molecular relationship between PD-1 signaling and IFNγ secretion is not clear, although TCR proximal signals driving IFNγ transcription are certainly involved, as PD-1 blockade has a rapid effect on IFNγ transcription (135). It is possible that PD-1 signaling blocks IFNγ shuttling to the target cell synapse, causing preformed intracellular IFNγ protein to freeze in transit for targeted release until PD-1 blockade unlocks the synaptic paralysis. Consistent with this model, PD-1 signaling has little effect on TNFα production (135), which is a non-synaptic multidirectional cytokine (101). That APCs play a significant role in regulating TCR–pMHC interaction outcomes during both the priming and effector phase is clear, yet how they modulate the quality of these interactions remains incompletely understood.

Regulatory T cells (Treg) are a class of $CD4^+$ T cells that by a variety of means suppress autoimmune and non-specific T-cell responses (reviewed in 139). Tregs are critical for survival (140) and have been shown to influence the immune response and outcomes to a number of viral infections (141). Mempel and colleagues (142) observed that the presence of antigen-specific Tregs inhibited the ability of CTLs to lyse target APCs within draining lymph nodes. Despite engaging in long-lived conjugates with their targets, the presence of Tregs prevented CTL lytic granule exocytosis in a TGF-β-dependent manner. Prolonged physical contact between the CTLs and Tregs was not required to suppress cytolysis, which is in accordance with Treg-mediated inhibition of $CD4^+$ T-cell responses in two autoimmune disease models (143, 144). Tregs interact with APCs to dissolve or prevent autoreactive $CD4^+$ T-cell clustering. The presence of Tregs appears to impair TCR signal integration such that the dynamic swarming behavior observed after successful TCR–pMHC signaling does not occur, and $CD4^+$ T cells fail to proliferate or acquire effector functions. Interestingly, it was also recently reported by Marangoni and colleagues (41) that Tregs can actually destabilize CTL interactions with primary tumor target cells in non-lymphoid tissue. However, it still remains unclear what mediators Tregs deploy to influence T cells or APCs in these different models to suppress T-cell responses.

Immunotherapeutic clearance of a persistent infection as model for regulated T-cell function

In addition to inducing lethal meningitis, LCMV can establish a carrier state of chronic infection (referred to as LCMV carrier mice) wherein animals remain viremic with high viral burden in all tissues, but have no overt signs of immunopathology (145–147). Although strong TCR–pMHC interactions in LCMV-infected secondary lymphoid tissues typically results in T-cell priming and expansion, the TCR–pMHC interactions of developing T cells in the thymus of neonatal carrier mice negatively selects LCMV-specific T cells from the T-cell repertoire, establishing immune tolerance

(57, 148). This negative selection is not complete and some LCMV reactive cells escape selection (149); however, the cells that do persist in the circulating T-cell pool are tolerized, rendering them incapable of clearing virus. Viral clearance from LCMV carrier mice can be achieved by an adoptive immunotherapy strategy in which memory T cells are transferred into carrier mice (150) (*Fig.* 2). Simultaneous transfer of both $CD8^+$ and $CD4^+$ T cells is required (151, 152) to rapidly control viremia (153) and purge virus from most peripheral organs; however, it takes much longer for CNS virus to be cleared (153, 154). Within the CNS of carrier mice, neurons bear a heavy viral burden (154, 155), which presents a conundrum to $CD8^+$ T cells. CTL lytic effector function typically destroys infected target cells (99). This cytolysis is acceptable in most peripheral organs with regenerative capacity like the liver but is not ideal within the CNS, because the majority of neurons are postmitotic and their lysis poses a significant risk to host fitness.

Uninfected neurons protect themselves by expressing little MHC class I, thus reducing the likelihood of direct, stable engagement by CTLs and potential cytolysis (104, 105). During some CNS viral infections, CTLs do employ lytic effector mechanisms; both perforin- and Fas-dependent neuronal killing have been reported (156, 157). Certain pathogens may antagonize cytokine-induced antiviral protein function, resulting in viral resistance and ongoing replication (158). The increased inflammation and pMHC expression associated with continued viral replication may override a T cell desire to act non-cytopathically. However, in most cases, CTLs do not destroy infected neuronal networks, but rely instead on non-cytopathic mechanisms (159) to clear viral infections. Interestingly, forced expression of neuronal MHC I leads to profound illness and death in LCMV carrier mice following adoptive immunotherapy (160). Nevertheless, adoptive immunotherapy requires $CD8^+$ T cells and does succeed in clearing neuronal viral reservoirs from wildtype LCMV carrier mice in a TCR–pMHC-dependent manner without induction of overt pathology (154, 155). The CNS must therefore impose specific regulation on CTL by providing activating signals

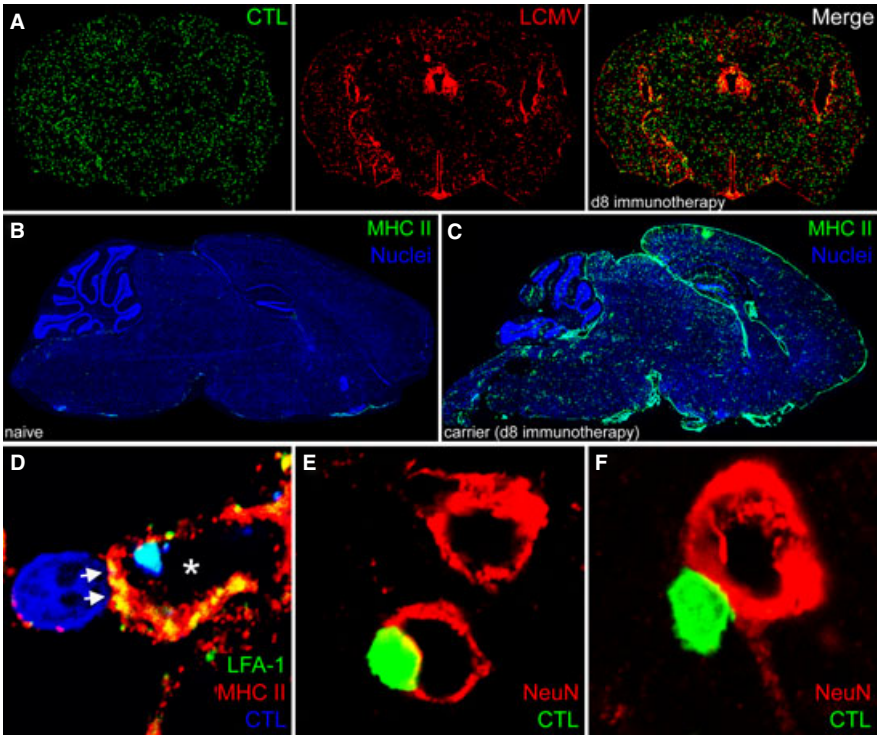

***Fig.* 2. Non-pathogenic $CD8^+$ T-cell interactions in the persistently infected brain.** Adoptive immunotherapy in persistently infected LCMV carrier mice was performed by intraperitoneally injecting 2×10^7 memory splenocytes from a LCMV immune animal. The memory splenocytes were seeded with GFP^+ P14 cells, which provided traceable representatives of immunotherapeutic CTLs as they engaged in clearance of the persistent viral infection. (A) At day 8 post-immunotherapy, P14 CTL (green) localized throughout the brain and meninges of LCMV (red) carrier mice. (B, C) Relative to naive (B) and untreated carrier control (not shown) mice, MHC II expression (green) was markedly upregulated in day 8 immunotherapy recipients (C). Cell nuclei are shown in blue. (D) Analysis of $CD8^+$ T-cell interactions revealed that P14 CTL (blue) engaged $MHC\ II^+$ APCs (red) at a LFA-1-rich (green) interface. (E, F) P14 CTLs (green) were also observed in juxtaposition with $NeuN^+$ neurons (red) at day 8 post-immunotherapy. CTL, cytotoxic T lymphocyte; LCMV, lymphocytic choriomeningitis virus; GFP, green fluorescent protein; LFA-1, leukocyte function-associated antigen-1; MHC, major histocompatibility complex.

such as pMHC without eliciting the negative consequences lytic function. Initial adoptive transfer of anti-LCMV memory cells into carrier mice results in robust secondary T-cell expansion, rapid clearance of peripheral virus, and T-cell trafficking into the virally infected CNS (*Fig. 2*). Our laboratory has shown that the arrival and dispersal of antiviral memory CTLs in the CNS dramatically increases the influx and activity of MHC II-expressing APCs (155) (*Fig. 2B–D*). Importantly, we were able to provide visualize evidence of CTLs interacting with these APCs during non-cytopathic clearance of the persistently infected brain (*Fig. 2D*). Although CNS-resident microglial cells as well as emigrating DCs and macrophages expressed antigen-presenting machinery, only DCs from immunotherapy recipients stimulated T cells to produce effector cytokines *ex vivo*. Intriguingly, brain-derived DCs from immunotherapy recipients elicited TNFα-biased cytokine production from antiviral $CD8^+$ T cells, which was in stark contrast to splenic DCs from carrier mice, which induced IFNγ-biased responses (155). This diametric change in cytokine production was crucial to successful viral clearance, as TNFα deficient memory T cells were unable to facilitate a reduction in viral load upon adoptive immunotherapy. Our data indicate that not only do transferred memory CTLs infiltrate the CNS of LCMV carrier mice, they also interact with CNS DCs to produce TNFα that is required for successful immunotherapy. How CNS DCs specifically suppress directionally secreted IFNγ production while still eliciting TNFα production is unknown, but it likely involves immunoregulatory molecules interacting with proximal TCR signaling to affect CTL secretory machinery.

LCMV clearance from the livers of immunotherapy recipients involves some degree of infected hepatocyte cytolysis, yet many cells are cleared non-cytopathically through memory T-cell cytokine production (161). Interestingly, Guidotti and colleagues (161) found that cytolysis was required to purge virus from non-parenchymal liver cells and splenocytes, indicating that host cell factors may intrinsically regulate which T-cell effector mechanism will most efficaciously clear a viral infection. CTL clearance of CNS coronavirus infection is also mediated by contrasting mechanisms. Bergmann *et al.* (162) reported that $CD8^+$ T-cell-derived cytolytic action (but not IFNγ) was required to clear astrocytes, whereas IFNγ alone could only inhibit viral replication in oligodendroglia. In these examples of liver and CNS viral clearance, T cells presumably produce IFNγ during contact with tissue-resident cell types, but somehow 'decide' when to abandon non-lytic effector mechanisms and resort to lysing infected cells. The mechanisms that guide these fate decisions remain unclear. Very little TCR stimulation is required to induce CTL cytotoxicity, whereas stronger antigen encounters (i.e. TCR–pMHC signaling) are needed to generate effector cytokine release (107). This model may explain why CTLs favor cytokine release following contact with infected hepatocytes that express high levels of pMHC; yet, this model is difficult to reconcile with observations in the CNS. Neuronal infection is certainly not associated with abundant MHC I presentation, which should favor engagement of lytic effector mechanisms, but these serial low peptide encounters within the CNS typically do not result in killing and instead bias antiviral T cells toward cytokine release and non-cytopathic clearance. Following adoptive immunotherapy in LCMV carrier mice, we have observed juxtaposed antiviral CTLs and neurons, suggestive of a productive interaction (*Fig. 2E, F*). However, our preliminary dynamic studies of CTL interactions with virally infected neurons suggest that the preponderance of these interactions is very rapid, and T cells for the most part remain highly motile (authors' unpublished observations). CXCL10, a CXCR3 ligand, is expressed by neurons in the virally infected CNS (163), and CXCR3 ligands suppress TCR activation and override stop signals to 'force' motility upon T cells (164). These findings might explain rapid migration along infected neurons with a lack of cytotoxicity, but it remains unclear how antigen-specific T cells produce antiviral effector cytokines under these conditions of enforced motility. We have shown that LCMV infection of mice with a restricted T-cell repertoire directed against OVA (OT-I mice) results in establishment of a novel carrier state in which viral tropism is expanded to include astrocytes and oligodendrocytes in addition to neurons (165). Immunotherapeutic memory T-cell transfer into OT-I carrier mice results in uncharacteristic illness and death during viral clearance. OT-I mice have Tregs with highly restricted TCR expression in addition to reduced Treg numbers. We found that co-transfer of Treg cells with antiviral memory T cells significantly dampened pathologic T-cell activity, while still allowing for eventual viral clearance in OT-I carrier mice (165). The role for Tregs in the TCR diverse LCMV carrier model is unknown, but it is possible that only interactions with certain infected cell types require mediation by Tregs, and normal neuronal clearance (as observed in wildtype carrier mice) can unfold safely. Mechanistically, the role for cell-mediated suppressive effects within the CNS remains unclear and in fact varies widely based on the model under investigation (139). How neurons dictate their preferred

effector mechanisms from CTLs is still a mystery, although TCR–pMHC interactions are a key element of the decision-making process. Further work is needed to elucidate how these non-pathologic T cells integrate the TCR–pMHC interactions in the CNS with potentially unique molecular queues that bias T cells away from cytotoxicity.

Concluding remarks

The life of a T cell revolves around TCR–pMHC interactions. In the beginning, the positive and negative selection synapses formed between T cells and thymic APCs (i.e. DCs and medullary thymic epithelial cells) result in clonal expansion of progenitors for further diversification and selection as well as clonal deletion, but do not result in effector differentiation or effector activity. TCR–pMHC interactions regulate these diverse outcomes, potentially through accessory molecules or differential signaling (166, 167). Once in the periphery, tonic TCR signaling due to interactions with self-peptides supports T-cell survival (168), until the TCR encounters activating pMHC complexes. Mature APC encounter leads to TCR–pMHC interactions that synergize with costimulatory molecules and the extracellular milieu to initiate the priming synapse and drive T-cell proliferation and acquisition of specific effector functions. Following a successful priming synapse, T cells can form effector synapses that result in authorization to execute an effector program through cytokine/chemokine production and cytolytic granule release. Bifurcation of freshly activated T cells into memory or effector precursor cells can result from asymmetric division (169, 170); however, it remains unclear what role TCR–pMHC signaling plays in directing asymmetric division. TCR–pMHC interactions drive cell division in peripheral tissues (48), yet it remains unknown whether these division events are asymmetric. If indeed asymmetric division does occur within peripheral tissues, it could play an underlying role in generating tissue-resident memory cells (171).

Successful entry into the memory pool finds T cells again awaiting pMHC encounter, at which point they form a secondary priming synapse that reinitiates the priming program in antigen-experienced memory T cells, likely with qualities different from the primary phase. Every time a TCR interacts with pMHC, the interaction results in information transfer through TCR proximal signals that depend on the TCR–pMHC affinity and synaptic partners. Controlling the outcome of TCR–pMHC encounters is paramount for pathogen clearance and immunopathology. In some target organs, CTLs kill directly without the requirement for APC interactions. Within the CNS, CTLs typically appear to favor non-cytotoxic effector mechanisms. Understanding how TCR signals integrate with immunomodulators and secreted factors in the milieu to deliver varied effector programs will allow for a greater potential to manipulate and tune T-cell responses to promote viral clearance while preventing undesirable immunopathology.

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