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CD8 T cell memory is defined by direct interactions with CD4 T cells and tissue localization during T cell activation and clonal expansion

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Doctor of Philosophy

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B.S. and M.S., Universidad Nacional de Cordoba, 1998

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An abstract of a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
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Abstract

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by Pablo A. Romagnoli

Immunological memory against intracellular pathogens is highly dependant on the number, quality and location of memory CD8 T cells, all features defined during CD8 T cell memory formation. In terms of quality formation, CD8 T cells need CD4 T cells to develop into effective memory CD8 T cells. Since murine CD8 T cells do not transcribe MHC class II genes, several models have proposed antigen-presenting cells (APCs) as intermediaries that allow CD8 T cells to receive CD4 T cell help. Here, we demonstrate that activated murine CD8 T cells possess MHC class II molecules acquired from APCs via a process called trogocytosis. Transferred MHC class II molecules are functionally competent and enable activated CD8 T cells to receive CD4 T cell help enhancing their recall responses upon challenge. These observations indicate that direct CD8:CD4 T cell interactions may significantly contribute to the development of memory CD8 T cells.

The location in which CD8 T cells get activated can also influence generation of CD8 T cell memory. Naive CD8 T cells responding to infection in different tissues integrate signals that determine their phenotype and functional properties as effector or memory CD8 T cells. Here, we show that CD8 T cells responding to acute LCMV infection leave blood to populate several lymphoid tissues (spleen, PLN, MLN and bone marrow). We find that while the phenotype of LCMV-specific CD8 T cells varies with the tissue, CD8 T cells from bone marrow display a memory precursor phenotype, $CD62L^{lo}CD25^{lo}Ly6C^{hi}$ different to the mixed phenotype observed in CD8 T cells from spleen or PLN 3 days post LCMV infection. Interestingly, T-bet is absent in LCMV-specific CD8 T cells from bone marrow, in contrast to the high T-bet expression found on LCMV-specific CD8 T cells in PLN and spleen. Differences in phenotype also correlate with the cytokine profile of CD8 T cells, detecting less IFNγ-producers in bone marrow than in spleen upon ex vivo stimulation. These findings suggest that memory precursors arise in bone marrow early during LCMV infection. Taken together, we show two unappreciated contributions to CD8 T cell memory development.

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Chapter 1

Defining moments in the life of a CD8 T cell

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1.1 Immunological Memory

The immune system provides a tremendous evolutionary advantage by allowing an organism to defend itself against agents that could endanger the survival and reproduction of its species. One of the most important components of this immune system is the immunological memory, a remarkable feature that ensures that, in most cases, an organism will never be affected again once it has successfully fought a life-threatening infection.

Several times in history it has been observed that the immune system learns with every challenge that it has successfully overcome. From Thucydides describing how nobody was attacked twice in the plague of Athens (1), to the reports of long-standing immunity against measles from Faroe Islands (2), to the first experimental vaccination recorded in our western modern history by Edward Jenner (3), every single one of these events highlights the importance of immunological memory.

It is then logical to hypothesize that manipulation of immunological memory could give evolutionary advantages against harmful environments; and that is exactly what vaccines hold in promise for humankind. Vaccines exploit immunological memory to preemptively mount defenses against pathogens that cause terrible scourges to our kind as they have in the past the likes of smallpox, measles or influenza. It is therefore very important to investigate the mechanism behind the generation of immunological memory to design better vaccines against HIV, dengue or Tuberculosis that could be considered today serious infectious challenges to our species.

Immunological memory is conferred by the presence of clonally expanded populations of pathogen-specific lymphocytes (4), called T and B cells. To properly

control and clear an an infection, both T and B cell subsets are important (5-7), but it is CD8 T cells who are critical against infections with intracellular pathogens like viruses (6), bacteria (8) or some parasites (9). Therefore, to design effective vaccines against intracellular pathogens, not only do we need to learn how to generate protective antibody responses, but also identify the mechanism by which memory CD8 T cells are developed and preserved over time (10).

1.2 CD8 T cell activation

CD8 T cells are generated in bone marrow to migrate into thymus, where they develop into naïve CD8 T cells (11). Upon exiting thymus, naïve CD8 T cells circulate through lymphoid tissues where they receive homeostatic survival signals (12, 13). Once encountering their cognate antigen, naïve CD8 T cells integrate more than one signal of activation to become fully functional (14-16). The first signal is provided by the T cell receptor (TCR) on CD8 T cells upon recognition of its cognate peptide presented in the groove of MHC class I molecules on the membranes of antigen presenting cells (APCs) (17), usually dendritic cells (DCs) in secondary lymphoid organs (SLOs) (18, 19). costimulatory molecules provide the second signal, typically members of the B7 family (20) or TNF superfamily (21) and/or chemokines (22), expressed on DCs that have sensed inflammatory pathogen-associated molecular patterns (PAMPs) (23, 24). Lastly, cytokines present in the surrounding inflammatory milieu (25-28) like interleukin-12 (IL-12) or Type I interferons (IFNα and IFNβ, IFNs) (29), provide a third signal whose timing (29) and magnitude (30) also has great impact in the differentiation program of the responding CD8 T cell. After expansion, 90-95 % of CD8 T cell will die in the contraction phase and the remaining cells will become memory CD8 T cells (31).

1.3 CD8 T cell memory

Currently, there are two proposed models to explain how CD8 T cell memory is generated. One is the progressive linear differentiation model (32), where all memory CD8 T cells are descendants of naïve CD8 T cells that where able to kill infected cells and secrete antiviral cytokines as effector CD8 T cells. On the other hand, there is a model that proposes the generation of separate lineages of effector and memory CD8 T cells from naïve CD8 T cells called bifurcative differentiation model (33). Controversy between these models continues to be reflected by evidence favoring one model (34-36) or the other (33). Therefore, identification of early memory precursors during an immune response could help to elucidate this controversy (37) and render more clues about the signals that participate in the differentiation of memory CD8 T cells.

Markers and functional properties can define CD8 T cell populations. Two subsets were first described in humans using CCR7 and L-Selectin (CD62L) (38). In mouse, two subpopulations of memory CD8 T cells can be found after an acute LCMV infection has been cleared (39): central memory (Tcm), characterized by high expression of CD62L; and effector memory (Tem), defined by low CD62L expression (38-40). Ly6C (41), CD27, CD43 and CXCR3 (42) have further defined these subsets. More importantly, IL-7 receptor alpha (CD127) (43) together with the killer cell lectin-like receptor subfamily G member 1 (KLRG1) (44) have allowed discrimination between memory precursor effector cells (MPECs) $CD127^{hi}KLRG1^{lo}$ from the short lived effector cells (SLECs), $CD127^{lo}$ $KLRG1^{hi}$. Interestingly, low expression of IL-2 receptor alpha (CD25) can also be used as a marker for precursors of memory CD8 T cell in the first days after acute LCMV infection (45). In addition to that, CD8 T cell subpopulations can

differ in functional properties, for example, it has been reported that central memory CD8 T cells can produce more IL2 and protect upon secondary challenge better than effector memory CD8 T cells (39).

CD8 T cell memory can also be defined by the presence of molecular markers. T-box transcription factors T-bet (tbx21) and Eomesodermin, have been found to be expressed in effector CD8 T cells, but inversely regulated by IL-12, which increases T-bet but represses Eomesodermin (46). In fact, expression of T-bet can discriminate between MPECs and terminally differentiated SLECs, which have been shown to posses high expression of T-bet (47). Another transcription factor associated with SLECs is BLIMP-1 (48). Additional transcription factors have been found including bcl-6 (49), critical for generation and maintenance of central memory CD8 T cells, Spi2A as a protective factor for memory CD8 T cell development (50) and two anti-apoptotic factors for effector CD8 T cells, Id2 (51) and Spi6 (50). Furthermore, future studies involving gene expression and epigenetics will certainly increased our knowledge about the transcriptional regulation of memory CD8 T cell development.

1.4 CD4 T cell help

To become functional long term memory CD8 T cells, additional signals from CD4 T cells are required (52). CD4 T cell help for CD8 T cells was first observed 35 years ago when in vitro amplification of CD8 T cells, Ly-23+ at that time, was abolished by excluding the participation of CD4 T cells, then Ly-1+ cells (53). CD4 T cell help for CD8 T cells was later observed in vivo using a system in which priming of CD8 T cells required linked recognition of alloantigens, Qa-1 for CD8 T cells and H-Y for CD4 T cells (54). These pioneer experiments validated the hypothesis that CD4 help for CD8 T

cells needed the formation of a three-cell cluster, in which CD8 T cells and CD4 T cells recognize their cognate antigen on the same APC (55). These results also highlighted that cooperation between CD8 T cells and CD4 T cells occurs only with low amounts of cognate antigen, as suggested yet by another report (56). In addition, IL-2 was proposed to mediate CD4 T cell help for CD8 T cell response (57).

In contrast, it was reported that CD4 T cells were not actually required for CD8 T cells to clear a viral infection (58, 59). At the time, current understanding about CD4 T cell help for CD8 T cells proposed that high amount of antigen present in acute or chronic viral infections was the reason behind the discrepancy between models of CD4 T cell help. However, a report showed that generation of primary CD8 T cell clearly depended on the presence of CD4 T cells in the immune response to herpes simplex infection (60). On the other hand, peptides derived from herpes simplex virus emulsified with Incomplete Freund Adjuvant (IFA) were able to generate CD8 T cell responses without the need for CD4 T cells (61). A report showing the importance of co-stimulation by B7 molecules on APCs to generate CD8 T cells in the absence of CD4 T cells (62) and the observation that costimulatory activity on APC was induced by influenza virus (63) led to the hypothesis that the need of CD4 T cell help for CD8 T cells depended solely on the amount of costimulatory molecules on APCs, later confirmed by a subsequent study (64).

A possible mechanism for CD4 T cell help was proposed based on CD40:CD40L interactions. An interesting report on mice deficient in CD40L, a TNF receptor family member, showed defects in antiviral immunity. Humoral responses were heavily compromised while efficient primary CD8 T cell response cleared the infection, but most surprisingly, CD8 T cell memory resulted impaired (65). These observations seemed to

indicate that CD40:CD40L interactions were required not only for B cell responses as reported (66) but also for the establishment and/or maintenance of memory CD8 T cells. Since a report showed no involvement of CD4 T cells in the maintenance of memory CD8 T cells (67), it was proposed by independent reports that CD40:CD40L interactions were involved in generation of memory CD8 T cells (68, 69). Concordantly, observations on immunized animals with no CD4 T cells resulted in reduced levels of memory CD8 T cells (70). In a apparently unrelated event, it was shown that CD8 T cells activated by peptides cross-presented on bone marrow-derived DCs required cognate CD4 T cell help (71, 72), supporting once more the hypothesis for a three-cell cluster. Not long after, these ideas were brought together by the licensing model of CD4 T cell help, where CD4 T cells were hypothesized to provide signals to activate DCs that will then stimulate CD8 T cells, forming a sort of temporal bridge (73, 74); and that this event was mediated by CD40:CD40L interactions (75-77).

However, several reports appeared to show that CD40:CD40L interactions were not indispensable to induce CD8 T cells (78-82), bringing back the idea that the activation status of APCs reflected by their costimulatory molecules dictated the need for CD4 T cell help through CD40-CD40L interactions (83). At the same time, additional ideas were proposed about the mechanism behind CD4 T cell help for CD8 T cells. One idea hypothesized that expression of CD40 on CD8 T cells allowed for direct interactions with CD4 T cells (84), but it was later challenged by experiments with CD40 KO CD8 T cells (85). Another idea proposed that the precursor frequency of CD8 T cells dictated the dependence on CD4 T cells, but its validity has been debated since it was based on

experiments involving the transfer of non-physiological high numbers of TCR transgenic CD8 T cells (86).

These controversies seemed to find common ground on a series of reports showing that potent inflammatory conditions created by an infection influenced APCs to upregulate enough costimulatory molecules to bypass the need of CD4 T cell help in mounting a primary CD8 T cell response (87-90). Most importantly, all these reports agreed that CD4 T cell help was required for secondary recall of memory CD8 T cell, with the caveat that no mechanism could be defined by which CD4 T cell help was delivered to CD8 T cells.

Following reports focused on different aspects of CD4 T cell help for CD8 T cells. Results describing the necessity of CD4 T cells (91) or not (92, 93) in the maintenance of memory CD8 T cells turn out to be very variable and mostly depended on the mouse model used for their experiments (94). Alternative signals involved in CD4 T cell help were proposed, CD27:CD70, found to support primary CD8 T cell response in the absence of CD4 T cells (95) or IL-15, proposed to be the mediator of CD4 T cell help (96). In addition, the idea that help is cognate or linked to the antigen recognized by CD8 T cells in need for help was resurfaced (97-99). More importantly, two-photon studies further supported the idea of DCs were licensed by CD4 T cells showing accumulation of cognate CD4 T cells in the same lymph node as CD8 T cells that were being primed by peptide-pulsed bone-marrow derived DCs (100, 101). These studies suggested that chemokines gradients guided cells into a two-step choreography where CD4 T cells activated DCs to secrete more chemokines to attract naïve CD8 T cells.

More recently, the role of IL-2 as mediator of CD4 T cell help has been explored in detail. Using a mouse model of mixed bone marrow containing wild type CD8 T cells and CD8 T cells with no CD25, the IL-2 receptor alpha chain, it was found that CD8 T cells without CD25 had an impairment in secondary recall (102). It was then hypothesized that IL-2 released by CD4 T cells had an impact in the programming of memory CD8 T cells. In contrast, reports have shown that CD25 expression (45) and IL-2 (103) favored terminal differentiation of CD8 T cells. Further studies supported the contribution of IL-2 for development of effector CD8 T cell responses showing that the functionality of effector CD8 T cells and generation of effector memory CD8 T cells was impaired in the absence of IL-2 (104). Concordantly, another report described a decrease of CD25 expression on CD8 T cells in the absence of CD4 T cells (105), which not only supported the idea that IL-2 is necessary for the rapid generation of effector cells in primary responses as well as in secondary responses but also suggested there might be signals other than IL-2 influencing CD8 T cell response. Reconciling these observations with previous reports, a recent publication showed that CD4 T cells could influence CD8 T cell response by two synergistic pathways. One pathway involved CD4 T cells activating APCs through CD40:CD40L interactions to induce secretion of IL12 that in turn increase CD25 expression on CD8 T cells, and the other pathway involving secretion of IL-2 by CD4 T cells for CD8 T cells in need of help (106). Even though several models proposed a third cell to bring CD4 T cells in proximity of CD8 T cells, further studies might be needed to understand how precisely CD4 T cells deliver their help to CD8 T cells when interacting at the initiation of an immune response.

Another aspect that has remained elusive is the exact molecular signature imprinted by CD4 T cell help on CD8 T cells. Several studies have attempted to measure how gene expression on activated CD8 T cells changes in the absence of CD4 T cells, but these results might not be applicable to all models of CD4 T cell help due to the diversity of conditions used in experiments. For example, early reports found apoptosis mediated by TRAIL in the secondary recall of helpless CD8 T cells (107), observation that was also replicated in experiments testing homeostatic proliferation of CD8 T cells in the absence of CD4 T cells (108). However, later reports could not find a direct correlation between TRAIL and absence of CD4 T cell help (109, 110). A partial view might be presented by studies in which gene expression have been measured using CD8 T cells lacking either CD25 (104) or CD40 (111), since one might have to assume the indispensable role for CD8 T cells of IL-2 or CD40L respectively received from CD4 T cells. T-bet has also been found to be highly expressed in helpless CD8 T cells (112), but it remains to be further investigated because it has also been implicated as a molecular switch between central and effector memory cell differentiation. In terms of epigenetic modifications, CD4 T cell help has been shown to influence methylation of IL-2 promoter and acetylation of histones of the IFNγ locus (113, 114). Although these studies shed light into possible mechanisms of long-term regulation of gene expression by CD4 T cells, one might suspect there will be plenty of regions in the CD8 T cell genome subjected to change that remain unexplored (115).

1.5 T:T interactions

Several cells of the immune system improve their contribution to an ensuing immune response after direct interaction with CD4 T cells; this has been called CD4 T

cell help. For example, macrophages are able to kill ingested bacteria more efficiently, B cells are able to switch immunoglobulin isotypes generating memory B cells and dendritic cells are able to increase their activation status to provide better costimulation, as well as to release inflammatory cytokines. These cells are considered to be specialized APCs that express αβ heterodimers called MHC class II molecules (116). These molecules present in their groove peptides derived from extracellular antigens that are recognized by the TCR on CD4 T cells (117). CD4 T cells are then activated by MHC class II molecules on APCs, which in turn receive help by direct contact, usually through CD40:CD40L interactions. It is then a common feature of CD4 T cell help for these APCs, the clear and measureable changes that can be observed on them before and after interacting with CD4 T cells (118).

In contrast, as mentioned in the previous section, the mechanism of CD4 T cell help for CD8 T cells is not well understood (52, 119). On one hand, CD8 T cells do not express MHC class II molecules in mouse models widely used to study CD4 T cell help (120), leading to the hypothesis that an intermediary cell is needed to facilitate this help (55, 73). On the other hand, there are no clear indications of changes in CD8 T cells that could mark the before and after of CD4 T cell help as an immunoglobulin switch is for B cells (118). In consequence, current consensus understands CD4 T cell help for CD8 T cells by effects observed in the absence of CD4 T cells like low or absent primary responses (54, 71, 121), defects in secondary recall (70, 87, 88, 90) or maintenance of CD8 T cells (91) in distinctively diverse models (52).

1.5.1 MHC class II and T cells

MHC class II molecules were detected on murine T cells more than 35 years ago when several reports described Ia antigens, today MHC class II molecules, in thymus-derived lymphocytes (122-125) or in vitro activated T cells (126, 127). These observations wonder about the functionality of Ia antigens on T cells (128), later fueled by reports showing "Ia-like" molecules on human T lymphocytes (129-132). Intriguingly, it was found that Ia molecules on T cells appeared only after activation (133, 134) and that these Ia antigens could clearly be detected on T cells in blood from patients suffering widespread immune activation caused by human autoimmune disorders like systemic lupus erythematosus (SLE), arthritis (135-137), and in a chronic infection like HIV (138-141).

Not surprisingly, Ia antigens detected on thymocytes were chemically indistinguishable from Ia antigens found on splenocytes (142), which suggested that Ia antigens could be functional, notion that was later confirmed by using Ia-positive T cell clones (143-146) and Ia-positive T cells from peripheral blood of SLE patients (147). As a consequence of having functional Ia antigens or MHC class II molecules, T cells were hypothesized to amplify an immune response as APCs (148-152). However, when studying T:T interactions, only few reports found an activating role in T cell presentation (151, 153-155) while many found them rather tolerizing (156-163). Intriguingly, CD4 T cells were reported to be polarized into a Th2 phenotype rather than into a Th1 phenotype when MHC class II was permanently induced on T cells, but the mechanism behind did not seem to involve T:T interactions (164).

An interesting twist to the story of Ia antigens on T cells was added by a report describing two types of Ia-positive T cells. One type was the blastoid T cell, responding from stimulation by mitogens, antigens and MLRs, while the other type was shown by smaller cells that were found in low levels in human normal peripheral blood and markedly elevated in various pathological states as described before (165). Also suggested by this report was the fact that Ia antigens could potentially be acquired from other cells, idea later supported by observations describing passive acquisition of Ia antigens by T cells (166). Moreover, it was reported that while human activated T cells can express MHC class II (167), mouse T cells could not expressed MHC class II due to the absence of MHC class II transactivator (CIITA) (120). It was later found that DNA methylation at the CIITA promoter III region (168) prevented expression of CIITA and therefore no MHC class II was expressed. Furthermore, evidence seems to suggest that transference from APCs to T cells must occur if MHC class II is found on the surface mouse T cells. To understand this surprising event, it is necessary to delve into how T cells interact with APCs in what has been called immunological synapse.

1.5.2 Immunological synapse

The recognition of a specific peptide in the groove of MHC class I complex (MHCp) by the TCR of a CD8 T cell trigger a series of events that result in CD8 T cell activation. The mechanism that allows the communication of signals between an APC and a naïve CD8 T cells has been termed Immunological Synapse (IS), which is an specialized cell-cell adhesive junction that features stability to sustain signaling and directed secretion (169).

The classical concept of IS is defined by a cell-cell interface in which leukocyte function-associated antigen 1 (LFA-1) interacts with intercellular adhesion molecule (ICAM-1) and talin to form a ring around a central cluster of TCR-MHCp and protein kinase-θ (170-172). These structures were defined as supramolecular activation clusters (SMACs). TCR-MHCp cluster indicated the central (c)SMAC, while LFA-ICAM-1 ring defined the peripheral (p)SMAC. It was then found that TCR signaling was initiated and sustained by cSMAC. Further studies indicated that TCR microclusters associated with MHC molecules were converging from the periphery of the "immature" synapse into the cSMAC, releasing these interactions from steric barrier created by large molecules like CD43 and CD45 (173-175). Additional molecules have also been identified as cSMAC constituents, including CD2, CD28, Lck, Fyn and CD8 (176).

It has been suggested that the main function of the immunological synapse, specially of the cSMAC, is to sustain TCR signaling (172, 177). However, TCR signaling augments phosphorylation that leads to its internalization and degradation (178) and since receptor down-regulation has been described to turn off signaling (179)**,** the cSMAC sustained signaling may facilitate TCR down-regulation (180). In that regard, TCR down-regulation upon activation of a CD8 T cell has been implicated in the surprising finding of acquisition of peptide-MHC complexes from the APC participating in the IS to activated CD8 T cells (181).

On the other hand, IS is not always the same, it depends on the differentiation status of the CD8 T cell interacting with an APC. When we focus on a mature CTL interacting with an APC, we find what it is called a cytotoxic IS, that differs from the classical mature IS structure (182). These structures serve more like a docking to the

directional secretion of cytokines and lysosomes containing Fas-ligand into the contact area between a CTL and its target (183, 184). The rapid delivery of CTL granules onto the target cell upon its detection will lead to what has been called, the “kiss of death” (185). Intriguingly, the cytotoxic IS not only contains a secretory domain, but also membrane bridges (186). These membrane bridges can allow protein transfer from the target cell to the CTLs, which could also participate in future interactions that these CTLs could have with other cells. A consequence of this phenomena has been used to detect specific CTLs with labeled protein that is transferred upon killing of the target cell (187, 188). In order to understand better the transference of protein occurring upon CD8 T cell activation and killing of a target cell, we have to describe the phenomena of intercellular transfer of protein.

1.5.3 Intercellular transfer of protein and Trogocytosis

Some decades ago, surprising observations described proteins specific of one cell type were detected in small quantities on surfaces of other cell types (189). Worth mentioning is the capture of B-cell surface immunoglobulin by T cells, later found to be antibodies against allotypic MHC acquired by these T cells (190, 191). It was also observed transference of antigens from macrophages to T cells (192), the acquisition of recipient MHC class I and class II proteins on donor thymocytes in bone marrow transplants experiments (124, 125) and the transfer of MHC class II protein from splenic cells to allogeneic T-cell clones (166). However, the significance of this interecellular transfer of proteins remained unkown.

Multiple studies have now shown that cells of the immune system can transfer surface molecules from one cell to another upon interaction (193). For example, a

bidirectional receptor-specific transfer of molecules was observed when natural killer (NK) cells interacted with their targets (194). In consequence, when the inhibitory killer immunoglobulin (Ig)-like receptors (KIRs) on NK cells interacted with MHC class I ligands on the target cells, MHC class I was absorbed by NKs (195-197) and KIRs appeared on the target cells (198). The antigen-specific TCRs on T cells not only acquire cell-associated ligands from APCs like MHC class II for CD4 T cells or MHC class I for CD8 T cells, but also bystander MHC molecules (181, 198), costimulatory proteins (199-201) and membrane fragments (186, 202). However, in the case of TCR-mediated absorption, it has been shown to occur in a unidirectional manner, which means that there is no reciprocal transfer of T cell surface molecules onto APCs (203).

As mentioned before, when TCR transgenic T cells recognize specific MHC-peptide complexes on APCs, the transference of MHC molecules onto the responding T cell appear to be mediated by TCRs (181, 200). Strikingly, this is not the only mechanism that can be accounted for absorption of molecules, since the culture of purified polyclonal T cells with syngeneic APCs in the absence of foreign antigen can also lead to fast transfer of MHC and other molecules from the APC, even when TCR blocking antibodies are present (200). This TCR-independent absorption is mediated in part by CD28 interaction with B7 molecules, like B7-1:CD80 and B7-2:CD86, suggesting that the level of CD28 in T cells has an important impact on a TCR-independent transfer. It was found that CD4 T cells and activated CD8 T cells acquired more B7 molecules on their surface than naïve CD8 T cells because they had higher expression of CD28. In addition, it has also be found some involvement of LFA-1 in the transfer of molecules using CD28

deficient T cells, but the level of transference detected was not as high as it was when CD28 was present (200, 204).

Interestingly, it has been reported the transfer of proteins that do not participate in ligand-receptor interactions, which might suggest that pieces of membrane are ripped off from the surface of the donor cells. This process has been called trogocytosis (188, 205) that derives from the Greek word "trogo", which means to gnaw or to nibble. This situation is especially applicable to cell-cell interactions that involve IS formation, where the associated cytoskeleton can augment and stabilize receptor/ligand interactions that can lead to membrane fusion (181, 186, 206-209), and the subsequent dissociation of the interacting cells might cause some membrane fragments to be transferred from one cell to another.

As an alternative model of transference at the immunological synapse, the molecules can be obtained from vesicles shed or secreted from the donor cells. This model was tested for T cells in transwell experiments where APC remained separated by porous membrane from T cells (200, 209). Little absorption by naïve T cells was observed, which infer the need of cell-cell contact, but it was increased if the T cells were activated previously to the cell culture, probably by increasing the level of uptake by CD28. On the other hand, many cell types can shed membrane fragments in the form of exosomes (193, 210, 211), including dendritic cells (212, 213), B cells (214, 215) and T cells (216, 217). These are transwell-permeable small membrane vesicles (50-100 nm) derived from fusion of multivesicular endosomes with the plasma membrane. Immature DCs release high amounts of exosomes (212, 213) that if loaded with peptides can become highly immunogenic when cross-presented by mature APCs (213). DCs can

uptake exosomes by using integrins, LFA-1 and ICAM-1 (211). Interestingly, while exosomes can bind to both naïve and activated T cells (218), binding of exosomes to naïve T cells can only happen when there is TCR-MHC-peptide interaction and presence of ICAM-1. Moreover, naïve CD8 T cells can only be stimulated by exosomes that contain B7 molecules.

The physiological relevance of intercellular transference is still in question. While transfer of molecules could merely be a by-product of high-avidity receptor/ligand interactions, many reports have described immunological consequences. It has been reported that specific peptide-MHC complexes on APCs might get ripped off when recognized by the TCR of CD8 T cells at early stages of an immune response to ensure that only high avidity T cells are stimulated (219). CD8 T cells can also become the target of fratricide by another CD8 T cell recognizing an specific MHC class I transferred from an APC (181) or interact with memory CD8 T cells (220). CD4 T cells have also been reported to interact with CD8 T cells by receiving bystander MHC class I upon their activation (221), but the validity of this model for CD4 T cell help could be questioned on grounds that CD4 T cells do not receive signals to deliver their help since MHC class II is not present on CD8 T cells. All these observations suggest that molecules transferred from APCs onto T cells upon activation can facilitate interactions between T cells, namely CD4 and CD8 T cells. In chapter 2, we investigated the plausibility of a model involving MHC class II bearing CD8 T cells and the possible interactions with CD4 T cells.

1.6 CD8 T cell migration

Many important advances in understanding immunology have been made by studying lymphocyte migration. The single most important and founding discovery of cellular immunology was the observation that lymphocytes were the units of the immune system providing protection (222). Cells from lymph nodes were found to transfer resistance to infection from immune animals to naïve animals (223) as predicted by the clonal selection theory (4). These cells were later found to be lymphocytes that circulated from blood into lymph nodes and then to the thoracic duct back to blood (224, 225). At this time, pioneer studies also described that T lymphocytes specific for an ensuing immune response will disappear from thoracic duct (226), and therefore from blood and spleen (227), the first days after injection of antigen. More than 30 years later, this sequestration was found to be induced by type I IFNs and/or TCR activation (228) and mediated by lymphocyte response to exit signals detected on sphingosine-1-phospate receptors (229).

As mentioned in previous sections, differentiation of memory CD8 T cells can be influenced by the location of cells responding to a viral infection. The anatomical environment in which CD8 T cells are located has been suggested to determine functional properties in viral immunity, being in the spleen (230) or lymph nodes (231), lung (232), gut (233) or bone marrow (234). For example, it has been found that the immunosuppression observed in the weeks after a viral infection was caused by the interruption of chemokine gradients that guide CD8 T cells into white pulp of spleen (230). A later report has found that early memory precursors or MPECs locate to the

white pulp of spleen attracted by the same chemokines, CCL19 and CCL21, while terminally differentiated effector cells or SLECs remained in red pulp (235).

CD8 T cells acquire specific integrins and chemokine receptors after activation that define their migration profile. Naïve CD8 T cells express CD62L and CCR7 that allow them to enter into lymph nodes passing through High Endothelial Venules (HEV) following chemokine gradients secreted by lymph node DCs (19, 183, 236, 237). Soon after activation, effector T cells that downregulate CD62L and CCR7 could nonetheless enter reactive lymph nodes using CXCR3 to kill DCs and stop antigen presentation (238). The same way, effector CD8 T cells can gain entry into gut by the expression of alpha4 beta7 integrin ($\alpha4\beta7$) and differentiate into an effector memory T cells influenced by cytokines in their environment like TGFβ (233, 239). In the case of bone marrow, it has been described that LFA-1 and alpha4 beta1 integrin ($\alpha4\beta1$) interacts with VCAM1 to allow CD8 T cells circulate into bone marrow (240) and CXCR4 expression can allow CD8 T cells to be retained when encountering a CXCL12 rich stromal niche that also contains IL-7 and IL-15 in bone marrow (241-246). In consequence, these observations suggest that the differentiation of a CD8 T cells could be shaped by the location in different tissues defined by migration. Since it is not known yet where CD8 T cells specifically locate when responding to viral infection in the first days after LCMV infection, we designed experiments to investigate CD8 T cells location, as well as resulting phenotype and functional properties as a measure for their differentiation in lymphoid tissues. Moreover, we placed special emphasis on bone marrow, found to host memory CD8 T cells, in comparison with other lymphoid tissues like spleen and PLN.

CD8 T cell presence in bone marrow has been observed in primary as well as in secondary immune responses. Bone marrow is an immunological niche that supports survival of memory CD8 T cells (247), plasma B cells (243) and memory CD4 T cells (248). In primary responses, priming can occur in bone marrow, even in the absence of any other lymphoid tissues (234) or when migration to lymphoid tissues has been disrupted (249) providing protection against viral infection. CD8 T cells generated in Bone Marrow have been also reported to induce milder Graft vs Host Disease (GVDH)(250) and shown to be specially effective against tumors (251), which might accentuate their unappreciated contribution in primary immune response. In the case of secondary responses, several groups have described preferential localization of memory CD8 T cells in Bone Marrow (244, 246, 252, 253), where they specifically home to compete for critical survival factors as mentioned before (254, 255).

In this thesis, we present results on the contribution to CD8 T cell memory formation provided by direct interactions between CD8 and CD4 T cells during the first days of LCMV infection (chapter 2) and results about the location CD8 T cells during the first days of LCMV infection (chapter 3). We also present conclusions that could be drawn from our results and their implications for future research (chapter 4).

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Chapter 2

CD8 T cell memory recall is enhanced by novel direct interactions with CD4 T cells enabled by MHC class II transferred from APCs

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2.1 Abstract

Protection against many intracellular pathogens is provided by CD8 T cells, and pathogen-specific CD8 T cells are thought to need CD4 T cell help to develop into effective memory CD8 T cells. Because murine CD8 T cells do not transcribe MHC class II genes, several models have proposed antigen presenting cells (APCs) as intermediaries required for CD4 T cells to deliver their help to CD8 T cells. Here, we demonstrate the presence of MHC class II molecules on activated murine CD8 T cells in vitro as well as in vivo. These CD8 T cells acquire MHC class II from their activating APCs, particularly CD11c positive dendritic cells (DCs), via a process called trogocytosis. Transferred MHC class II molecules on activated murine CD8 T cells were functionally competent and could directly stimulate specific indicator CD4 T cells. CD8 T cells that were "helped" in vitro and subsequently allowed to rest in vivo showed enhanced recall responses upon challenge compared to "helpless" CD8 T cells. In contrast, no differences were seen upon immediate challenge. These data indicate that direct CD8:CD4 T cell interactions may significantly contribute to help for CD8 T cells. Furthermore, this mechanism may enable CD8 T cells to communicate with different subsets of interacting CD4 T cells that could modulate immune responses.

2.2 Introduction

Immunological memory is defined by the ability of the immune system to respond faster and more effectively to a pathogen that it has previously encountered and eliminated. This ability is conferred by the presence of long-lived, clonally expanded populations of pathogen-specific T and B lymphocytes (1). While both subsets are important to control and clear pathogens (2-4), CD8 T cells are critical for the control of infections with intracellular pathogens (3, 5, 6). Therefore, in order to improve quality and effectiveness of vaccines for such pathogens, it is essential to define the precise mechanism by which memory CD8 T cells are generated and preserved over time (7).

CD8 T cells must receive more than one signal of activation to become fully functional (8-10). Signal 1 is provided when the T cell receptor (TCR) on CD8 T cells recognizes its cognate peptide presented in the groove of MHC class I molecules on the membranes of antigen presenting cells (APCs) (11), usually a dendritic cell (DC) in secondary lymphoid organs (SLO) (12, 13). Signal 2 is provided by costimulatory molecules (14-16), typically members of the B7 family or TNF family or chemokines, expressed on the same DCs if they have been activated by inflammatory pathogen-associated molecular patterns (PAMPs) present during the innate response (17, 18). Lastly, a third signal provided by cytokines present in the surrounding inflammatory milieu (19, 20) completes the priming phase of a nascent CD8 T cell response.

In addition to the signals described above, to become functional long term memory cells, CD8 T cells require additional signals from CD4 T cells (21). It has been reported that when CD4 T cells are depleted or absent, memory recall responses by CD8 T cells are impaired (22-26). However, whereas some of the signals involved in the CD4

T cell help have been identified (27-36), the precise mechanism by which CD4 T cells provide help for CD8 T cells remains poorly understood.

A major conceptual roadblock to understanding how CD4 T cells provide help to CD8 T cells is that while all other immune cells that require help – e.g. B cells and macrophages – transcribe and translate MHC class II, murine CD8 T cells do not. Further complicating matters, mice are one of the few species in which CD8 T cells do not transcribe MHC class II genes, an effect that has been tied to the hypermethylation in promoter III of the transcription factor MHC class II Trans Activator (CIITA) (37). In contrast, in humans, it has been shown that activated CD8 T cells express MHC class II (38) and even co-stimulatory molecules such as CD86 (39), a B7 family member, though the immunological significance of these observations has never been satisfactorily addressed.

While the data on the failure of murine CD8 T cells to transcribe MHC class II appears to be very solid, scattered reports over the course of 30 years have described MHC class II on mouse T cells (40-45) and have suggested that the cells acquire MHC class II from other cell types by a membrane transfer mechanism termed trogocytosis (46-50). In this report we further verify that activated CD8 T cells do become MHC class II positive during the early stages of antigen recognition and that the MHC class II molecules are derived from APCs, principally CD11c+ DCs. We also show that the transfer of MHC class II with their peptide ligands endows CD8 T cells with the ability to interact directly with helper CD4 T cells which in turn deliver signals that confer to the activated CD8 T cell the ability to become a long term memory cell.

2.3 Material and Methods

2.3.1 Mouse strains

Wild type (WT) C57BL6, CIIKO, B6.Thy1.1 and OTII mice purchased from The Jackson Laboratories (Bar Harbor, ME). P14 and SMARTA mice were obtained from Dr. Rafi Ahmed (Emory University, Atlanta, GA). OTI mice Rag KO were obtained from Shivaprakash Gangappa (Emory University, Atlanta, GA). F5 mice were obtained from Demetrius Moskophidis (Medical College of Georgia, Augusta, GA), with permission from Dimitris Kioussis (NIMR, London, UK). SM1 mice were obtained from Marc K. Jenkins (University of Minnesota, Minneapolis, MN). I-Ab-gfp mice were obtained from Dr. Hidde Ploegh (MIT, Cambridge, MA). P14xCIIKO were generated by backcrossing into CIIKO mice more than 10 generations confirming genotype by PCR and phenotype by flow cytometry (data not shown). F1 ($I\text{-}A^b$ x $I\text{-}E^k$) hybrids were created by breeding AND (B10.A) mice, obtained from Dr. Brian Evavold (Emory University, Atlanta, GA), with C57BL/6J from Jackson Laboratories. All mice were maintained under specific pathogen-free conditions at the Emory Vaccine Center, Yerkes National Primate Research Center, Atlanta, GA.

2.3.2 Pathogens and infections

Age-matched sex-matched animals were injected with either $2x10^5$ plaque forming units (p.f.u.) intraperitoneally (i.p.) or $2x10^6$ p.f.u. intravenously (i.v.) of LCMV Armstrong 53b (Arm) virus or $5x10^4$ c.f.u. i.v. of Recombinant *L. Monocytogenes* containing gp33 peptide (rLM-gp33) obtained from Dr. Rafi Ahmed (Emory University, Atlanta, GA). LMCV Arm and rLMgp33 have been described previously (51, 52). Bacteria numbers

were determined by plating serial dilutions of spleen homogenates on PBS 0.05% Triton X-100 as described (53).

2.3.3 Cell suspensions and flow cytometry

Single cell suspensions were prepared using 70 μm cell strainers or by collagenase treatment performed as described (54) using Collagenase 4 (Worthington Biochemical Corp., Lakewood, NJ). Red Blood Cells were lysed using Red Blood Cell Lysing Buffer (Sigma-Aldrich, St Louis, MO) and washed with RPMI. Fluorochrome-conjugated antibodies were obtained from BD Pharmingen (San Diego, CA), eBiosciences (San Diego, CA) and BioLegend (San Diego, CA). Intracellular cytokine staining was performed following manufacturer's protocol with either 2.5 μM of peptides or anti-CD3 (145-2C11) plus anti-CD28 (37.51) both from BD Biosciences Pharmingen (San Diego, CA), for 6 hrs in the presence of Brefeldin A (1 μl/ml GolgiPlug, BD Pharmingen, San Diego, CA). Tetramers were prepared as described (55) and tetramer enrichment protocol was performed as described (56). Live-dead discrimination was performed using an in-house developed protocol based on Alexa Fluor 430 dye (Molecular Probes, Eugene, OR). Multiparameter analysis of samples was performed on LSRII flow cytometer (BD Biosciences) and results were analyzed using FlowJo software (TreeStar, Ashland, OR).

2.3.4 Peptides

Peptides gp33-41 (KAVYNFATM), Ova257-264 (SIINFEKL), np366-374 (ASNENMDAM), gp61-80 (GLNGPDIYKGVYQFKSVEFD), Ova323-339 (ISQAVHAAHAEINEAGR), FliC427-441 (VQNRFNSAITNLGNT) were synthesized by Microchemical Facility Core (Emory University, Atlanta, GA).

2.3.5 Cell culture

CD8 T cells, CD4 T cells, FMS-like tyrosine kinase 3 ligand (flt3L)-DCs, B cells and splenic macrophages were purified using autoMACS system per manufacturer's protocol (Miltenyi Biotech, Auburn, CA). CD8 T cells and CD4 T cells were purified by negative selection. Flt3L-DCs, B cells, splenic dendritic cells or splenic macrophages were purified by positive selection. T cells and flt3L-DCs were cultured in 48 flat-bottom well plates at a 2:1 ratio (T:DC) in complete RPMI media [RPMI-1640 media (Life Technologies) supplemented with 10% FBS, 50 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin G, 100 μg/ml streptomycin, 10 mM HEPES buffer, 1 mM L-glutamine and 0.5 mM sodium pyruvate] at 37°C in a 5% CO_2 incubator. Flt3L was obtained from Dr. Bob Mittler (Emory University, Atlanta, GA) and flt3L-DCs were generated as described (57). Concanavalin A was purchased from Sigma-Aldrich (St Louis, MO).

2.3.6 In vitro CD4 T cell differentiation

CD4 T cells (OTII, SM1 or SMARTA) were magnetically isolated and cultured with flt3L-DCs at a 5:1 ratio in flat bottom 6-well plates overnight with their specific peptide (ova323-339, Flic427-441 or gp61-80, respectively). Activated CD4 T cells were separated by density using Lympholite M (Cedarlane, Hornby, Ontario, Canada) and incubated in complete RPMI medium supplemented with IL-2, IL-7 (10 ng/ml, eBioscience, San Diego, CA) and IL-15 (10 ng/ml, R&D Systems, Minneapolis, MN) changing media every 2 days as described before (58). After 4 days of culture, CD4 T cells are again magnetically isolated and cultured overnight in complete RPMI media. Finally, Live CD4 T cells were depleted of dead cells using Dead Cell Discrimination kit (Miltenyi Biotech, Auburn, CA).

2.3.7 Adoptive Transfer

CD8 T cells and CD4 T cells purified as described above were counted with hemocytometer by optical microscopy. Purity was calculated by staining with antibodies specific for the Vα and Vβ TCR domains and acquisition using FACScalibur (Becton Dickinson). Purified cells were labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Invitrogen, CA) at 2.5 μM as described before (59, 60) and adoptively transfer as described (61).

2.3.8 Statistical Analysis

Statistical significance was determined by ANOVA, using Prism 5 (Graphpad Software, La Jolla, CA).

2.4 Results

2.4.1 MHC class II is present on recently activated murine CD8 T cells in vitro as well as in vivo

Although it is known that murine CD8 T cell can not transcribe MHC class II genes (37), some reports have described the presence of MHC class II on activated CD8 T cells after interaction with APCs (46, 47). To verify this, we incubated magnetically sorted P14 TCR transgenic CD8 T cells (P14 cells) with flt3L in vivo expanded CD11c-enriched DCs (flt3L-DCs) pulsed with one of the following: vehicle, control peptide (Ova257-264), the mitogen Concanavalin A (Con A), or the stimulatory cognate peptide (LCMV.gp33-41). We found that MHC class II was displayed on the surface of CD8 T cells activated with either their cognate peptide or with Con A (Fig. 2.1a). In contrast, MHC class II was not observed in conditions where CD8 T cells were not activated (no peptide or control peptide).

To determine if a similar event occurs upon in vivo activation, P14 cells ($1x10^6$) were adoptively transferred into WT mice that were infected one day later with $2x10^5$ p.f.u. of LCMV Arm i.p.. At two days post-infection (p.i.) in the draining mesenteric lymph node (MLN), MHC class II was detected on transferred cells that had upregulated CD54 (ICAM-1), a marker of activation, but not on the approximately 50% of transferred cells which remained $CD54^{lo}$ (Fig. 2.1b); detection of MHC class II on the $CD54^{hi}$ cells was transient and disappeared by 6 days post-infection. Similar results were found in the spleen and peripheral lymph nodes (PLN) (Fig. 2.1c, Fig. 2.6), but with slightly different kinetics.

As expected, MHC class II was not detected on bystander CD8 T cells in vitro or in vivo (Fig. 2.7). Intriguingly, transferred CD8 T cells never displayed MHC class II on their surface in blood (Fig. 2.1c and 2.6). Together, these results definitively show the presence of MHC class II on recently activated CD8 T cells responding to an infection in vivo.

To investigate if non-transgenic CD8 T cells display MHC class II when responding to an infection, WT mice were infected with $2x10^6$ p.f.u. of LCMV Arm i.v.. Spleens, PLNs and MLNs were collected and LCMV-specific CD8 T cells were enriched magnetically using D(b)/LCMV.gp33-41(KAVYNFATM) tetramers. At 2.5 days post-infection, enriched cells that expressed CD25 –indicating in vivo activation– also had detectable MHC class II, which was not found on $CD25^{neg}$ cells (Fig. 2.1d). As expected, Kb/Ovalbumin.ova257-64 (SIINFEKL) tetramer enriched CD8 T cells did not show any MHC class II staining since they all remained naïve ($CD25^{neg}$) and were not responding to infection (Fig 2.8). Taken together, both in vitro and in vivo results show that recently activated CD8 T cells display MHC class II on their surface.

2.4.2 MHC class II is transiently present on responding transgenic CD8 T cells after infection

To determine the kinetics of MHC class II presence on CD8 T cells in vivo, CFSE-labeled P14 cells ($1x10^6$) were adoptively transferred into WT mice, and were infected one day later with $2x10^6$ p.f.u. of LCMV Arm i.v.. As shown in Fig. 2.2a, the transferred transgenic CD8 T cells in the spleen start to increase detectable MHC class II as soon as 12 hours p.i., even before they begin dividing. Division commences between

24 and 36 hours, with the fraction of cells with detectable MHC class II peaking between 36-42 hours post-infection and decreasing through 62 hrs p.i.

We compared the presence of MHC class II and two markers of T cell activation (CD69 and CD54) on transferred CD8 T cells in spleen, PLN and MLN as a function of time after intravenous infection with LCMV Arm (Fig. 2.2b). All markers of activation on transferred CD8 T cells, including MHC class II, appeared faster in the spleen than in PLN and MLN. MHC class II on transferred P14 T cells continued to rise in PLN and MLN at time points (50 and 62 hours post-infection) when it was already falling in the spleen.

No significant MHC class II presence was detected at 36 hours post-infection when CD8 T cells were activated in MHC class II KO (CIIKO) mice (Fig. 2.2c), consistent with the observation that mouse activated CD8 T cells can not transcribe and translate their own MHC class II genes (37).

2.4.3 MHC class II is transferred onto CD8 T cells from APCs

Since mouse CD8 T cells can not express MHC class II, we decided to test the idea that CD8 T cells could acquire these MHC class II molecules by trogocytosis. For that reason, CD8 T cells were activated in vitro in the presence of flt3L- DCs from either CIIKO, B6 or an F1 B6xB10.A mouse. As shown in Fig. 2.3a, CIIKO DCs lacked MHC class II expression, B6 DCs expressed their strain specific MHC class II (I-A^b) and the F1 hybrid DCs expressed both MHC class II molecules derived from its breeder strains (I-A^b and I-E^k).

P14 cells cultured with DCs pulsed with their cognate peptide but not with an irrelevant peptide (ova257-264) or no peptide, displayed the MHC class II that is present

on the DC (Fig. 2.3b). Since P14 cells are on the B6 background and do not have the H-2^k alleles, this indicates that the I-E^k observed on the activated P14 cells is derived from the F1 B6xB10.A APCs. To further test for the source of the MHC class II observed on in vitro activated P14 cells, we performed an experiment in which the MHC class II locus was knocked out (CIIKO) on the T cells, the APCs, or both. Maximal detection of MHC class II on activated P14 CD8 T cells was observed only on T cells cultured with wild type DCs, again suggesting that most of the MHC class II observed on the T cells was derived form the DCs (Fig. 2.3c); no differences were seen when WT or CIIKO T cells were stimulated with WT DCs. However, we cannot completely rule out a very low level of expression of MHC class II by the T cells themselves, since WT CD8 T cells stimulated with CIIKO DCs had a very small frequency of cells with detectable MHC class II compared to CIIKO T cells stimulated with CIIKO DCs. Similar results were seen in experiments in which the P14 cells were replaced with OT-I TCR transgenic CD8 T cells (data not shown).

To determine what type of APC was best able to transfer MHC class II molecules, we isolated three major populations of APCs from WT mice using magnetic enrichment, B220+ containing mostly B cells, CD11b+ containing myeloid DCs, macrophages, monocytes and granulocytes and, CD11c+ containing lymphoid and myeloid DCs (Fig. 2.3d). Even though all the enriched populations possessed high amounts of MHC class II (Fig. 2.3e), CD11c+ enriched population transferred the most MHC class II onto CD8 T cells upon in vitro activation compared to B220+ and CD11b+ magnetically enriched populations (Fig. 2.3f and g). These results further support the fact that MHC class II is

transferred from APCs onto activated CD8 T cells and that the APC subset that transfers most effectively are CD11c+ DCs.

2.4.4 MHC Class II on activated CD8 T cells mediate direct stimulation of experienced CD4 T cells.

LCMV specific P14 CD8 T cells were activated as described in Fig. 2.1a with cognate peptide in addition to MHC class II peptides, ova323-339 for OTIIs (OTIIp) or FliC427-441 for SM1s (SM1p). After activation, CD8 T cells were isolated magnetically (purity ~85%, data not shown) and tested for their ability to stimulate in vitro primed OTII or SM1 CD4 T cells (see Material and Methods), measured by intracellular staining of CD4 T cells for TNFα and IL2. As shown in Fig. 2.4a, CD8 T cells presenting MHC class II molecules, obtained from DCs that had been pulsed with MHC class II restricted cognate but not with control peptide, were able to stimulate indicator transgenic CD4 T cells as measured by intracellular staining for TNFα and IL2. In these assays, the antigen presenting CD8 T cells were as potent as peptide-pulsed CD11c+ DCs. Intracellular cytokines were not detected when CD4 T cells were incubated in the absence of antigen presenting CD8 T cells or DCs, and strong responses were seen when CD4 T cells were stimulated with the combination of plate-bound antibodies anti-CD3 and anti-CD28. These results demonstrate MHC class II/peptide complexes obtained by CD8 T cells from DCs retain full capacity to be recognized by CD4 T cells.

To exclude the possibility that the above results were due to DCs contaminating the purified activated CD8 T cells population, two approaches were used. In the first, prior to purification, the CD8 T cell population was spiked with DCs that had been pulsed with the MHC class II restricted peptide that is recognized by CD4 T cells. In these

experiments, the level of contaminating DCs is below the limit of detection by flow cytometry assay for CD11c+ cells (Fig. 2.9a) and insufficient to provide significant stimulation of the indicator CD4 T cells (Fig. 2.9b).

In a second approach, we determined how many DCs contaminating either primed or naïve CD8 T cells are required to provide significant stimulation to indicator CD4 T cells. As shown in Fig. 2.9c, significant CD4 T cells responses were not seen until the ratio of DCs to CD8 T cells exceeded 1:40. Intriguingly, we noticed that DCs added to the culture containing activated CD8 T cells did not stimulate CD4 T cells as much as they did when added to naïve CD8 T cells. In light of these results, we can conclude that the cytokine release by the indicator CD4 T cells is not caused by DC contamination of the purified activated CD8 T cell population.

2.4.5 In vitro interactions between MHC Class II+ activated CD8 T cells and experienced CD4 T cells improve the in vivo recall response of the CD8 T cells

To test the hypothesis that CD4 T cells can provide help to MHC class II+ CD8 T cells via direct T:T interactions, we developed a model in which we tested the in vivo recall responses of in vitro "helped" CD8 T cells. Prior to transfer into naïve congenic Thy1.2 hosts, naïve Thy1.1 P14 CD8 T cells were primed on DCs pulsed with both the cognate MHC class I restricted peptide (LCMV.gp33-41) and an MHC class II restricted peptide (either LCMV.gp61-80 or control ova peptide), purified, cultured with in vitro primed SMARTA CD4 T cells, and re-purified. After transfer, recipients were further split into two groups. One group (day +1) was immediately challenged with a recombinant *Listeria monocytogenes* strain expressing the peptide recognized by the CD8 T cells but not the CD4 T cells (Lm.gp33), while the other group was rested for 30 days

prior to challenge with the same pathogen (day +30). Mice were sacrificed 4 days after challenge and their spleens were analyzed for numbers of Thy1.1+ CD8+ events (P14 CD8 T cells) (Fig. 2.5).

In the groups challenged immediately after transfer (day +1), there was no difference in expansion of P14 cells that had or had not received help in vitro. In contrast, when mice were rested for 30 days after transfer (day +30), P14 cells expansion was significantly greater in the mice that had received in vitro "helped" P14 cells compared to mice that received "unhelped" P14 cells. Preliminary results suggest that this difference in expansion was not due to differences in survival of the transferred cells prior to the challenge (Fig. 2.10). These results suggest that memory recall of CD8 T cells is significantly improved by direct interactions with experienced CD4 T cells that are mediated by MHC class II transferred from activating APCs.

2.5 Discussion

Current consensus is that CD4 T cell help is required for differentiation of CD8 T cells into memory cells that have strong proliferative potential upon secondary challenge (22-25). Dendritic cells that present both class I and class II MHC restricted antigens on the same cell are a central feature of all models for delivery of help to CD8 T cells. At the level of cellular interactions, there are two alternative models for how help is delivered. In the "licensing model" (34), CD4 T cells deliver signals that lead to differentiation of a dendritic cell into a mature stage that is subsequently able to provide adequate differentiation signals to CD8 T cells that are stimulated by the matured DC at a later time (32, 33, 35). In contrast, the "paracrine" model invokes three-cell clusters including an antigen-bearing DC, a specific CD4 T cell, and a specific CD8 T cell, with the release of CD4 T cell derived "helper" factors in close proximity to the CD8 T cell (36, 62, 63). Models incorporating direct antigen-specific interactions between the CD4 and CD8 T cells have generally not been contemplated, largely because CD8 T cells in the mouse do not synthesize the class II MHC molecules and therefore present the relevant peptides that would allow them to stimulate a CD4 T cell. In this paper, we present data that supports the plausibility of a unique model in which direct CD4:CD8 T cell interactions can contribute to the delivery of help to CD8 T cells. In our model, this interaction is enabled by class II MHC/peptide complexes on the CD8 T cell that were obtained by trogocytosis.

Trogocytosis has emerged as a mechanism by which cells that do not directly transcribe and translate mRNA for specific membrane-associated proteins can nevertheless acquire those proteins from other cells. The history of trogocytosis begins

more than 35 years ago with observations describing the acquisition of allo-antigens on the surface of activated allo-specific T cells (64), and in 1982, Fitch's group described the passive acquisition of I-A antigens by alloreactive T cells (45). Nearly 20 years later, Sprent's group demonstrated that transfer of cell surface molecules to CD8 T cells required signaling from either TCR or CD28 on the surface of the T cell, and at least some of the transferred molecules were not obviously directly involved in ligand interactions between the two cells, including class II MHC (47). The term trogocytosis itself was coined by Hudrisier and Joly (65), and has since been extensively studied by that group (66-77). More recently, transfer of membrane proteins from APC—CD45.1, allo-class I MHC, and biotinylated molecules from surface-biotinylated DCs—to virus-specific TCR transgenic CD8 T cells has been seen in the acute phase of the T cell response following infection of mice with viruses expressing the antigen recognized by the T cells, but no functions were ascribed to the transferred molecules (78). Acquisition of class I MHC peptide complexes by trogocytosis may provide antigenic targets for fratricide among CTL clones (48) or may simply reduce the concentration of antigen on the surface of APCs, thereby leading to a reduction in T cell stimulus (79). Xiang et al (80) proposed that class I MHC molecules obtained by CD4 T cells via trogocytosis formed the basis for a novel and direct CD4:CD8 T cell interaction that results in the provision of help for the CD8 cells, but this model fails to account for signals that maintain effector functions of the CD4 T cells themselves because the CD8 T cells in this interaction do not present MHC class II/peptide complexes.

The function of class II MHC on T cells, either expressed directly as in human T cells or acquired by trogocytosis, has been even more of a mystery. Some investigators have

proposed that acquisition of class II MHC on T cells enables them to act as an APC (T-APC), resulting in amplification of CD4 T cell responses by increasing total numbers of effective APCs (81-83). Others have proposed that class II MHC on T cells provides either a tolerogenic signal (84-86) or a signal that directs the development of CD4 T cell cytotoxic function (87). It is also possible that class II MHC molecules on T cells serve as ligands for T_{regs}, though this appears not to have been investigated thoroughly (88). As far as we know, we are the first to propose and demonstrate the plausibility of a model in which class II MHC acquired by CD8 T cells via the process of trogocytosis enables them to receive help from specific CD4 T cells.

The sequence in our model begins when DCs acquire antigens—either by direct infection or by phagocytosis—and present peptides from those antigens on class I and class II MHC molecules. In the absence of inflammatory signals that activate the DC, CD4 T cells must first license the DC before it can activate a CD8 T cell; when inflammatory signals are present, licensing of the DC is CD4-independent. Importantly, even in the case where licensing is CD4-dependent, the signals delivered via this CD4:DC interaction are distinct from those that are subsequently delivered via a putative direct CD4:CD8 T cell interaction. During its interaction with a licensed DC, a responding CD8 T cell will acquire class II MHC proteins from the DC, and some of these class II molecules will be loaded with peptides that are both derived from the same antigen source that provided peptides recognized by the CD8 T cell and that stimulated the CD4 response. After the CD8 T cell has acquired class II MHC peptide complexes from the DC, it can then present them to antigen-specific CD4 T cells, which deliver helper signals that endow the CD8 T cell (and/or its progeny) with the ability to

subsequently proliferate rapidly in a secondary recall immune response.

The events in this model take place in a window of time that is restricted to the relatively short period in which the CD8 T cells acquire and retain the class II molecules and associated peptides from the DC. In our experiments, using a relatively large number of adoptively transferred, CFSE labeled transgenic T cells, after infection with LCMV, the CD8 T cells acquire class II MHC molecules even before the first cell division, and begin to lose class II molecules starting at about the 5th cell division, between 42-50 hours post-infection. In the absence of adoptive transfer, our tetramer-enrichment experiments (Fig 2.1d) demonstrated that endogenous T cells also have class II MHC molecules 2.5 days post infection. We believe that the onset of the decline in class II MHC molecules on CD8 T cells is likely to be coincident with the clearance of antigen and the consequent loss of antigen-specific contacts between CD8 T cells and APC. It is well-established that once recruited into a response, CD8 T cells are programmed to continue dividing for a finite number of divisions even after antigen is cleared (89). This antigen-independent division—together with the normal turnover of class II MHC that takes place even on a non-dividing cell—will lead to partition of a limited quantity of class II MHC between daughter cells and its eventual loss from all cells in the population, much as CFSE is lost in standard turnover experiments. Though we have not measured viral titers in these experiments, it is likely that virus will be cleared substantially sooner in mice that received large numbers of naive transgenic T cells specific for a viral antigen than unmanipulated mice.

The kinetics of acquisition of class II MHC peptide complexes by CD8 T cells observed here are similar to the kinetics of other key events in regulation of CD8 T cell

memory development that have been described by others. Using the same LCMV model as we employed here, Kalia and colleagues demonstrated that the kinetics of CD25 expression on adoptively transferred transgenic CD8 T cells following infection are very similar to the kinetics of class II MHC acquisition by CD8 T cells (Fig. 2.1c) (90); observations we have also reproduced (data not shown). More importantly, these investigators also found that IL-2 levels in the spleen peak between days 1-3 post-infection (though they did not identify the source of the IL-2). Therefore, it is only during this short, critical time window that CD8 T cells possess the molecules that they need to both stimulate a CD4 T cell and to efficiently respond to IL-2 produced by the same CD4 T cell. The kinetics of CD8 T cell CD25 expression and class II MHC acquisition is also similar to the kinetics of co-accumulation of cognate CD4 and CD8 T cells around DC in draining lymph nodes that has been observed by intravital microscopy (36, 63). While these investigators have focused upon a paracrine mode of delivery of IL-2 from CD4 to CD8 T cells in these CD4:DC:CD8 clusters, Huse and colleagues have shown that IL-2 is secreted in a synaptic rather than multidirectional fashion (91, 92), suggesting that IL-2 delivery is more carefully targeted than is suggested by a standard paracrine model. Our proposal that CD4 T cells can be directly stimulated by CD8 T cells that have acquired class II MHC peptide complexes from DC provides a resolution to these apparent contradictions.

The model for delivery of CD4 T cell help to CD8 T cells that we have proposed in this paper was based upon experiments in the mouse, one of the few mammalian species that does not directly express class II MHC on activated CD8 T cells (93). How might this model work in humans? Expression of MHC class II on activated human CD8

T cells has been demonstrated in an enormous number of studies, including direct ex vivo analyses of CD8 T cell responses to vaccinia virus and the live-attenuated Yellow Fever Virus vaccine (94). While these studies demonstrate that class II MHC is present on antigen-specific CD8 T cells during a primary response as soon as they are detectable in the blood, we remain blind to the phenotype of these cells during priming, both because they are below the limits of detection and because they are probably sequestered in experimentally inaccessible secondary lymphoid tissues. It is possible that during priming, human CD8 T cells both express their own class II MHC and/or acquire it from APC by trogocytosis, with either enabling interactions with CD4 T cells that could provide help signals to the CD8 T cells, just as in the model presented in this paper. This model does not address the immunological function of class II MHC that is detected on T cells in the blood at the peak of the primary effector response (94) or on bulk CD8 T cells in the blood of patients during chronic immune activation such as is associated with HIV infection (95), arthritis and lupus (96); the function of class II MHC on CD8 T cells during the latter stages of an immune response remains a mystery.

2.6 Figures

Figure 2.1

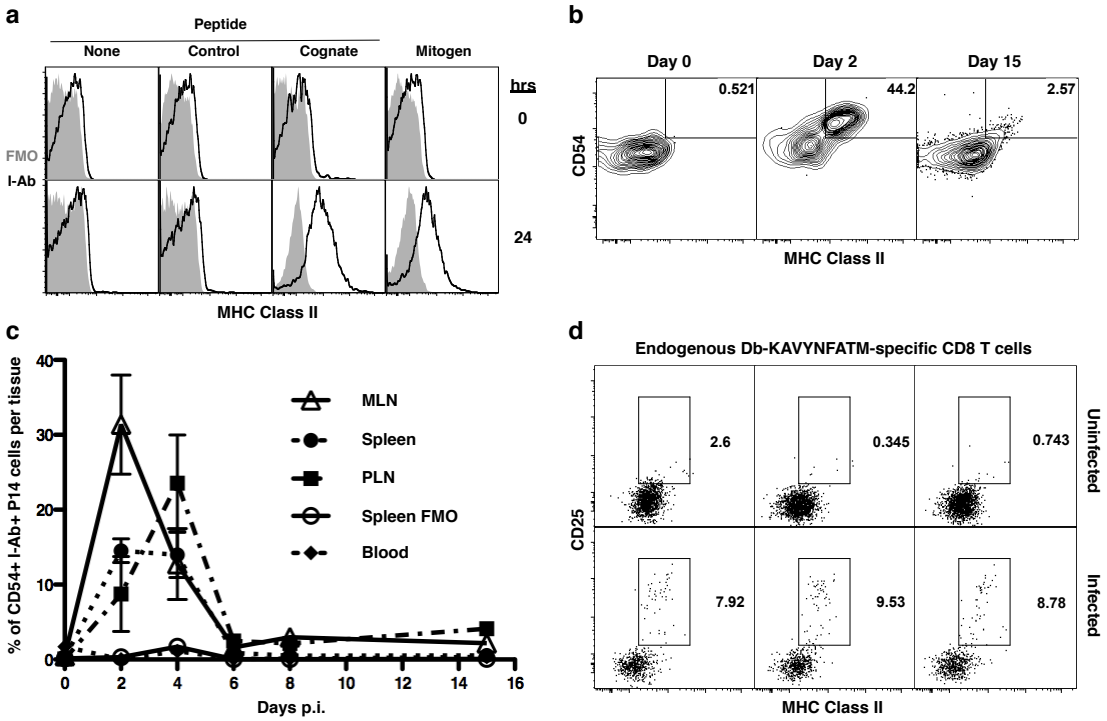

Figure 2.1: MHC class II is present on recently activated CD8 T cells in vitro as well as in vivo

a: MHC class II is present on CD8 T cells upon in vitro activation. MHC class II staining on Tg CD8 T cells (P14 cells) shown at times 0 and 24 hrs after in vitro incubation with WT flt3L-DCs pulsed with vehicle, control peptide (ova257-64), cognate peptide (gp33-41) or mitogen (Concanavalin A). Empty histograms: I-A^b-FITC stained. Solid histograms: FMO control. Events were gated on CD3+CD8+ singlets.

b: MHC class II is present on CD8 T cells upon in vivo activation. CD54 and MHC class II (I-A^b) staining on Tg CD8 T cells in MLN at days 0, 2 and 15 after infection with

$2x10^5$ p.f.u. of LCMV Arm i.p. Plots are representative of triplicates of one experiment. Events were gated on live CD19-Thy1.1+CD8+ singlets.

c: MHC class II on in vivo activated CD8 T cells peaks around Day 2, 3 and 4 in MLN, Spleen and PLN respectively. Percentage of CD54+ and I-Ab+ P14 cells at different times (0, 2, 4, 6, 8 and 15 days) after infection with $2x10^5$ p.f.u. of LCMV Arm i.p. Each point is represented by the mean and SEM of triplicates of one experiment.

d: MHC class II is present on endogenous CD8 T cells responding to LCMV infection in vivo. MHC class II ($I\text{-}A^b$-gfp) vs CD25 staining on activated D(b)/LCMV.gp33-41 (KAVYNFATM) tetramer enriched CD8 T cells 2.5 days post-infection with $2x10^6$ of LCMV Arm i.v. One graph per mouse. Triplicates are representative of two independent experiments. Events were gated on live CD19-CD11b-CD4-CD8+ KAVYNFATM-tetramer+ singlets.

Figure 2.2

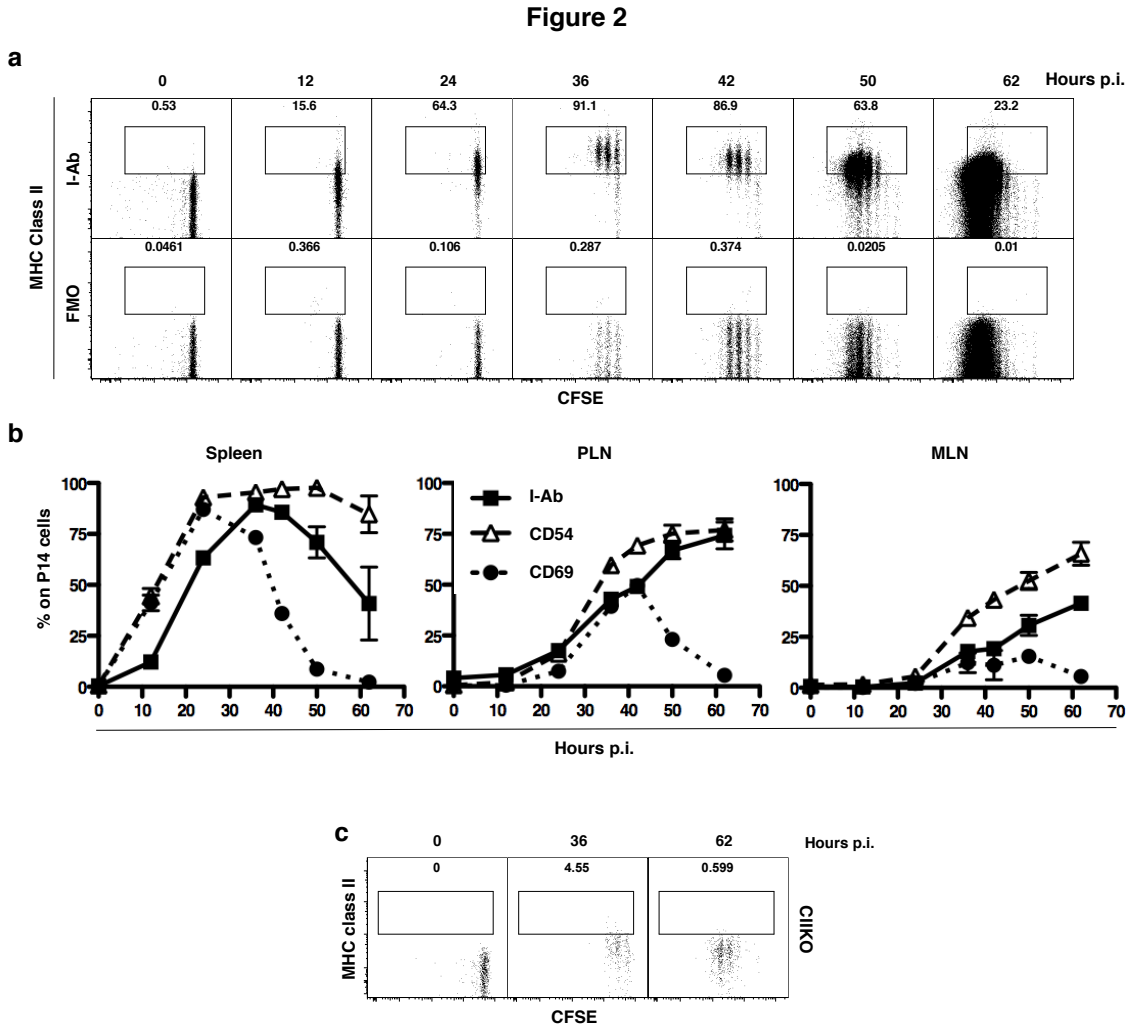

Figure 2.2: MHC class II is transiently present on responding transgenic CD8 T cells after infection.

a: MHC Class II is present on CD8 T cells as they divide in spleen between days 2 and 3 after in vivo activation. MHC class II staining vs CFSE dilution on CD8 T cells at different times (0, 12, 24, 36, 42, 50 and 62 hrs) after infection with $2x10^6$ p.f.u. LCMV Arm i.v.. Plots are representative of duplicates. First row: I-A^b-APC staining. Second row: FMO control. Events were gated on live CD19-CD11c-Thy1.1+CD8+ singlets.

b: MHC class II presence coincides with CD54 and CD69 expression on CD8 T cells in several tissues upon in vivo activation. (b) spleen, (c) PLN and (d) MLN. Each point is represented by the mean and SEM at different times (0, 12, 24, 36, 42, 50 and 62 hrs) after infection with $2x10^6$ p.f.u. LCMV Arm i.v..

c: CD8 T cells show no significant MHC class II presence when responding to an infection in mice lacking MHC class II. MHC class II staining vs CFSE dilution on CD8 T cells (P14) in MHC Class II KO (CIIKO) mice at 0, 36 and 62 hrs after infection with $2x10^6$ p.f.u. LCMV Arm i.v.. Plots are representative of triplicates. Events were gated on live CD19-CD11c-Thy1.1+CD8+ singlets.

Figure 2.3

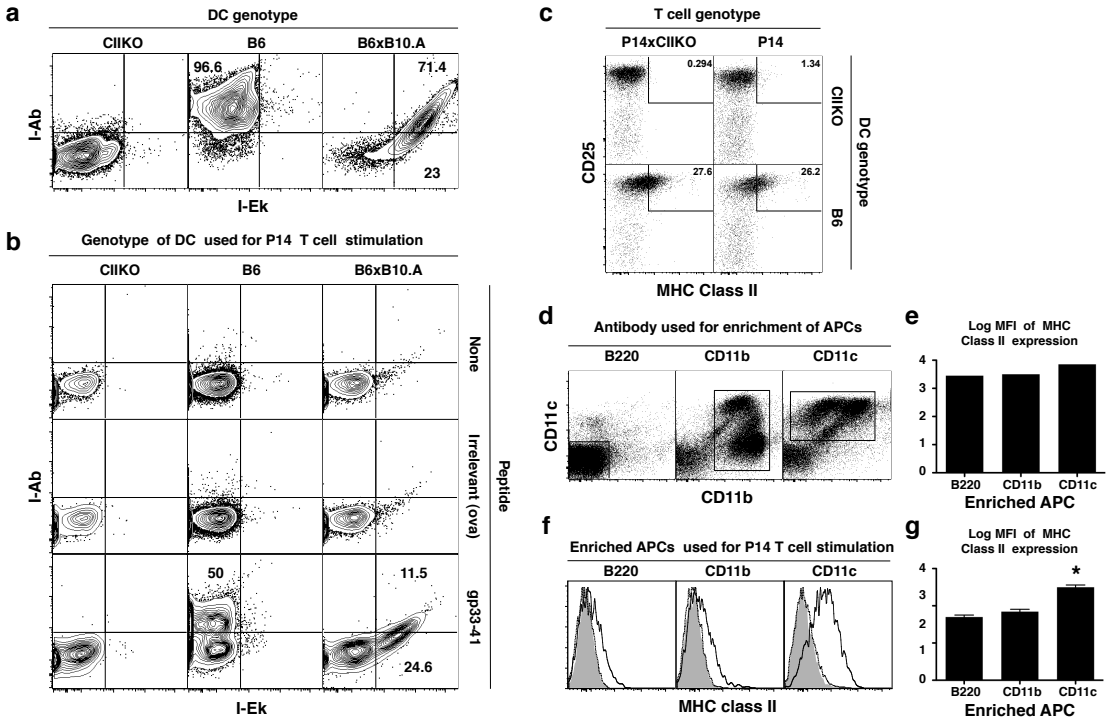

Figure 2.3: MHC class II is transferred onto CD8 T cells from APCs

a: MHC class II expression on Flt3L-DCs from CIIKO, B6 and B6xB10.A mice. I-A^b and I-E^k staining on magnetically enriched Flt3L-DCs. Events were gated on CD11c+ singlets.

b: MHC class II presence (None, I-A^b or both I-A^b and I-E^k) on activated CD8 T cells depends exclusively on the flt3L-DCs used for in vitro stimulation. I-A^b vs I-E^k staining on activated Tg CD8 T cells (P14) after 24 hrs of in vitro culture without any peptide, with an irrelevant peptide (ova257-64) or cognate peptide (gp33-41). Events were gated on live CD11c-Thy1.1+CD8+ singlets.

c: MHC class II is not expressed by activated CD8 T cells but transferred from APCs. MHC class II and CD25 staining on Tg CD8 T cells (P14 or P14xCIIKO) cultured in

vitro for 24 hrs with cognate peptide using flt3L-DCs from either CIIKO or WT mice. Events were gated on live CD19-CD8+ singlets.

d: CD11c vs CD11b define magnetically enriched APC populations (B220+, CD11b+ or CD11c+) cultured in vitro with CD8 T cells. Events were gated on live singlets.

e: Comparable amounts of MHC Class II on magnetically enriched APC populations (B220+, CD11b+ or CD11c+) cultured in vitro with CD8 T cells. MFI values of I-Ab-APC, calculated on events gated respectively on CD19+, CD11b+ or CD11c+ live singlets.

f and g: CD11c enriched population transferred the most of MHC Class II molecules onto activated CD8 T cells. d) Tg CD8 T cells (P14) were cultured in vitro with an control (ova257-64, solid histogram) or cognate (gp33-41, empty histogram) peptide for 24 hrs using different magnetically enriched APCs (B220+, CD11b+ and CD11c+). Events were gated on live CD19- Thy1.1+CD8+ singlets. e) Mean Fluorescent Intensity of MHC Class II ($I\text{-}A^b$) on activated CD8 T cells portrayed in d. Events were gated on live CD19- Thy1.1+CD8+ singlets. *p=0.0157.

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Figure 2.4

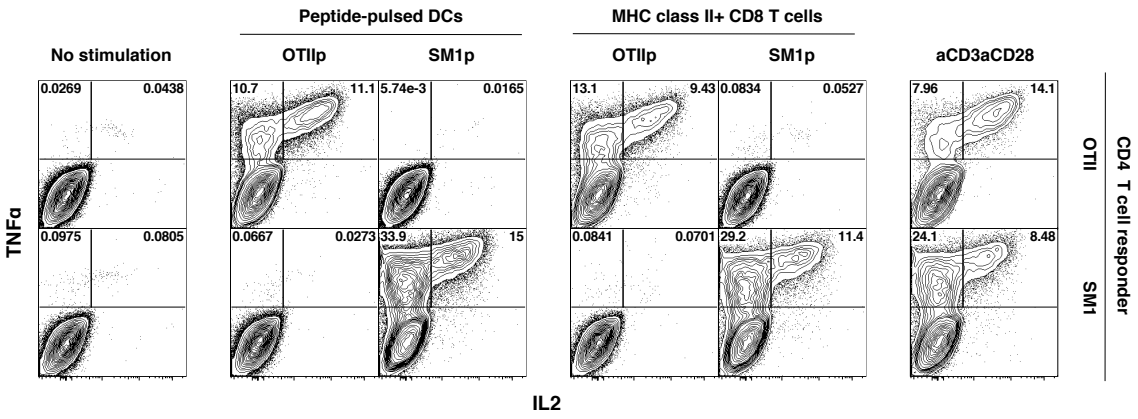

Figure 2.4: MHC class II on activated CD8 T cells mediate direct stimulation of experienced CD4 T cells.

CD8 T cells directly stimulate experienced CD4 T cells. TNFα vs IL2 expression detected using intracellular cytokine staining by flow cytometry. Experienced CD4 T cells generated by priming and differentiation with IL2, IL7 and IL-15, are stimulated (OTII, upper row; SM1, lower row) by no peptide, DCs or activated Tg CD8 T cells, loaded with either ova353-369 (OTIIp) or FliC427-441 (SM1p) peptide. Events were gated on live CD8-CD4+ singlets.

Figure 2.5

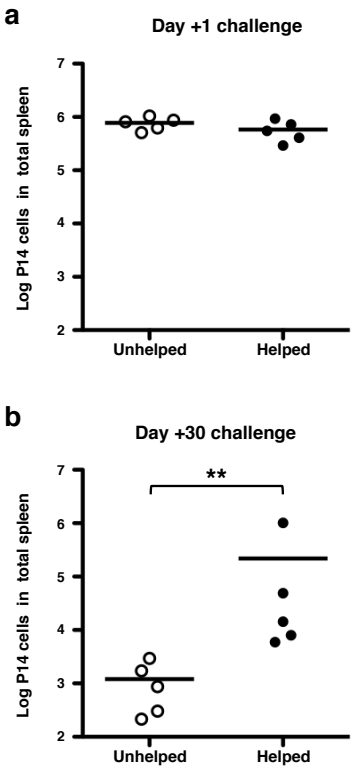

Figure 2.5: In vitro interactions between MHC class II+ activated CD8 T cells and experienced CD4 T cells improve the in vivo recall response of CD8 T cells.

Memory recall is improved on CD8 T cells by direct interaction with experienced CD4 T cells. P14 cells were specifically activated in vitro using gp33-41 peptide in the presence of either ova323-339 (OTII) or gp61-81 (SMARTA) peptide for 24hrs. Activated CD8 T cells were then magnetically isolated and cultured with experienced CD4 T cells for another 24 hrs. Activated CD8 T cells were again magnetically isolated and adoptively transferred into WT mice. Animals were challenged with $5x10^4$ c.f.u. of Lmgp33 i.v. at **a)** 1 day (day +1) or **b)** 30 days (day +30) after transfer. Stimulation for ICS and flow cytometry analysis was performed on spleens taken 4 days after Lmgp33 challenge. Events were gated on live CD19-Thy1.1+CD8+ singlets. **p=0.0079

Figure 2.6

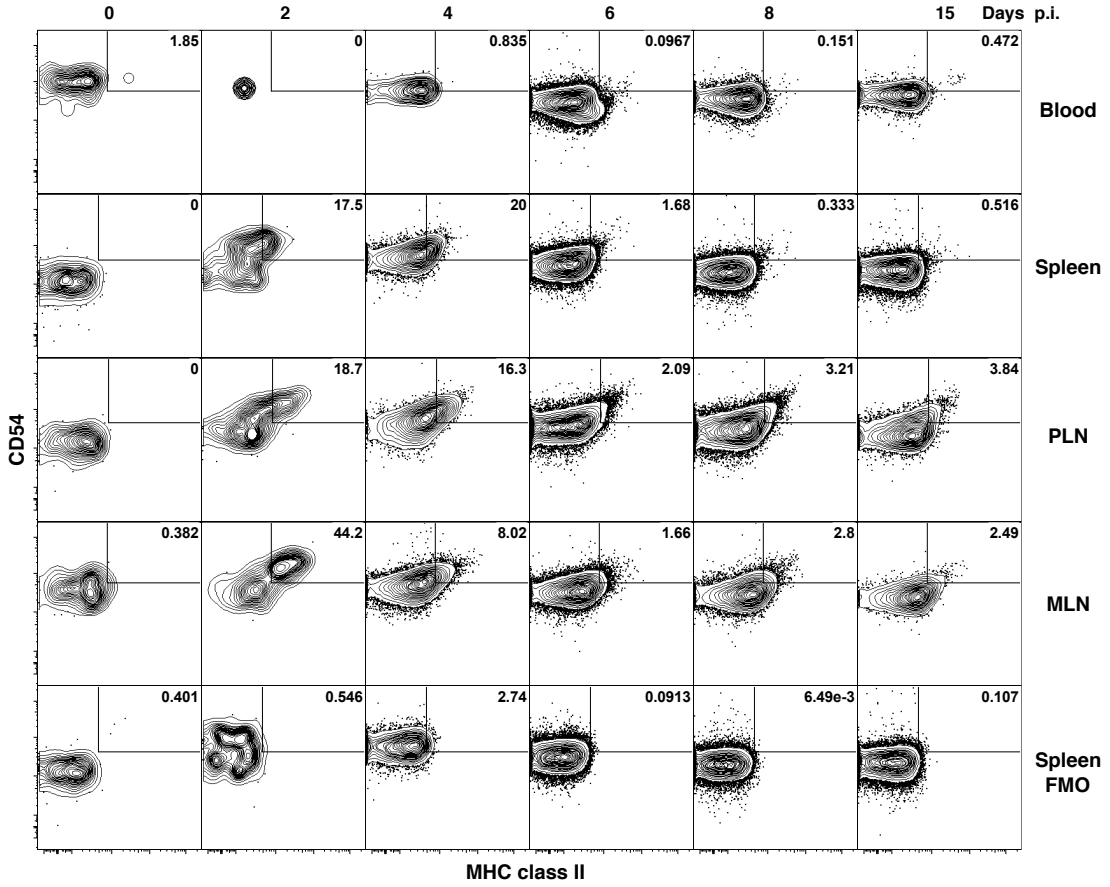

Figure 2.6: MHC class II and CD54 is present around Day 2, 3 and 4 on gp33-specific CD8 T cells in MLN, spleen and PLN respectively after LCMV infection

CD54 and MHC class II (I-Ab) staining on Tg CD8 T cells in MLN at days 0, 2, 4, 6, 8 and 15 after infection with $2x10^5$ p.f.u. of LCMV Arm i.p. Plots are representative of triplicates. Blood, Spleen, PLN and MLN and Spleen FMO. Events were gated on live CD19-Thy1.1+CD8+ singlets.

Figure 2.7

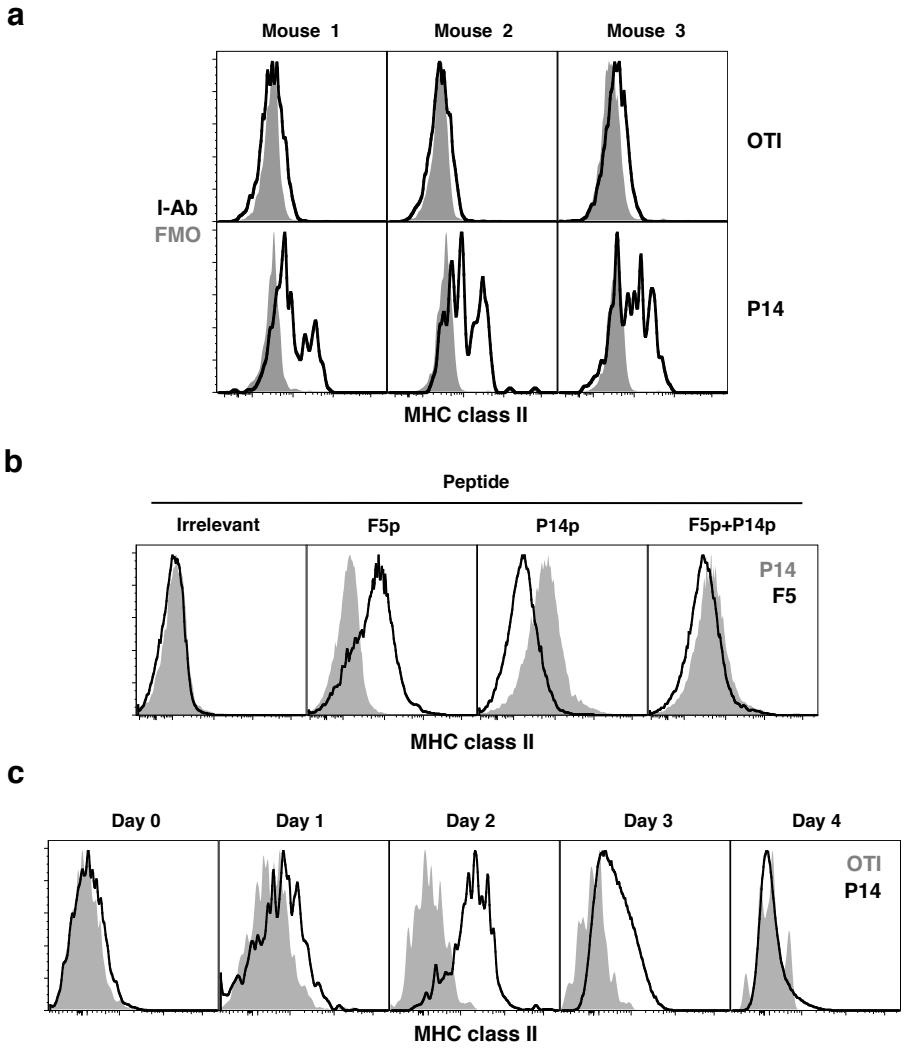

Figure 2.7: MHC class II is present only on specific CD8 T cells after activation in vitro and in vivo

a: MHC class II staining on adoptively transferred OTI (ova257-64 specific) or P14 (gp33-41-specific) in spleen after 32 hrs of infection with $2x10^6$ p.f.u. LCMV Arm i.v.. Each histogram represents one mouse, plots are representative of one experiment. Events were gated on live Thy1.1+CD8+ singlets.

b: MHC class II staining on CD8 T cells, P14 and F5 cells, together in culture for 24 hrs with flt3L-DCs pulsed with an irrelevant peptide (ova257-264), np366-374 (F5p), gp33-41 (P14p) or both np366-374 and gp33-41 (F5p+P14p). Plots are representative of replicates in one experiment. Events are gated on either CD8+Thy1.1+ singlets (P14 cells) or CD8+Thy1.2+ singlets (F5 cells).

c: MHC class II staining on adoptively transferred OTI and P14 cells, together in the same mouse, detected in spleen at days 0, 1, 2, 3 and 4 after infection with $2x10^6$ p.f.u. LCMV Arm i.v. Plots are representatives of duplicates in one experiment. Events were gated on live Thy1.1+CD8+ CTFR+ (OTI) or CTFR- (P14) singlets**.**

Figure 2.8

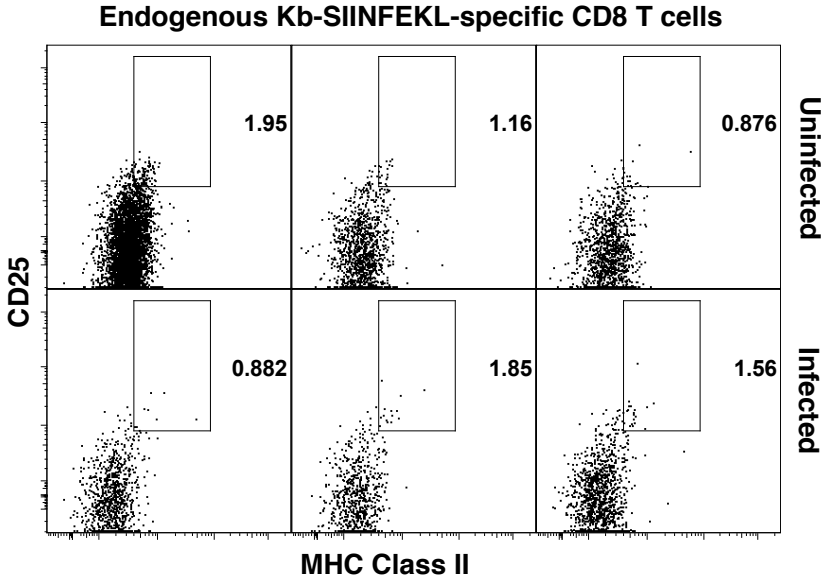

Figure 2.8: MHC class II is not present on endogenous CD8 T cells that do not respond to LCMV infection in vivo

MHC class II (I-Ab-gfp) vs CD25 staining on K(b).Ovalbumin257-64 (SIINFEKL) tetramer enriched CD8 T cells 2.5 days post-infection with $2x10^6$ of LCMV Arm i.v. One graph per mouse. Events were gated on live CD19-CD11b-CD4-CD8+ SIINFEKL-tet+ singlets. Plots are representative of one experiment.

Figure 2.9

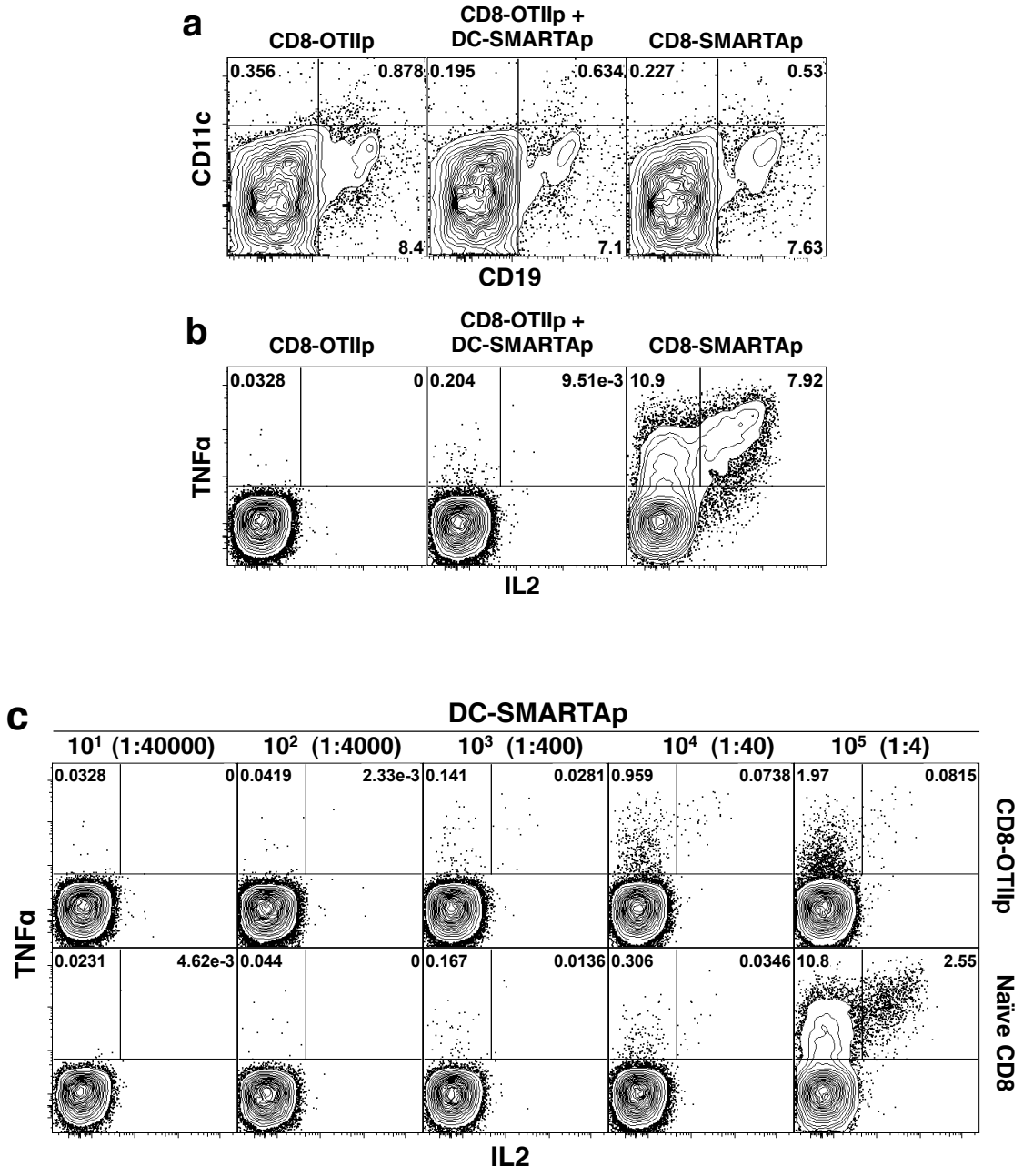

Figure 2.9: CD4 T cells stimulation with activated CD8 T cells is not due to DC contamination.

a: Little DC contamination can be detected on purified activated CD8 T cells. CD11c vs CD19 on ungated total cells from magnetically purified CD8 T cells after 24 hrs of in

vitro activation with flt3L-DCs. CD8 T (P14) cells were primed in the presence of gp33-41 peptide either with ova323-339 (OTIIp) or with gp61-80 (SMARTAp). flt3L-DCs cultured for 24 hrs in the presence of SMARTA peptide were added to activated CD8 T cells loaded with OTII peptide to control for possible DC contamination. Events were gated on live singlets.

b: Residual DC contamination after magnetic isolation of activated CD8 T cells is not responsible for CD4 T cell stimulation. TNFα vs IL2 expression detected using intracellular cytokine staining by flow cytometry. Direct CD4 T cell stimulation by activated CD8 T cells isolated as described in Fig. 2.9a. Contaminating DCs are added to activated CD8 T cell before magnetic isolation. Events were gated on live CD19-Thy1.2+CD4+ singlets.

c: CD4 T cell responses are mainly caused by peptides presented by CD8 T cells. TNFα vs IL2 expression detected using intracellular cytokine staining by flow cytometry. Ratio of flt3L-Dcs to both CD8 T cells and CD4 T cells ($1x10^5$ cells per well, 1:1 CD8 to CD4 T cell ratio). Events were gated on live CD19-Thy1.2+CD4+ singlets.

Figure 2.10

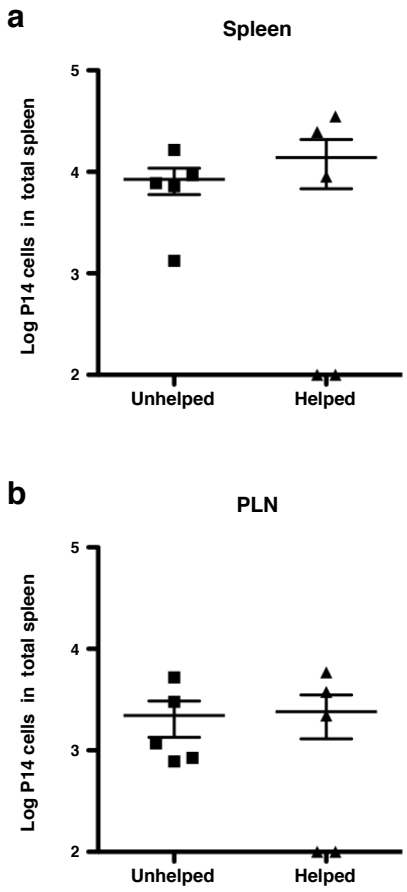

Figure 2.10: No difference in absolute numbers between adoptively transferred helped or unhelped CD8 T cells before delayed challenge

There is no significant statistical difference between helped vs unhelped CD8 T cells 30 days after in vivo transfer. Absolute numbers of P14 cells in **a)** spleen, **b)** PLN. Plots are representative of one experiment. Events gated on live CD19-Thy1.1+CD8a+KAVYNFATM+ singlets.

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2.8 Footnotes

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Chapter 3

Memory precursor phenotype is detected early on CD8 T cells responding to acute LCMV infection in bone marrow

Unpublished results by

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3.1 Abstract

Understanding the mechanism behind the generation of memory CD8 T cells is critical to generate potent vaccines against intracellular viruses. Upon exiting thymus, naive CD8 T cells migrate through different lymphoid tissues where they can be activated in response to infection. These CD8 T cells responding to infection will then integrate signals from tissue environments in which they are located that will determine their phenotype and functionality as effector or memory CD8 T cells. Here, we show that CD8 T cells responding to an acute LCMV infection leave the blood during the first 48 hours to populate several lymphoid tissues (spleen, PLN, MLN and bone marrow). We find that phenotype of LCMV-specific CD8 T cells varies with the tissue, but most importantly, that CD8 T cells isolated 3 days post infection from bone marrow displayed a memory precursor phenotype, $CD62L^{lo}CD25^{lo}Ly6C^{hi}$ different to the mixed phenotype observed in LCMV-specific CD8 T cells from spleen or PLN at the same time post infection. Interestingly, T-bet, a T-box transcription factor found predominantly in terminally differentiated effector CD8 T cells, is almost absent in LCMV-specific CD8 T cells from Bone Marrow; in contrast to the high T-bet expression found on LCMV-specific CD8 T cells in PLN and spleen. Furthermore, we observed that differences in phenotype correlate with the cytokine profile of CD8 T cells, detecting less IFNγ-producers within the LCMV-specific CD8 T cells in bone marrow than in spleen upon ex vivo stimulation. These findings highlight the need for further investigation to test memory potential of CD8 T cells responding to an infection from different tissues, particularly from bone marrow, to characterize what signals are being integrated into their differentiation program when responding to infection.

3.2 Introduction

Immunological memory defines the ability of an immune system to respond faster and more effectively to a pathogen that has previously encountered and eliminated. This ability of the immune system resides in the persistence of long-lived, clonally expanded, populations of pathogen-specific B and T cells (1). Both subsets are important to control and clear pathogens (2-4), but in the case of intracellular pathogens like bacteria and viruses (4-6), CD8 T cells become critical component of the immune response. Therefore, deciphering the precise mechanisms behind the development of CD8 T cell memory is of utmost importance to develop effective vaccines.

CD8 T cells arise from precursors in bone marrow that home to thymus, where they differentiate into mature naïve CD8 T cells (7). Once they leave thymus, naïve CD8 T cells circulate through the body by blood, entering mostly lymphoid tissues to receive homeostatic signals to survive (8, 9). To become activated, naïve CD8 T cells need to receive at least three signals. The first one, given by an antigen presenting cell (APC), is the recognition of an specific peptide in the groove of an MHC class I molecule by their T cell receptor (TCR) (10). The second signal is delivered by costimulatory molecules from the B7 family or the TNF superfamily of co-receptors and chemokines (11-13). This second signal depends on the type and activation status of the APC defined by signals present in the local environment (14, 15). A third signal is given by the inflammatory milieu, containing IL-12 or type I IFNs, present during activation of CD8 T cells and their subsequent clonal expansion (16-18). After expansion, 90-95 % of CD8 T cell will die in the contraction phase and the remaining cells will become memory CD8 T cells (19).

In an attempt to define memory CD8 T cell development, two models have been proposed. The progressive linear differentiation model (20) propose that all memory cells descend from naïve cells that became effectors, which are cells that kill infected cells and secrete antiviral cytokines. In contrast, the bifurcative differentiation model contemplates the generation of separate lineages from naïve cells (21), where some cells will become effectors and others will become memory CD8 T cells. Therefore, it is critical to identify memory precursors at the earliest time during an immune response to finally understand this process and characterize the signals that participate in the differentiation of memory CD8 T cells.

CD8 T cells have been defined by phenotype and functionality. In humans, two subsets were described based upon their expression of CCR7 and L-Selectin (CD62L). In the case of mouse CD8 T cells, memory CD8 T cells generated after an acute LCMV infection can be divided into two subpopulations (22): central memory (Tcm), which are characterized by high expression of CD62L and CD44; and effector memory (Tem), defined by low expression of CD62L with high CD44 expression (22-24). A series of other markers have further defined these subsets, Ly6C (25), CD27, CD43 and CXCR3 (26). Most importantly, the presence of IL7 receptor alpha (CD127) on CD8 T cells can identify early memory precursors (27), and when used in conjunction with the killer cell lectin-like receptor subfamily G member 1 (KLRG1) (28), can discriminate between memory precursor effector cells (MPECs) $CD127^{hi}KLRG1^{lo}$ from the short lived effector cells (SLECs), $CD127^{lo}$ $KLRG1^{hi}$. In addition, expression of a T-box transcription factor T-bet can also discriminate between MPECs and terminally differentiated SLECs (29). More recently, it has been reported that low expression of IL2 receptor alpha (CD25) can

be considered as a marker of CD8 T cell memory precursors as early as 3.5 days after acute LCMV infection (30).

Since CD8 T cells migrate throughout different tissues, the development of memory CD8 T cells can be influenced by the different environments in which they respond to an infection (31). In this chapter, we present results from experiments designed to investigate whether CD8 T cells had different phenotypes and functionality depending on their physical location in the first three days of an acute LCMV infection. Here, we show that CD8 T cells disappear from blood at day 1 after LCMV infection to be activated in several tissues showing distinct differentiation phenotypes. More importantly, we find that, as early as 3 days after LCMV infection, CD8 T cells from Bone Marrow display a distinct phenotype and functional features of an early CD8 T cell memory precursor.

3.3 Material and Methods

3.3.1 Mouse strains

Wild type (WT) C57BL6, B6.Thy1.1 and OTII mice purchased from The Jackson Laboratories (Bar Harbor, ME). P14 mice were obtained from Dr. Rafi Ahmed (Emory University, Atlanta, GA). OTI mice Rag KO were obtained from Shivaprakash Gangappa (Emory University, Atlanta, GA). All mice were maintained under specific pathogen-free conditions at the Emory Vaccine Center, Yerkes National Primate Research Center, Atlanta, GA.

3.3.2 Pathogens and infections

Age-matched sex-matched animals were injected with either $2x10^5$ plaque forming units (p.f.u.) intraperitoneally (i.p.) or $2x10^6$ p.f.u. intravenously (i.v.) of LCMV Armstrong 53b (Arm) virus obtained from Dr. Rafi Ahmed (Emory University, Atlanta, GA). LMCV Arm has been described previously (32).

3.3.3 Cell suspensions and flow cytometry

Single cell suspensions were prepared using 70 μm cell strainers or by collagenase treatment performed as described (33) using Collagenase 4 (Worthington Biochemical Corp., Lakewood, NJ). Red Blood Cells were lysed using Red Blood Cell Lysing Buffer (Sigma-Aldrich, St Louis, MO) and washed with RPMI. Fluorochrome-conjugated antibodies were obtained from BD Pharmingen (San Diego, CA), eBiosciences (San Diego, CA) and BioLegend (San Diego, CA). Intracellular cytokine staining was performed following manufacturer's protocol with either 2.5 μM of peptides for 6 hrs in the presence of Brefeldin A (1 μl/ml GolgiPlug, BD Pharmingen, San Diego, CA). Tetramers were prepared as described (34, 35) and tetramer enrichment protocol was

performed as described (36, 37). Live dead discrimination was performed using an in-house developed protocol based on Alexa Fluor 430 dye (Molecular Probes, Eugene, OR). Intracellular staining of T-bet, Eomesodermin and Granzyme B was performed using Foxp3 Staining Buffer Set from eBiosciences (San Diego, CA). Multiparameter analysis of samples was performed on LSRII flow cytometer (BD Biosciences) and results were analyzed using FlowJo software (TreeStar, Ashland, OR).

3.3.4 Peptides

Peptides gp33-41 (KAVYNFATM) and Ova257-264 (SIINFEKL) were synthesized by Microchemical Facility Core (Emory University, Atlanta, GA).

3.3.5 Adoptive Transfer

CD8 T cells were purified by negative selection using autoMACS system per manufacturer's protocol (Miltenyi Biotech, Auburn, CA). Cells were counted with hemocytometer by optical microscopy and purity was calculated by staining with antibodies specific for the Vα and Vβ TCR domains and acquisition using FACScalibur (Becton Dickinson). Purified CD8 T cells from P14 and OTI mice were labeled with 5-(and-6)-carboxyfluorescein diacetate (CFSE, Molecular Probes, Invitrogen, CA) at 2.5 μM as described before (38, 39). In addition, CD8 T cells from OTI mice were costained with CellTrace^{TM} Far DDAO-SE (Molecular Probes, Invitrogen, CA) at 3.75 μM and adoptively transfer as described (40).

3.3.6 Statistical Analysis

Statistical significance was determined by ANOVA, using Prism 5 (Graphpad Software, La Jolla, CA).

3.4 Results

3.4.1 Responder CD8 T cells locate to different lymphoid tissues than bystander CD8 T cells and divide up to 10 times during the first three days of LCMV infection.

It has been reported that signals received by CD8 T cells early during an immune response determine the quality of the CD8 T cell memory that is generated (41). Since these signals might be different depending on where CD8 T cell activation and clonal expansion is occurring (31), we decided to determine numbers and location of CD8 T cells responding to acute LCMV infection in lymphoid tissues compared to bystander CD8 T cells. We adoptively transfer magnetically isolated TCR transgenic CD8 T cells, one specific for LCMV.gp33-41 peptide presented by MHC class I molecule D(b) (P14 cells, responders) and another one specific for ovalbumin.ova257-264 peptide presented in MHC class I K(b) (OTI cells, bystanders) into C57BL6 mice that were infected the day after transfer with $2x10^6$ p.f.u. of LCMV Arm i.v.. Using flow cytometry, we detected and quantified adoptively transferred cells in blood as well as in spleen, peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and bone marrow during the first three days of LCMV infection.

As shown in Figure 3.1a, adoptively transferred CD8 T cells were detected in blood and Bone Marrow, but predominantly in spleen, PLN and MLN prior to infection (Fig. 3.1b). Both responder and bystander adoptively transferred CD8 T cells disappeared from blood upon infection until day 3 post LCMV infection as expected based on previous reports (42, 43). In contrast, adoptively transferred CD8 T cells remain detectable in spleen, PLN and MLN during the first three days while steadily increasing in Bone Marrow (Figure 3.1a and 3.1b).

While bystander OTI cells show no CFSE dilution in all tissues, responder P14 cells start to divide between day 1 and day 2 post LCMV infection and reach up to 10 cell divisions at day 3 post LCMV infection (Figure 3.1a). These observations correlate with an increase in numbers detected 3 days post LCMV infection in all lymphoid tissues sampled (Figure 3.1b). Interestingly, responder CD8 T cells did not show as much CFSE dilution in Blood and Bone Marrow (Figure 3.1a).

In terms of lymphoid tissue location, bystander CD8 T cells remained present in high numbers in PLN as well as in MLN, while decreasing in spleen and almost undetectable in Bone Marrow in the first three days of LCMV infection. In contrast, responder CD8 T cells are decreased at Day 2 post LCMV infection in PLN and MLN while present in spleen and increasing in Bone Marrow. At day 3 post LCMV, responder CD8 T cells are increased in all lymphoid tissues, predominantly in spleen, PLN and MLN but most notably in Bone Marrow (Figure 3.1a and 3.1b).

As shown in Figure 3.1b, numbers of total host CD8 T cells do not change drastically in all sampled tissues. However, it is possible to appreciate a trend that is follow by the non-responders OTI cells, which might highlight changes in migration that are not due to TCR activation but to inflammatory signals as reported before (43).

3.4.2 Responder CD8 T cells from bone marrow display a memory precursor phenotype early during LCMV infection.

To investigate if the phenotype of responder CD8 T cells depends on the lymphoid tissue they are located in after 3 days of LCMV infection, magnetically isolated P14 cells were adoptively transferred into WT mice that were infected the day after transfer with 2 $x10^6$ p.f.u. of LCMV i.v..

As shown in Figure 3.2a, CD62L, CD25, Ly6C and CD44 markers can be used to define responder CD8 T cell phenotype. Intermediate or low CD62L staining is shown on responder CD8 T cells in spleen and bone marrow, while it remains mostly high in PLN. As a control, adoptively transfer bystander OTI cells showed similar CD62L staining in spleen, PLN, MLN and bone marrow after 3 days post LCMV infection (Fig. 3.5). Also, responder CD8 T cells displayed high staining of CD25, an activation marker, in spleen and PLN while showing an intermediate to low CD25 staining in Bone Marrow (Figure 3.2a). While no adoptively transferred responder P14 cells could be detected in lung or Peyer patches (data not shown), detected responder CD8 T cell showed similar high staining of CD25 in MLN as well as in Liver (Fig. 3.6). Responder CD8 T cells showed two populations in spleen when stained for Ly6C, high and low; in contrast, PLN showed $Ly6C^{hi}$ with fewer cells $Ly6C^{int}$, while strikingly bone marrow only showed $Ly6C^{hi}$ cells.

While no differences on CD127 and KLRG1 staining of responder CD8 T cells could be detected regardless of tissue (Fig 3.7), responder CD8 T cells from the bone marrow interestingly displayed decreased or absent T-bet staining in contrast to the positive T-bet staining detected on responder CD8 T cells from spleen and PLN (Fig. 3.2b).

3.4.3 Endogenous CD8 T cells from bone marrow responding to LCMV infection also display lower CD25 expression.

To ensure our observations were not an artifact of using high numbers of adoptively transferred TCR transgenic cells, we looked at the phenotype of endogenous CD8 T cells responding to LCMV infection using tetramers and magnetic enrichment. WT mice were infected with LCMV Arm i.v. in high dose, $2x10^6$ p.f.u., as well as with a

low dose i.p., $2x10^5$ p.f.u. Two different doses of LCMV were employed to control that these observations were not caused by the use of high dose LCMV infection. As shown in Fig. 3.3, endogenous CD8 T cells magnetically enriched using D(b)/LCMV.gp33-41(KAVYNFATM)-tetramer (analogous to responder P14 cells) showed the previously observed high CD25 staining in spleen, PLN and MLN combined as well as the low CD25 staining detected in bone marrow in both doses of LCMV infection. As a control, endogenous bystander CD8 T cells magnetically enriched using K(b)/Ovalbumin.ova257-264(SIINFEKL)-tetramer (analogous to bystander OTI cells), showed the same staining on CD25 and CD44 regardless of tissue location or infection (Fig 3.8).

3.4.4 Lower percentage of IFNγ-producer responder CD8 T cells are found in Bone Marrow than in spleen.

To investigate if the phenotype observed in P14 cells from different tissues was reflective of their functionality, we decided to assay cytokine expression by ex vivo stimulation. As shown in Fig. 3.4, P14 cells from bone marrow show lower percentage IFNγ-producers when compared to P14 cells from spleen.

3.5 Discussion

Understanding the precise mechanism of memory CD8 T cell differentiation is critical to design better protective vaccines. This process is defined by several factors that including the nature and amount of the infectious agent, the activation status of APCs (14, 15) and the inflammatory milieu present together with the environmental cues like cytokines (44) or the presence of CD4 T cells (45). Most importantly, all of these factors depend invariably of the tissue in which CD8 T cells develop into effector and memory CD8 T cells (46). In consequence, the integration of multiple signals from different environments into the programming of CD8 T cells results in the reported heterogeneity observed in the memory CD8 T cell population (47). Therefore, studying how and where memory precursors arise in a immune response can give us valuable information about the process of differentiation of memory CD8 T cells. Here, we report differences in phenotype and functionality of CD8 T cells responding to an acute infection that not only highlight heterogeneity of CD8 T cell populations but also identify a population CD8 T cells in Bone Marrow displaying hallmarks of an early CD8 T cell memory precursor.

Throughout their development, T cells differentiate in response to signals from the environment they are located in. For example, early T cell populations originate from multipotent hematopoietic stem cells (HSCs) in fetal liver (48). Later, neonate T cell precursors differentiate in bone marrow and migrate into thymus to mature as naïve T cells (7). During homeostasis, naïve CD8 T cells follow chemokine gradients to find their activating DCs in lymph nodes (49-51). It is then of no surprise that naïve CD8 T cells will also integrate the surrounding signals when differentiating into effector and memory CD8 T cells upon recognition of its cognate antigen in peripheral tissues (33, 52, 53). In

this scenario, the programming of a CD8 T cell population depends mostly of the tissue in which environmental signals are sensed.

From the first observations describing cellular immunity, pioneer experiments have reported the migrational nature of lymphocytes (54). This nature allows naïve CD8 T cells to circulate throughout many tissues, mostly lymphoid tissues, to receive survival signals (55). When infection occurs, the circulation of naïve CD8 T cell is altered by either the recognition of their cognate antigen (42) or inflammatory signals that cause cell arrest (43). At this time, cells will get activated in tissues where cognate antigen is presented by APCs. This recognition will initiate their differentiation into effector and memory CD8 T cells that will subsequently be reflected in their phenotype and functional properties.

Here we show that responder CD8 T cells from the bone marrow are different in phenotype and function from responder CD8 T cells detected in spleen, PLN and MLN. As shown in Figure 3.1a, we detected the disappearance from blood of adoptively transferred naïve CD8 T cells upon LCMV infection. When looking for differences on CD8 T cells at the tissues where responder CD8 T cells located to, we found that responder CD8 T cells in spleen, PLN and MLN were dividing at a faster pace than those in the bone marrow. This difference in responder CD8 T cell proliferation suggests either more abundance of antigen in tissues such as the spleen or a higher ability to divide as proposed for early effector CD8 T cells (21). Using known markers of differentiation, we can notice particular phenotypes in responder CD8 T cells from different tissues. Although CD62L staining on responder CD8 T cells does not reveal differences between tissues, responder CD8 T cells from spleen and PLN show higher amounts of IL-2 alpha

receptor, CD25, reported to favor terminal-effector differentiation (30, 56) in contrast to the lower expression of CD25 observed in responder CD8 T cells from bone marrow. On the other hand, CD8 T cells from bone marrow are preferentially high on Ly6C, a molecule found to play a role in clustering LFA-1 (57, 58) to allow entrance of CD8 T cells into lymph nodes and found to be high on central memory CD8 T cells (59). Taken together, low CD25 expression coupled to high Ly6C expression and a possible decreased early proliferation suggests that responding CD8 T cells found early in bone marrow could be early memory precursors. Moreover, while CD127 vs KLRG1 staining suggest no differences between responder CD8 T cells from different tissues, the notion that memory precursors could arise in bone marrow is supported by an unexpected low to absent signal detected in T-bet staining on these responder CD8 T cells (Figure 3.2b) in contrast to the high T-bet expression typical of terminally differentiated effectors (29) detected on responder CD8 T cells from Spleen and PLN. Furthermore, as shown in Figure 3.4, we were able to detect a lower percentage of IFN γ producing cells in responder CD8 T cells from bone marrow when compared to responder CD8 T cells from spleen, which might also suggest that responder CD8 T cells could be memory precursors.

CD8 T cell presence in the bone marrow has been observed in primary as well as in secondary immune responses. In primary responses, priming can occur in the bone marrow, even in the absence of any other lymphoid tissues (60) or when migration to lymphoid tissues has been disrupted (61) providing protection against viral infection. CD8 T cells generated in the bone marrow have also been reported to induce mild Graft vs Host Disease (GVDH) (62) and shown to be particularly effective against tumors (63).

This data suggests that the bone marrow is an important site of CD8 T cell activation during a primary response. In the case of secondary responses, several groups have described preferential localization of memory CD8 T cells in bone marrow (64-67), where they specifically home to and compete for critical survival factors (68, 69). Here, we find CD8 T cells in bone marrow with phenotype and functional features of memory precursors early during the primary response to LCMV infection. Intriguingly, preliminary results shown in Figure 3.9 find no difference between responder CD8 T cells from the bone marrow, spleen or PLN when stained for Eomesodermin, a transcription factor reported to be critical for seeding CD8 T cell memory precursors into the bone marrow upon infection clearance (70). All in all, these observations suggest that the bone marrow is indeed an immunological niche that not only supports survival of memory CD8 T cells (71), as it is definitively for long-lived plasma B cells (72) and memory CD4 T cells (73), but more importantly, could provide appropriate signals to drive the differentiation of CD8 T cells responding to an infection into early memory CD8 T cells precursors.

The phenotype and functionality of CD8 T cells are the consequence of a complex process in which gene expression is changed (74, 75) and epigenetic signatures are imprinted (76-79) during immune response to eliminate the infectious agent. It would be of great interest to isolate cells responding to infection from different tissues to assess secondary recall properties, their gene expression profile and the ensuing epigenetic changes to understand the mechanism of CD8 T cell memory generation. Furthermore, it will be interesting to investigate how preventing entrance with blocking antibodies or the absence of cytokines in different tissues, particularly bone marrow, can impact the

differentiation of memory CD8 T cells. Understanding this process will aid to the development of better and more protective vaccines.

3.6 Figures

Figure 3.1

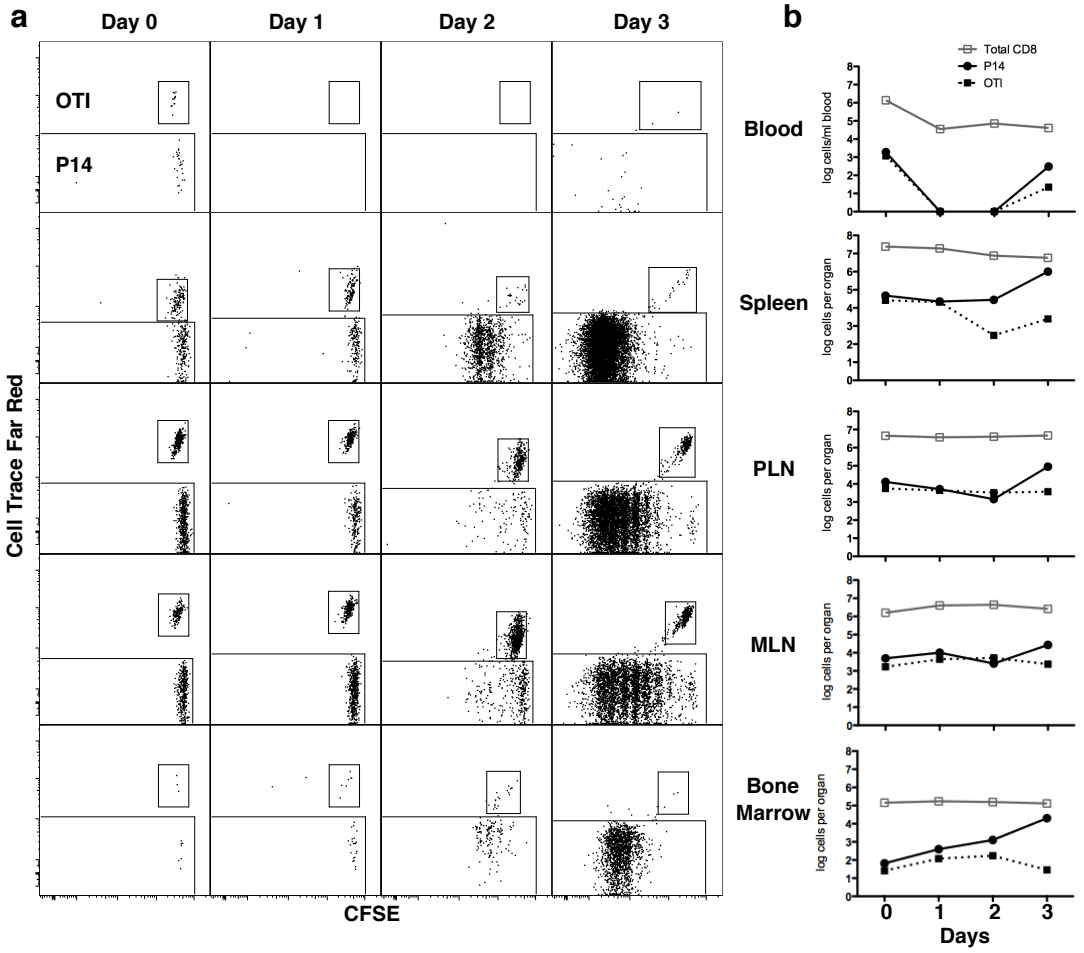

Figure 3.1: Responder CD8 T cells locate to different lymphoid tissues than bystander CD8 T cells and divide up to 10 times during the first three days of LCMV infection.

a. Cell Trace Far Red vs CFSE portrays OTI (bystander, Cell Trace Far Red+) or P14 (responders, Cell Trace Far Red-) CD8 T cells in Blood, Spleen, PLN, MLN and Bone Marrow during days 0, 1, 2 and 3 after LCMV Arm $2x10^6$ i.v. infection. Plots are

representative of replicates from two separate experiments. Events were gated on CD19-Thy1.1+CD8+ live singlets.

b. Absolute number of Total host CD8 T cells (empty squares, grey), P14 (filled circle, black solid line) or OTI (filled squares, black doted line) cells in Blood, Spleen, PLN, MLN and Bone Marrow during days 0, 1, 2 and 3 after LCMV Arm $2x10^6$ i.v. infection. Plots are representative of replicates in one of two experiments. Total host CD8 T cells were gated on CD19-Thy1.1-CD8+ live singlets. P14 and OTI were gated on CD19-Thy1.1+CD8+ live singlets.

Figure 3.2

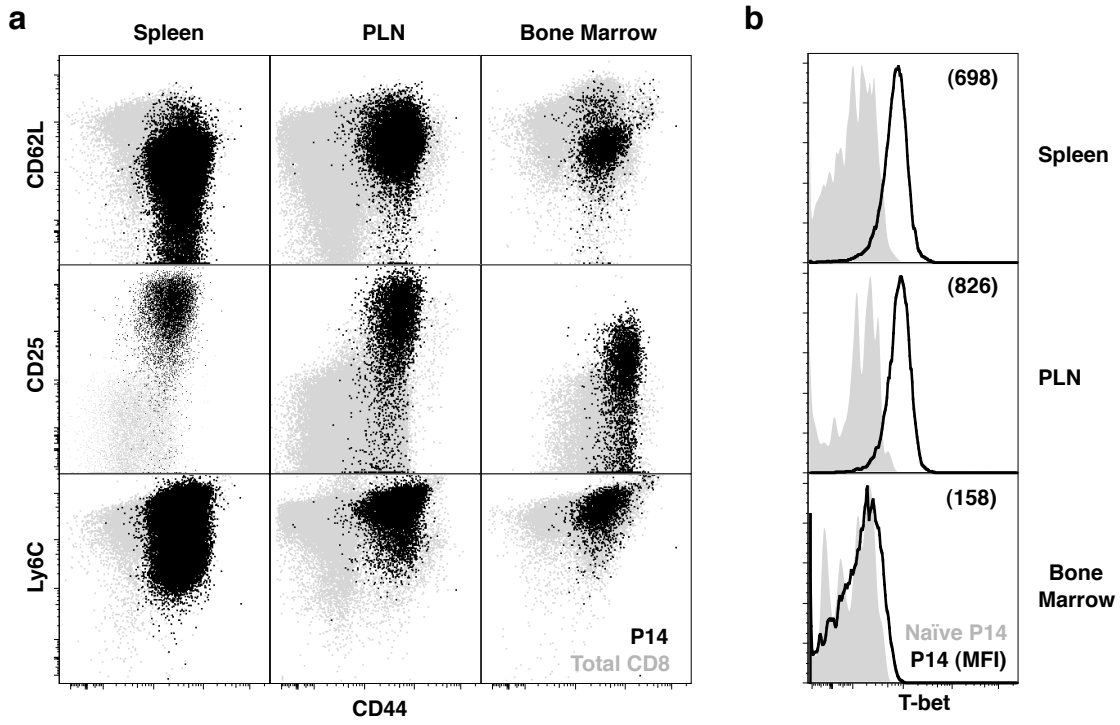

Figure 3.2. Responder CD8 T cells from bone marrow display a memory precursor phenotype early during LCMV infection.

a. CD62L, CD25, Ly6C and CD44 staining on Total host CD8 T cells (grey) vs responder P14 cells (overlaid black) in Spleen, PLN and Bone Marrow after 3 days of infection with LCMV Arm $2x10^6$ i.v.. Plots are representative of replicates in two separate experiments. Total host CD8 T cells were gated on CD19-Thy1.1-CD8+ live singlets. P14 and OTI were gated on CD19-Thy1.1+CD8+ live singlets.

b. T-bet staining on responder P14 cells at day 0 (solid gray) or day 3 (black) post LCMV infection in Spleen, PLN and Bone Marrow. Mean Fluorescent Intensity (MFI) of T-bet staining on responder P14 cells after 3 days of infection with $2x10^6$ p.f.u. of LCMV Arm i.v.. Plots are representative of replicates from two separate experiments. Events were gated in CD19-Thy1.1+CD8+ live singlets.

Figure 3.3

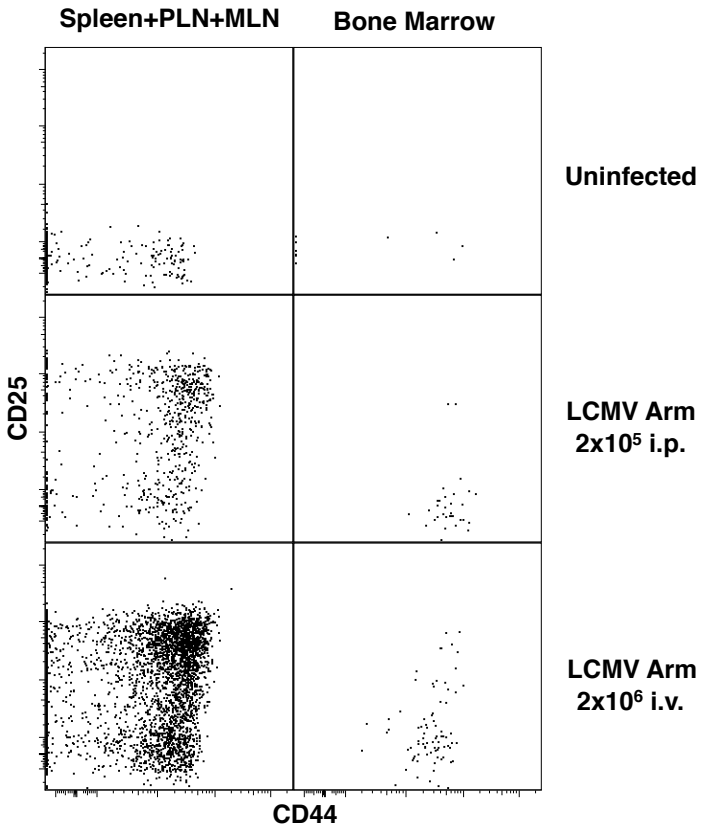

Figure 3.3: Endogenous CD8 T cells from bone marrow responding to LCMV infection also display lower CD25 expression

CD25 and CD44 staining on KAVYNFATM-tetramer enriched CD8 T cells from either Spleen, PLN and MLN, all combined in one, or Bone Marrow at day 0 or after 3.5 days of infection with LCMV Arm at doses $2x10^5$ i.p. or $2x10^6$ i.v., respectively. Each graph represents one mouse. Plots are representative of duplicates in one experiment. Events were gated on live CD19-CD11b-CD4-CD3+CD8+ KAVYNFATM-tetramer+ singlets.

Figure 3.4

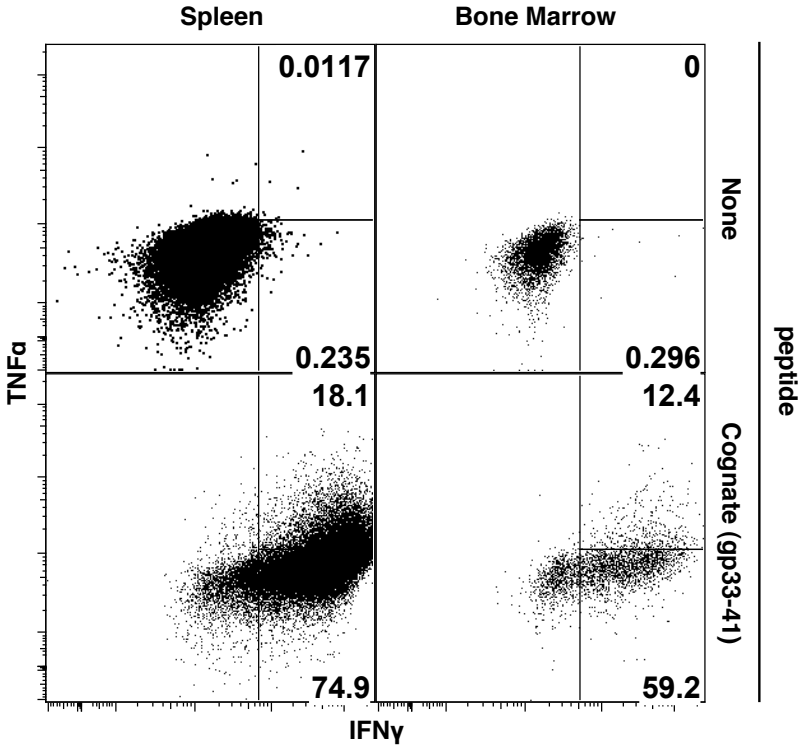

Figure 3.4: Lower percentage of IFNγ-producer responder CD8 T cells are found in Bone Marrow than in spleen

TNFα vs IFNγ intracellular cytokine staining by flow cytometry. Plots are representative of duplicates in one experiment. Events were gated on CD19-Thy1.1+CD8+ live singlets.

Figure 3.5

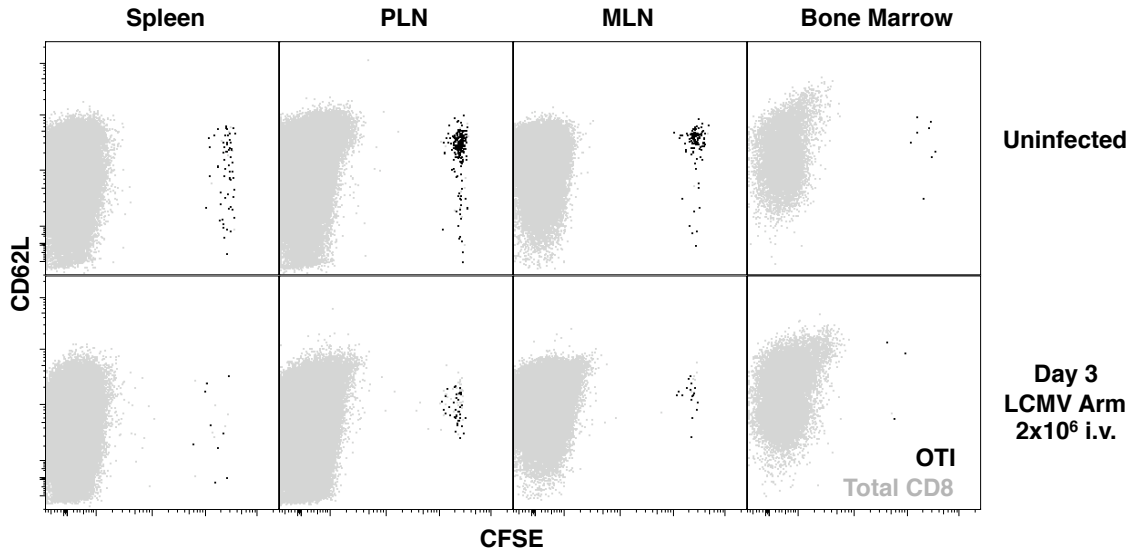

Figure 3.5: Phenotype of bystander CD8 T cells remains unchanged after 3 days of LCMV infection

CD62L staining vs CFSE on Total host CD8 T cells (grey) vs OTI cells (overlaid black) in Spleen, PLN, MLN and Bone Marrow at days 0 and 3 after infection with LCMV Arm $2x10^6$ i.v. Plots are representative of replicates in two separate experiments. Total host CD8 T cells were gated on CD19-Thy1.1-CD8+ live singlets. OTI cells were gated on CD19-Thy1.1+CD8+ live singlets.

Figure 3.6

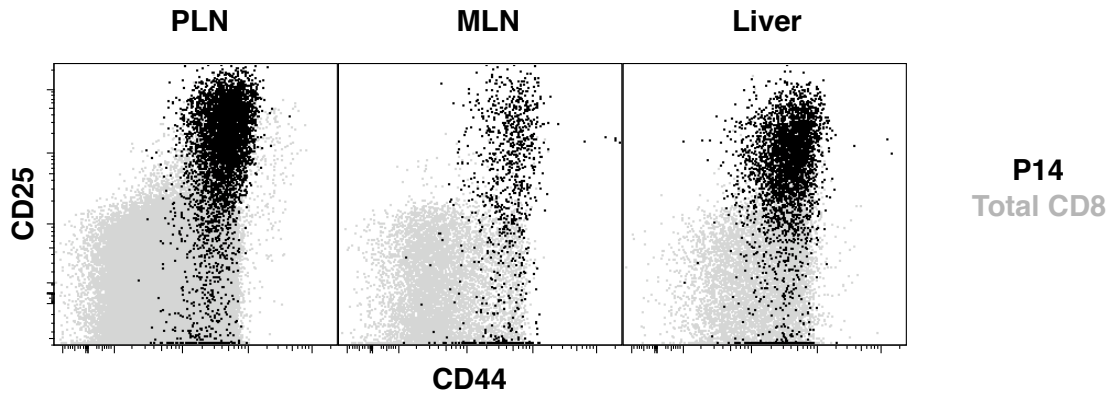

Figure 3.6: Responder CD8 T cells show similar activation phenotypes in PLN, MLN and Liver after 3 days of LCMV infection.

CD25 and CD44 staining on Total host CD8 T cells (grey) vs responder P14 cells (overlaid black) in PLN, MLN and Liver 3 days after infection with LCMV Arm $2x10^6$ i.v. Plots are representative of replicates in two separate experiments. Total host CD8 T cells were gated on CD19-Thy1.1-CD8+ live singlets. P14 cells were gated on CD19-Thy1.1+CD8+ live singlets.

Figure 3.7

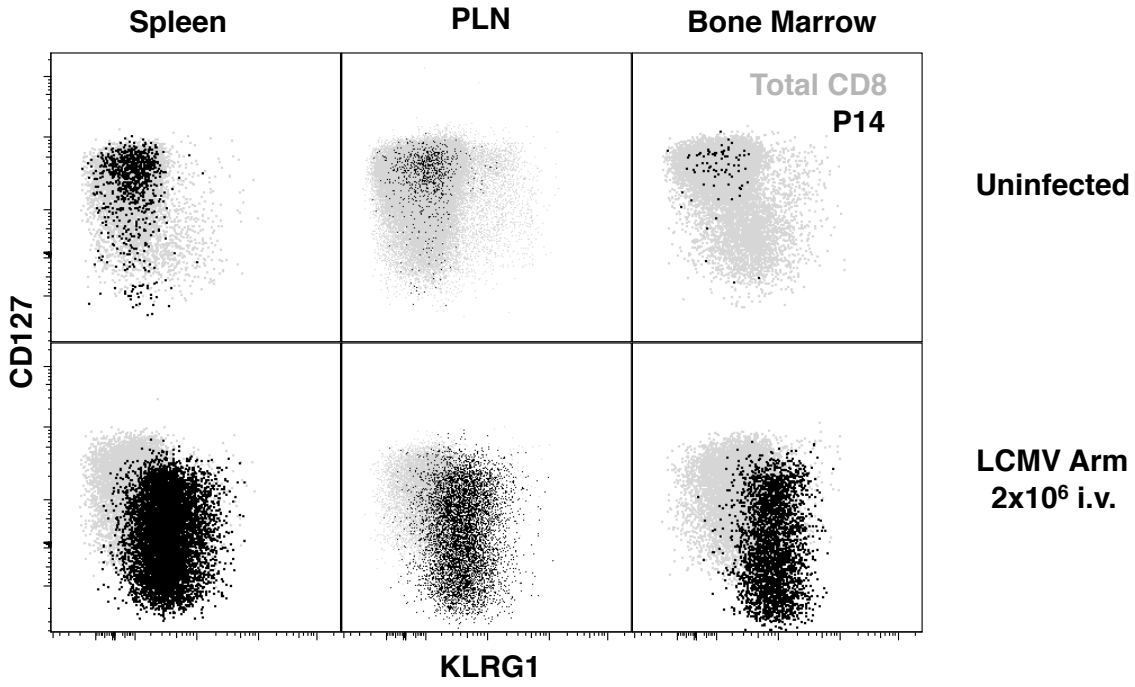

Figure 3.7: CD8 T cells responding to LCMV show similar differentiation markers in Spleen, PLN and Bone Marrow

CD127 and KLRG1 staining on Total host CD8 T cells (grey) vs responder P14 cells (overlaid black) in Spleen, PLN and Bone Marrow at day 0 or 3 days after infection with LCMV Arm $2x10^6$ i.v. Plots are representative of replicates in two separate experiments. Total host CD8 T cells were gated on CD19-Thy1.1-CD8+ live singlets. P14 and OTI were gated on CD19-Thy1.1+CD8+ live singlets.

Figure 3.8

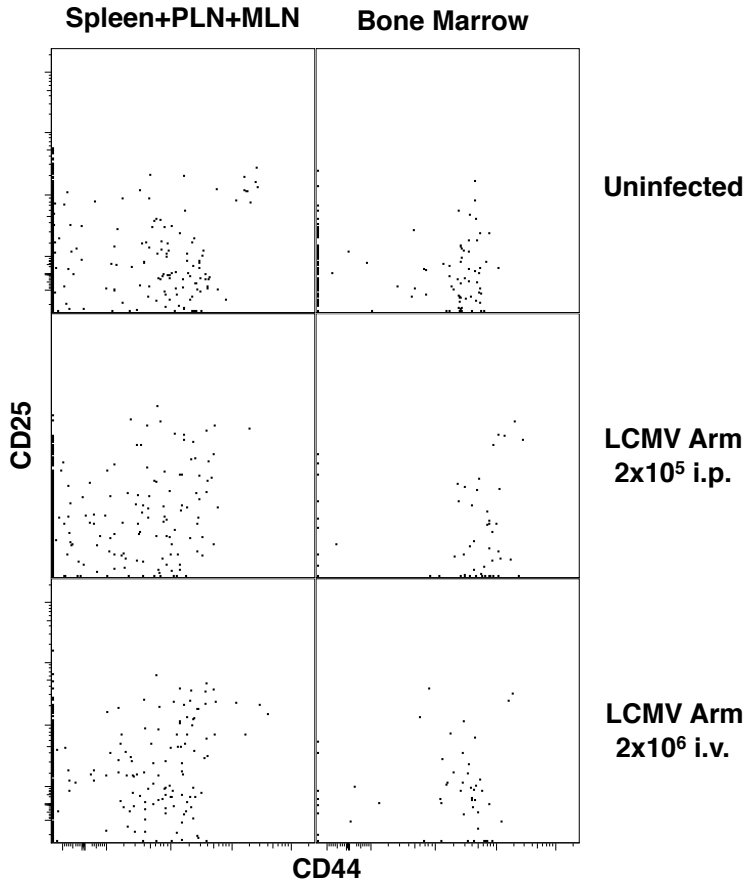

Figure 3.8: Phenotype of endogenous bystander CD8 T cells remains unchanged after 3 days of LCMV infection regardless of tissue location

CD25 and CD44 staining on endogenous bystander CD8 T cells enriched with KAVYNFATM-tetramer from Spleen, PLN and MLN, all combined in one, or Bone Marrow at days 0 or 3.5 after infection with LCMV Arm at doses $2x10^5$ i.p. or $2x10^6$ i.v. Each graph represents one mouse. Plots are representative of duplicates in one experiment. Events were gated on live CD19-CD11b-CD4-CD3+CD8+ KAVYNFATM-tetramer+ singlets.

Figure 3.9

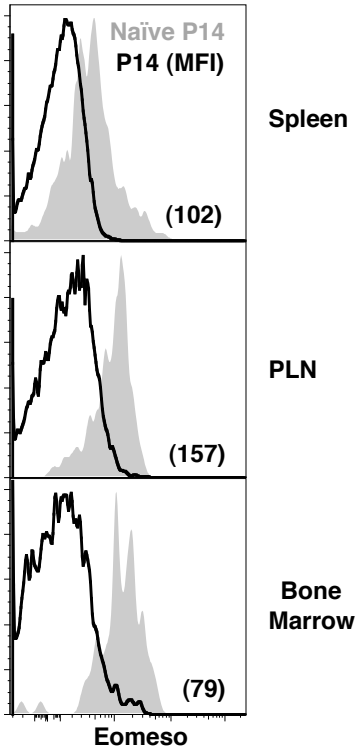

Figure 3.9. Responder CD8 T cells display similar Eomesodermin in different lymphoid tissues after 3 days of LCMV infection

Eomesodermin staining on responder P14 cells at day 0 (solid gray) or day 3 (black) post LCMV infection in Spleen, PLN and Bone Marrow. Mean Fluorescent Intensity (MFI) of Eomesodermin staining on responder P14 cells after 3 days of infection with $2x10^6$ p.f.u. of LCMV Arm i.v.. Plots are representative of replicates from one experiment. Events were gated on live CD19-Thy1.1+CD8+ singlets.

3.7 Acknowledgements

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3.8 Footnotes

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Chapter 4

Conclusions

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4.1 Why research on Immunological Memory is important

The immune system plays an important role in evolution. The way an organism defends itself against agents that could endanger the survival and reproduction of its species is no doubt part of evolution, it provides an evolutionary advantage against competing species. The immune system has many components that contribute to the defense against harmful pathogens; and immunological memory is one of them. This remarkable feature ensures that an organism that has successfully fought a life-threatening infection would, in most cases, never be affected again, giving an enormous survival advantage to this organism.

It is possible to argue that manipulating immunological memory could improve the quality of life of species under constant infectious challenges, and that is exactly what vaccines can achieve for humankind. Vaccines can generate immunological memory to provide defense against pathogens that have caused terrible epidemics in the past, for example smallpox, measles or influenza. For that reason, understanding how immunological memory develops will help to design better vaccines for the new infectious challenges as HIV, HCV, Tuberculosis or dengue are today.

4.2 Memory CD8 T cells and our research findings

Immunological memory depends on the number, quality and location of cells of the immune system. When an organism is infected, multiple arms of the immune system are recruited. In the case of intracellular pathogens like viruses (1), bacteria (2) and some parasitic infections (3), CD8 T cells are critical components of the immune system that control infection and then become part of this immunological memory. Upon re-infection, memory CD8 T cells ensure faster clearance of the infection by having higher

number of responding cells available, displaying enhanced functional properties to control the infection and by being strategically located upon pathogen re-entry. Recently, there have been tremendous advances in our understanding on memory CD8 T cells generation thanks to technological breakthroughs: Multicolor flow cytometry analysis and sorting, T cell receptor transgenic (TCR) mice that also allows adoptive transfer experiments (4-6), mouse strains in which a selected gene is knock-out, either irreversibly (7-11) or upon a certain condition (12), the use of MHC class I and class II tetramer technology (13, 14) and the possibility of magnetically enrichment of otherwise undetectable CD8 and CD4 T cell populations (15, 16). In addition, the use of immunofluorescence microscopy taken one step further with confocal and two-photon technology to perform live imaging of cells giving insight into antigen presentation (17-19) and lymphocyte trafficking in vivo (20). All these tools allow us to ask exciting new questions about how is it that memory CD8 T cells is generated from many levels, starting with the entire body of an organism, to its organs, to the cellular and even to a molecular level.

Using an animal model of acute LCMV infection known to generate CD8 T cell memory (21), we decided to study the contribution to CD8 T cell memory provided by novel direct interactions between CD8 and CD4 T cells and by the activation of CD8 T cells in different lymphoid tissues during the first days of LCMV infection. In both cases, we have found unappreciated mechanisms involved in CD8 T cell differentiation that could have a great impact in our understanding of CD8 T cell memory generation.

4.3 How direct CD8:CD4 T cell interactions impact the quality of CD8 T cell memory

CD4 T cell help can be defined by the enhanced contribution to an ensuing immune response that several cells of the immune system provide after direct interaction with CD4 T cells. It has been reported that upon CD4 T cell help macrophages can become activated more efficiently (22, 23), B cells are able to switch immunoglobulin isotypes (24) generating memory B cells and dendritic cells are able to mature providing better costimulation as well as releasing inflammatory cytokines (25). In all these examples, CD4 T cell help is triggered by MHC class II antigen presentation by these cells to receive in turn help by direct contact with CD4 T cells. This help is usually through CD40:CD40L interactions and cytokine secretion. Interestingly, another feature of CD4 T cell help for these cells is the measurable change in phenotype and/or functionality of these cells before and after receiving CD4 T cell help.

In contrast, the mechanism of CD4 T cell help for CD8 T cells is not entirely understood. One model proposes that CD4 T cells interact with DCs to properly mature them so they can provide, in a subsequent step, appropriate signals for CD8 T cells to differentiate into functional effector and/or memory cells (26, 27). Another model contemplates the simultaneous presentation to CD4 T cells and to CD8 T cells on the same cell, ensuring proximity so CD4 T cells can deliver signals to CD8 T cells (28). These two models have been proposed in part for the fact that murine CD8 T cells do not express MHC class II molecules (29), as described in previous sections, and therefore in response to the need of an intermediary cell expressing not only MHC class I for CD8 T cells but also MHC class II to activate CD4 T cells to deliver their help. However, the precise mechanism by which CD8 T cells receive CD4 T cell help when in proximity of each other is not well understood. Furthermore, there are no clear immediate indications

of phenotype and/or functionality change in CD8 T cells after receiving CD4 T cell help. In consequence, we can understand CD4 T cell help by observations made on CD8 T cells in the absence of CD4 T cells in distinctively diverse models (30), for example low or absent CD8 T cell primary responses (31-33), defects in secondary recall (34-37) or maintenance of memory CD8 T cells (38). In our observations, we provide evidence that MHC class II is indeed present on mouse CD8 T cells after T cell activation, after its transference from APCs by trogocytosis, enabling direct interactions with CD4 T cells. Moreover, we demonstrate as a proof of concept, that these direct interactions can improve secondary recall of CD8 T cells as an observed feature of CD4 T cell help for CD8 T cells (35-37).

The effects of CD4 T cell help on the transcriptional regulation of CD8 T cell are still not entirely known. Reported markers for CD8 T cell differentiation like CD127 (39), KLRG1 (40) or for CD8 T cell exhaustion like PD-1 (41) have been found using gene expression microarrays after acute (42, 43) or chronic infection (44). Intriguingly, it has been difficult to precisely define the molecular imprint that CD4 T cell help leave on CD8 T cells. As mentioned in chapter 1, TRAIL was proposed to mediate apoptosis in the secondary recall of CD8 T cells that did not receive CD4 T cell help (45), observation that was also reproduced using a model of CD8 T cell homeostatic proliferation in response to lymphopenia (46). However, this finding was challenged by reports that argued that TRAIL deficiency only delayed apoptosis (47) and did not rescue impaired memory CD8T cells generated in the absence of CD4 T cells (48). These differences could be the result of using diverse animal models to study CD4 T cell help. Another study looking at gene expression and CD4 T cell help (49) was based on the assumption

that CD4 T cells interacted directly with CD8 T cells through CD40:CD40L (50), notion that has also been challenged by report arguing that CD40 deficient mice did not show any impairment in CD8 T cell memory (51). In another model, CD25 deficient CD8 T cells replicated the secondary recall impairment observed in CD4 T cell deficient mice (52). However, it has been found that CD25 is needed to generate effector CD8 T cells (53) and effector memory CD8 T cells (54). Therefore, it is not clear if gene expression results obtained using this model could be considered an accurate reflection of all the possible effects of generating memory CD8 T cells in the absence of CD4 T cells, specially considering that central memory CD8 T cells proliferate more vigorously upon secondary recall than effector memory CD8 T cells (55). On the same notion, T-bet has been found to be highly expressed on helpless CD8 T cells (56), but since it has also been proposed as a molecular switch between central and effector memory CD8 T cell differentiation (57), and a marker for terminal differentiation (58), further studies would be needed to confirm its involvement in CD4 T cell help. Furthermore, studies have proposed that CD4 T cell help induce epigenetic changes in CD8 T cells, influencing the methylation of IL-2 promoter and histone acetylation on the IFNγ locus (59, 60). We believe our model of direct interactions between CD4 and CD8 T cells can be useful to investigate changes that occur in CD8 T cells before and after CD4 T cell help, either by measuring gene expression with microarrays or detecting of epigenetic modifications, that could be influencing secondary recall a month later. Moreover, it could serve as a tool to identify what are the signals CD4 T cells deliver to CD8 T cells that can improve their functionality and in consequence, to use them in future vaccination strategies.

In addition, it has been hypothesized that CD8 T cells could acquire different functionality depending on the subpopulation of CD4 T cells providing help. It has been reported that CD8 T cells display impaired killing functions when influenced by the phenotype of CD4 T cells, being Th2 (61, 62) affecting the outcome of leprosy, Th17 (63) or Tregs that could both diminish CTL activity (64, 65). We believe our model could also help to investigate possible consequences of direct interactions between activated MHC class II bearing CD8 T cells and different subpopulations of CD4 T cells.

4.4 How location impacts on CD8 T cell memory formation

Like every cell in our body, cells of the immune system differentiate by integrating signals they receive from their surrounding environment. From hematopoiesis to maturation into naïve cells, B and T cells develop stimulated by membrane-bound or soluble factors and recognition of self-antigen by their cell receptors. Once matured, naïve B and T cells circulate through periphery receiving survival signals until encountering their cognate antigen. In the case of CD8 T cells, the recognition of antigen and the amount of costimulation and/or inflammatory milieu present at the same time will determine the number, quality and location of the resulting memory CD8 T cells. It has been reported that the programming of CD8 T cell occurs early in the immune response (66-70). Therefore, since CD8 T cells don't seem to circulate in blood upon antigen recognition during the first days of an immune response, we could hypothesize that subpopulations of effector and memory CD8 T cell would arise differently in response to the local environment of the tissue they are present in. In consequence, detection of early differences in phenotype and functionality might help identify critical steps in the generation of memory CD8 T cells.

Previous studies have found that anatomical location determine phenotype and functional properties of CD8 T cells responding to an infection. Naïve CD8 T cell migration into spleen and lymph nodes is modulated by chemokines CCL19 and CCL21. It has been reported that this chemokine gradient is decreased upon infection (71) and, as a consequence, naïve CD8 T cells can not reach white pulp in spleen and therefore they can not respond to a new infection while this is occurring. This observation highlights the importance of tissue location for CD8 T cell activation. More recently, it has been shown that MPECs locate in white pulp of spleen in contrast to SLECs which can be observed in the red pulp upon LCMV infection, supporting again the notion that location within a tissue is critical of CD8 T cell differentiation (72). Lymph nodes have also a big role in the differentiation of CD8 T cells since it has been shown that the generation of CD8 T cells is impaired in the absence of lymph nodes (73). Non-lymphoid mucosal sites like lung (74) or gut (75) can also determine the differentiation of CD8 T cells upon infection. Differences observed in phenotype and functionality between tissues could be induced by the cytokines present in the tissue. For example, it has been shown that a particular effector memory phenotype is acquired by CD8 T cells in gut after LCMV infection induced by TGFβ present in the tissue (76). In consequence, to understand how the memory precursor phenotype arise in CD8 T cells responding to LCMV infection in bone marrow, it would be of great importance to find out the cytokines present as well as the adhesion molecules involved in their migration into bone marrow.

The expression of adhesive molecules like alpha 4 beta 7 in the case of gut or alpha 4 beta 1 and LFA-1 in the case of bone marrow allows entrance of CD8 T cells into tissues that can shape their differentiation (76, 77). Studies focused on migration of CD8

T cell into bone marrow have found that E- P- L-selectins, VCAM1 and alpha4 beta 1 and CXCR4 responding to CXCL12 chemokine gradient to be important for recruitment into bone marrow (78, 79). Another adhesion molecule that have been implicated in T cells from bone marrow has been CD44 (80), which it was found really high on CD8 T cells from bone marrow in our experiments. Intriguingly, central memory CD8 T cells seemed to be specially retained in bone marrow acquiring an activation state (81, 82) that could be explained by environmental cues received like CXCL12 or IL-7 (83) for which they also compete (84). It has been reported that Eomesodermin could be a critical factor for CD8 T cells to compete for space in bone marrow (85), although we could not detect Eomesodermin by flow cytometry on CD8 T cells from Bone Marrow.

In our observations, we were able to characterize a previously unreported distinct population of CD8 T cells in bone marrow. This CD8 T cell population from bone marrow expressed the same amount of CD127, KLRG1 markers as observed in CD8 T cells from spleen or PLN, but distinguish itself by displaying homogeneously high Ly6C and lower CD25. Moreover, in contrast to the high expression of T-bet, a marker associated with terminal differentiation observed in spleen and PLN, CD8 T cells from bone marrow displayed low to absent T-bet expression. These findings might indicate that CD8 T cells responding to LCMV in bone marrow are the earliest memory precursor detected so far. Furthermore, we detected less IFNγ-producers within LCMV-specific CD8 T cells from bone marrow than from spleen, which might also indicate these cells could be early memory precursors.

It is possible then that bone marrow not only harbors memory CD8 T cells, but also is capable of inducing CD8 T cells to differentiate into memory CD8 T cells early

during an infection. Long-lived plasma B cells (86), memory CD4 T cells (87) and memory CD8 T cells (88) have been found to reside in bone marrow. It was reported that all subsets of CD8 T cells, naïve, effector or memory CD8 T cells, can migrate into bone marrow, however only central memory CD8 T cells seemed to be preferentially retained (79). Based on our results, we could hypothesize that CD8 T cells responding to their cognate peptide in bone marrow acquire central memory features influenced by signals within local environment rather than migration since these events occur in a time where not many CD8 T cells circulate in blood. To test this idea, we could experimentally interrupt migration into bone marrow using CXCL12 blocking antibody as described before (79) and investigate which membrane-bound (CXCL12) or soluble factors (IL-7 or IL-15) in bone marrow could drive CD8 T cells in bone marrow into early memory precursors (86, 89). Moreover, the isolation of this early CD8 T cell memory precursors from bone barrow could shed light into the gene expression and epigenetic changes as well as secondary recall properties to define their potential to develop into memory CD8 T cells.

4.5 Conclusions

Taken together, our observations constitute an important step to further understand immunological memory and its early development during immune responses. We have proposed and tested a new model for CD4 T cell help where direct interactions between CD8 and CD4 T cells occur enabled by transferred MHC class II molecules that enhances secondary recall of CD8 T cells. We have also discovered a previously unreported population of CD8 T cells in bone marrow with a memory precursor

phenotype. We believe our results would open the door to more exciting experiments that would help to design better and more protective vaccines.

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