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The early generation of a heterogeneous CD4+ T cell response to *Leishmania major*[1]

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Abstract

$CD4^+$ T cells are an essential component of both the primary and secondary immune response against the intracellular protozoan parasite *Leishmania major*. Our lab has previously shown that $CD62L^{high}$ $IL7R^{high}$ central memory T (T_{CM}) cells mediate protective immunity following secondary challenge. To determine when T_{CM} cells develop, we examined the phenotype of *Leishmania*-specific $CD4^+$ T cells in the first two weeks following infection. As expected, we identified a population of $CD4^+$ T cells present in the draining lymph node with the characteristics of effector T cells. However, in addition, a second population phenotypically resembling T_{CM} cells emerged coincident with the effector population. These T cells, expressing CD62L, CCR7, and the IL7R, failed to produce IFN-γ but had the capacity to give rise to IFN-γ-producing effector cells. Our studies also demonstrated that the degree of proliferation and the timing of lymph node entry impact T_{CM} cell development. The early generation of T_{CM} cells following *L. major* infection indicates that T_{CM} cells may not only control secondary infections, but may also contribute to the control of the primary infection.

Keywords

Leishmania major; $CD4^+$ T cells; Memory; CD62L expression

Introduction

A heterogeneous population of memory T cells with distinct phenotypes and functional responses are generated during an immune response. The subset of central memory T (T_{CM})4 cells maintains the ability to home to the lymph nodes (LNs) due to their expression of CD62L and CCR7 (1-3). T_{CM} cells serve as a reservoir of antigen (ag)-specific T cells that can proliferate and become effector cells upon secondary challenge. A second population of cells, termed effector memory T (T_{EM}) cells, can migrate through the tissues and upon encountering antigen rapidly produce effector cytokines such as IFN-γ or IL-4. Therefore, T_{EM} cells may be poised to immediately respond to eliminate pathogens. How and when these memory T cells are generated remains unclear, and several models have been proposed to account for the heterogeneity in memory T cell populations (4-11). In contrast to models of memory generation following acute infection, it has been proposed

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that the lack of pathogen clearance may impair long-term memory development through the repeated stimulation of ag-specific T cells (12-15). Recent studies have suggested that the early $CD4^+$ and $CD8^+$ T cell response is heterogeneous, with some cells exhibiting the characteristics of memory T cells (or their precursors) while others have the phenotype of effector T cells (16-18). The majority of these studies have been done with pathogens that are rapidly cleared such as LCMV or *Listeria monocytogenes*, and whether the early heterogeneity observed in some of these studies will be evident with an infection where the pathogen is never cleared is unknown. Here we have used experimental infection with *Leishmania major* to ask whether $CD4^+$ T cells with the phenotype of memory T cells develop early after infection.

C57BL/6 mice resolve a primary infection with the intracellular protozoan *Leishmania major*, although they maintain low levels of persisting parasites. Following resolution of the primary infection, mice are subsequently immune to rechallenge. This immunity is mediated by both $CD4^+$ and $CD8^+$ T cells, although $CD4^+$ T cells alone can transfer resistance (19). We have identified three populations of $CD4^+$ T cells that can participate in both the control of persistent parasites and resistance to reinfection: $CD62L^{low}$ interleukin-7 receptor $(IL7R)^{low}$ T-bet^{high} cells, $CD62L^{low}IL7R^{high}$ T-bet^{high} cells, and $CD62L^{high}IL7R^{high}$ T-bet^{low} cells (20). The latter subset has the characteristics of T_{CM} cells since they migrate through LNs, can be maintained without antigen, and, upon restimulation, differentiate into Th1 cells that migrate to the site of infection and mediate parasite control (19). The ability of $CD4^+$ T_{CM} cells generated during *L. major* infection to differentiate into Th1 cells is dependent upon the presence of IL-12 since T_{CM} cells from immune mice transferred into *L. major* infected IL-12p40−/− mice develop into Th2 cells (21). Thus, the flexible nature of the T_{CM} cells induced by *L. major* infection suggests that they are not committed to a particular lineage and may develop prior to the acquisition of effector function. If this is the case, T_{CM} cells could be generated early following infection and provide a pool of ag-specific T cells that upon further activation augment the effector T cell pool.

To determine if the early $CD4^+$ T cell response to infection with *L. major* involved generation of cells with the characteristics of T_{CM} cells, we examined the responses of both transgenic and polyclonal $CD4^+$ T cells during the early weeks of *L. major* infection. We found that IL7R, CD62L and CCR7 expression defined two distinct populations of responding $CD4^+$ T cells that could be observed by two weeks post-infection. One population expressed low levels of CD62L, CCR7, and the IL7R, while the other population expressed elevated levels of these molecules, characteristic of T_{CM} cells. The T_{CM} cells did not produce the effector cytokine IFN-γ directly *ex vivo* but could efficiently give rise to IFN-γ-producing Th1 effector cells following restimulation *in vivo*. We used a combination of LN-homing blockade, CFSE-labeling, and BrdU administration to show that the

[4]Abbreviations used in this paper:

T_{CM}	central memory T
T_{EM}	effector memory T
Th1	T helper type 1
Th2	T helper type 2
pi	post-infection
dLN	draining lymph node
ndLN	non-draining lymph node
WT	wild-type

CD62L^{high} cells are stimulated in the draining LN within the first few days following infection but many of the T_{CM} cells soon thereafter cease proliferation. These data show that the early CD4$^+$ T cell response to *L. major* is heterogeneous and suggest that the generation of T_{CM} cells is concurrent with the initiation of the primary immune response to *L. major*.

Materials & Methods

Mice

C57BL/6, B6.PL-*Thy1*^{a}/CyJ (Thy1.1), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1), and C57BL/6-Tg(TcraTcrb)425Cbn/J (OTII) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Fredericksburg, MD). Thy1.1$^+$ OTII mice were bred and maintained in our facility. Animals were maintained in a pathogen free environment, and experiments were performed in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Parasites and infection

L. major (MHOM/IL/80/Friedlin) and Leish-OVA parasites were grown in Schneider's insect medium supplemented with 20% heat-inactivated FBS and 2mM glutamine (22). Infectious stage metacyclic parasites were enriched using density gradient centrifugation (23). Mice were infected in the hind footpad or the ear dermis with 1-2 × 10^6 parasites.

Adoptive transfers and in vivo antibody treatment

CD4$^+$ T cells were enriched from the lymphoid tissue of donor mice prior to transfer using MACS (Miltenyi Biotec). Where indicated, CD4$^+$ cells were sorted from transgenic donor mice prior to transfer on a FACS Aria (Becton Dickinson). All cells were CFSE-labeled (1.25μM) (Molecular Probes), and 5 × 10^6 cells were transferred to congenic recipients unless otherwise noted in figure legends. In all experiments, the inguinal, axillary, and brachial lymph nodes were harvested and pooled together as representative peripheral LNs (also referred to as non-draining LNs) from individual naïve or infected mice. For detection of low numbers of transferred cells, the harvested tissues were first enriched by negative selection (MACS) for the donor marker prior to analysis by flow cytometry (24). Briefly, samples were incubated with biotinylated antibodies against CD45.1 followed by incubation with anti-biotin microbeads according to the manufacturer's instructions (Miltenyi). The CD45.2$^+$ donor cells were then negatively selected using LS columns. Blocking antibody against CD62L (MEL-14) was a gift from S. Reiner (University of Pennsylvania, Philadelphia, PA). 250μg of antibody was administered i.p. at the indicated time points.

Flow cytometry and BrdU treatment

The following antibodies used to detect cell surface markers were purchased from eBioscience: CD4, Thy1.2, Thy1.1, CD45.2, CD45.1, B220, CD11b, NK1.1, CD127 (IL7Rα) (PE or allophycocyanin only), and CD62L. For detection of CCR7, cells were incubated with CCR7 antibody (PE or allophycocyanin only) for 1hr at 37°, washed, and then stained for any remaining surface antigens as above. For intracellular detection of cytokines, cells were stimulated with PMA, ionomycin, and Brefeldin A for 4 hours *in vitro* and fixed with 2% paraformaldehyde in PBS. Cells were then permeabilized with 0.2% saponin and stained with IL-2-allophycocyanin and IFN-γ-PE-Cy7 (eBioscience). BrdU was purchased from BD Pharmingen, and 1 mg BrdU was administered i.p. every 12 hrs for 3 days (6mg total per mouse). Cells were permeabilized and stained for flow cytometry per the manufacturer's instructions. Data was acquired on an LSR II or a FACS Canto (Becton Dickinson). Analysis was performed using FlowJo software (Tree Star, Inc.). For all

samples, gating was established using a combination of isotype and fluorescence-minus-one (FMO) controls.

Statistics

Statistical analysis was performed using Prism (GraphPad Software, Inc.). For all graphs, data is presented as the mean ± SEM. One-way ANOVA was used to establish significance in combination with the indicated post test, and a p-value of < 0.05 was considered significant.

Results

IL7R expression on CD4$^+$ T cells defines two populations of T cells responding to *L. major* infection

In order to monitor the ag-specific CD4$^+$ T cell response to *L. major*, we adoptively transferred CFSE-labeled OTII T cells into congenic C57BL/6 mice and challenged the recipient mice with *L. major* parasites expressing OVA (Leish-OVA) (22). Proliferation and IL7R expression on the ag-specific OTII cells in the LN draining the site of infection (dLN) was assessed on days 3, 7 and 14 following infection (Fig. 1A & 1B). We initially chose to focus on the expression of the IL7R since its expression is required for the survival of memory T cells (25-27). As early as 3 days post infection (pi) there was a robust proliferative response of the OTII cells. Almost all of the proliferating cells downregulated the IL7R, which is consistent with previous studies (25,28-37). However, we observed two distinct populations of responding cells by 7 and 14 days pi. One group of proliferating CD4$^+$ T cells expressed a low level of the IL7R, a phenotype suggestive of an activated effector T cell that has recently been stimulated via the TCR (38). The other population of responding cells expressed the IL7R at levels equivalent to that of naïve T cells. We also observed that by day 7, proliferated OTII cells could be detected within other peripheral (non-draining; nd) LNs. Most likely these T cells proliferated in the dLN and then migrated to the ndLNs. Interestingly, only the IL7R-expressing T cells were found in ndLNs suggesting that these two T cell populations may exhibit differences in their migratory potential or that migration away from the inflammatory site allows for the upregulation of the IL7R. While parasites can disseminate through the blood to distant sites (39), the lack of an IL7R^{low} population in the ndLNs could suggest that priming within the ndLNs is minimal or that the activation of CD4$^+$ cells under such conditions generates cells with a T_{CM}-like phenotype. Thus, based upon expression of the IL7R, within two weeks of *L. major* infection, two distinct populations of CD4$^+$ T cells are evident.

Expression of LN homing molecules by CD4$^+$ T cells responding to *L. major* infection

The expression of CD62L and CCR7 is required for efficient entry of T cells into LNs, and their differential expression has also been used to define memory T cell subsets (1). We have previously reported that CD62L^{high} CD4$^+$ T_{CM} cells are present in mice that have resolved a primary infection with *L. major* (referred to as 'immune mice') (19), and we wanted to determine if cells with a similar phenotype were present early after infection. Therefore, we again adoptively transferred CFSE-labeled OTII cells to naïve recipients that were subsequently challenged with Leish-OVA and asked if the T cells responding to infection exhibited differential expression of CD62L and CCR7. As shown in Fig. 2, a pattern similar to that observed with IL7R expression was seen in proliferating (CFSE^{dim}) OTII T cells. Thus, two populations of responding T cells were present early after infection: one population of cells expressed CD62L and CCR7, while the second did not (Fig. 2A and 2C). Furthermore, the CD62L^{low} cells expressed uniformly low levels of the IL7R while the population of CD62L^{high} cells contained both IL7R^{high} and IL7R^{low} cells (Fig. 2B). We confirmed these results with another TCR transgenic mouse (termed ABLE) that recognizes

a *Leishmania*-derived peptide ($LACK_{156-173}$; *Leishmania* homolog of receptors for activated C kinase) in the context of MHC II I-A^d and found similar results (data not shown).

Recent work has demonstrated that adoptive transfer of large numbers of transgenic T cells can impact the emergence of memory T cells (40-43). Thus, to determine if the generation of these two different cell types was due to the transfer of non-physiologic numbers of T cells with the same specificity, we repeated the above experiment but transferred reduced numbers of TCR transgenic cells (10^5, 10^4, and 10^3). Due to the loss of cells following transfer, the actual numbers present in spleen and LN tissues following transfer corresponds roughly to 10^4, 10^3, and 10^2, respectively (44). We then infected the recipient mice with Leish-OVA and isolated the dLN after 10 days. We found that regardless of the initial number of cells transferred, two populations of cells could be defined by CD62L expression, and the relative frequency of each population was the same (Fig. 3A & 3C). However, when lower numbers of cells were transferred, the extent of CFSE dilution was increased as only a small percentage of OTII cells remained $CFSE^{bri}$ after 10 days, and the fold increase in proliferating ag-specific cells over input was significantly increased (Fig. 3A and data not shown). These data are consistent with previously published findings that proliferation is dramatically influenced by the frequency of T cells recognizing a particular epitope (41).

To further confirm that our results reflected the naturally occurring immune response following infection, we adoptively transferred CFSE-labeled polyclonal $CD4^+$ T cells from naïve conventional C57BL/6 mice to congenic recipients (CD45.1) that were subsequently infected with wild-type *L. major* parasites. This allows us to monitor naturally occurring *Leishmania*-specific $CD4^+$ T cells at normal physiologic frequencies within a given pool of naïve T cells based upon their proliferation and dilution of CFSE. We isolated the cells from the dLN after 2 weeks and found that similar to the T cells from the TCR transgenic mice, polyclonal $CD4^+$ T cells from conventional mice exhibited the same degree of heterogeneity, generating both $CD62L^{low}$ and $CD62L^{high}$ populations of responding $CD4^+$ T cells (Fig. 3B & 3C). These results further support our findings that infection with *L. major* rapidly generates a heterogeneous population of $CD4^+$ T cells. Thus, we can conclude that the early $CD4^+$ T cell response to *L. major* infection generates at least two populations of cells that can be distinguished based on their expression of both the IL7R and LN-homing molecules and that one of these populations has the phenotype of T_{CM} cells.

Proliferative arrest generates $CD62L^{high}$ T cells early following infection

In addition to the differential expression of the IL7R and molecules associated with LN-homing, we noticed that the $CD4^+$ T cells responding to infection could be characterized by how many cell divisions they had undergone with a substantial enrichment in the $CD62L^{high}$ cells in divisions 1 through 5 (Figs. 2 & 3). This enrichment varied, as is evident when looking at Figures 3A and 3B. One cause for such variation could be the specificity of the monoclonal versus polyclonal cells. Alternatively, the dLNs were collected at different times (d10 versus d14 pi), and as the infection proceeds, one might anticipate that the $CD62L^{high}$ cells would have an increased opportunity to be restimulated and further dilute CFSE. By focusing on these early time, points we can examine how cell division might contribute to the generation of the heterogeneous $CD4^+$ T cell response. Since division was assessed by CFSE dilution after 7-14 days of infection, we could not determine when over the course of that time the cells in division 1-5 had actually divided. The cells in divisions 1-5 could represent (1) cells that had only recently begun to divide or (2) T cells that received a signal to proliferate early following infection but had ceased dividing. To distinguish between these two possibilities, we transferred CFSE-labeled OTII cells into congenic recipients that were given BrdU at different times pi to assess when the $CFSE^{dim}$ cells had proliferated. One group of mice was given BrdU from days 1 to 3, and a second group of mice received BrdU from days 7 to 9. Thus, if the cells were proliferating just prior to sacrifice, the

CFSE^{dim} OTII cells in divisions 1-5 would also be BrdU^{+} in mice in mice receiving BrdU from days 7 to 9. However, we found that only when BrdU was administered during the first 3 days following Leish-OVA infection was a large percentage of BrdU^{+} cells detected in the dLN on day 10 pi (Fig. 4). Interestingly, many of these BrdU^{+} cells were also detected in the ndLNs, suggesting that these cells proliferated in the first 3 days, likely in the dLN, but then migrated to other lymphoid tissues. When the BrdU was given just prior to the end of the experiment (days 7 to 9), the small percentage of cells that were BrdU^{+} were present in those cells that had undergone >5 divisions. Thus, through the combination of CFSE dilution and the incorporation of BrdU, we can conclude that many of the cells with a T_{CM} phenotype that are observed early following infection received a signal to proliferate in the first 3 days following infection but then underwent an active cessation in proliferation.

The timing of T cell arrival in the draining LN influences their ability to respond

The above results indicate that only some CD4^{+} T cells continue to divide following infection with *L. major*, and those with reduced proliferation exhibit the phenotype of T_{CM} cells. In previous studies, we found that naïve T cells proliferated poorly when transferred into already infected mice, suggesting that when T cells are recruited into the immune response could influence how well they respond (45). Similarly, studies in mice immunized with peptide showed that early arriving T cells proliferated more while late arriving T cells proliferated less and were directed towards a T_{CM} fate (46). Thus, we considered the possibility that one difference between the cells that had divided >5 times and those that had ceased proliferating was when they entered the dLN. To determine if this was the case, we assessed the response of T cells that were resident in the dLN at the time of infection by blocking the entry of additional recruited T cells into the LN using monoclonal antibodies against CD62L. In one group of mice, anti-CD62L mAb was administered at the time of Leish-OVA infection (one day after the transfer of OTII cells). A second group of mice did not receive anti-CD62L mAb treatment until 3 days pi, which allowed for transient recruitment of cells into the dLN. Because anti-CD62L mAb treatment prevents surface staining for CD62L, we used expression of CCR7 to differentiate between the two T cell populations. We found that very few cells were in divisions 1-5 when LN recruitment was blocked at the time of infection (Fig. 5A). Most of the cells found in the dLN at day 10 pi had divided >5 times and expressed low levels of CCR7 (Fig 5A & 5B). However, a population of T cells in divisions 1-5 and expressing CCR7 were present in the dLNs of mice when antibody treatment was withheld until day 3. These data suggest that the T cells resident in the dLN at the time of infection are more likely to differentiate into effector T cells, especially in the absence of newly recruited cells, which are more likely to develop a T_{CM} phenotype. In addition, our results suggest that recruited cells can be directed towards a T_{CM} fate within the first 3 days following infection.

CD62L^{high} T cells can become IFN-γ producing T cells upon restimulation

The lack of IFN-γ production by T_{CM} cells is one of the critical characteristics that was shown to distinguish T_{CM} cells from effector cells following *L. major* infection (19). Therefore, we next assessed the ability of the different types of early responding CD4^{+} T cells to produce IFN-γ directly *ex vivo*. Naïve polyclonal CD4^{+} T cells were enriched from the spleens and LNs of C57BL/6 mice, labeled with CFSE, and transferred to naïve congenic recipients. The recipient mice were infected with *L. major* the following day, and at 2 weeks pi, the dLN was isolated to determine cytokine production by responding (CFSE^{dim}) CD4^{+} T cells in the dLN. Because *in vitro* stimulation with PMA and ionomycin can influence surface expression of CD62L, we used the extent of CFSE dilution to compare T_{CM} cells in divisions 1-5 to the heterogeneous population of CD62L^{high} T_{CM} and CD62L^{low} effector T cells in >5 divisions. While both populations had the capacity to make IL-2, IFN-γ was only produced by cells that had divided more than 5 times (Fig. 6A). Thus,

similar to the T_{CM} cells that we observed >12 weeks pi, the early responding T cells that have ceased dividing and adopted a T_{CM} phenotype fail to make IFN-γ. To determine if the cells with a T_{CM} phenotype that emerge in the early weeks following infection could differentiate into IFN-γ-producing cells upon restimulation, we purified $CD4^+$ $CD62L^{high}$ cells from mice that had been infected with *L. major* for only 2 weeks. Based on the results shown in Fig. 3B, this population of donor cells would include a mixture of *Leishmania*-specific cells that have undergone varying rounds of division. We then CFSE-labeled the cells and adoptively transferred them into congenic recipients that were subsequently challenged with *L. major*. As controls, we also purified cells with this phenotype from naïve and immune mice. We isolated the dLNs and spleens after 2 weeks and compared the frequency of IFN-γ-producing cells within the total population of $CFSE^{dim}$ cells. This comparison allowed us to normalize for the expected differences in ag-specific cells that would be present in these three different populations of donor T cells. We found that while a small percentage of cells derived from naïve mice could produce IFN-γ after 2 weeks, this percentage was increased ~2-fold when the donor cells were derived from mice that had been infected for 2 weeks prior to isolation, transfer, and rechallenge (Fig. 6B). Thus, we found that at least some of the $CD62L^{high}$ cells generated early after infection had the capacity to differentiate into Th1 cells, similar to the T_{CM} cells we characterized in immune mice. Using current protocols, it is unknown whether the Th1-generating cells had undergone more or less rounds of division prior to restimulation. The increase in the percentage of IFN-γ-producing T cells by donor-derived cells from 2-week infected mice compared with naïve donor cells, in combination with the lack of IFN-γ production by cells in divisions 1-5, suggests that while the $CD62L^{high}$ cells may not produce IFN-γ directly *ex vivo*, some of the cells may already be predisposed along a pathway towards Th1 cytokine production. This would be consistent with our recent findings that some $CD62L^{high}$ cells, while not making IFN-γ, express T-bet, the transcription factor required for IFN-γ production (20).

Discussion

The hallmark of adaptive immunity is the ability to develop immunologic memory, such that following a primary infection we are better able to respond when re-exposed to the same pathogen. While immunologic memory is a critical component of the immune response, how and when memory develops is still not completely understood. We previously demonstrated that following infection with *L. major*, C57BL/6 mice develop $CD4^+$ T_{CM} cells that can mediate resistance to reinfection (19). Here, we find that $CD4^+$ T cells with a T_{CM} phenotype develop within the first few weeks following *L. major* infection, coincident with the development of $CD4^+$ Th1 effector cells. These T_{CM} cells expressed high levels of CD62L and CCR7, which promote migration through lymphoid tissues, and high levels of the IL7R, which is required for the long-term survival of memory T cells. Cytokine production by these early emerging T_{CM} cells was limited to IL-2 and required antigen-induced proliferation in order to give rise to IFN-γ-producing Th1 cells. Lastly, we provided evidence that the phenotype of the $CD4^+$ T cells is influenced by the timing of their arrival in the dLN and involves the proliferative arrest of ag-specific T cells, despite the continued presence of parasites. Taken together, these results indicate that $CD4^+$ T cells with a T_{CM} phenotype develop during the early stages of an immune response.

There are several models that attempt to explain how memory T cells develop, and while some postulate that memory T cells slowly develop after the pathogen has been cleared, others propose that memory T cells–or their precursors–develop very soon after infection (4-11). Our data indicate that $CD4^+$ T cells with a T_{CM} phenotype ($CD62L^{high}$ $CCR7^{high}$ $IL7R^{high}$) develop quite rapidly following infection with *L. major*. These cells do not immediately produce IFN-γ upon restimulation, suggesting that they develop prior to

becoming effector cells. These results are consistent with our previous studies indicating that T_{CM} cells in immune mice do not make IFN-γ and that, in the absence of IL-12, they can differentiate into Th2 cells (19,21). Catron *et al.* described a similar population of $CD62L^{high}$ $CD4^+$ T cells that were generated following peptide immunization (46), suggesting that these early emerging T_{CM} cells are not unique to leishmaniasis and may represent a normal component of the $CD4^+$ T cell response. Thus, while cytokine-producing cells can contribute to the pool of long-lived memory T cells (47), our data suggests that progression through an effector phase is not a requirement for T_{CM} generation. It is also possible that depending on the nature of the infection, T_{CM}-like cells might develop both prior to and after becoming effector cells (48).

The mechanism(s) driving the heterogeneity of the early T cell response is not well understood. The initial interaction between a T cell and the APC can lead to asymmetric cell division, such that one daughter cell becomes an effector cell while the other a less differentiated cell that exhibits a memory phenotype (18). Alternatively, Lanzavecchia and Sallusto proposed the signal strength hypothesis as a mechanism to explain the heterogeneity of the $CD4^+$ T cell response (49). Their hypothesis suggests that those cells receiving weaker signals will become T_{CM} cells while those cells receiving the strongest signals will become fully differentiated effector cells that exert their function and then die. Support for this hypothesis was provided by Wu *et al.* who showed that acquisition of IFN-γ production by multiple rounds of stimulation with antigen led to the inability of a $CD4^+$ T cell to survive long-term (50). However, more recent studies have shown that IFN-γ-producing cells can contribute to the pool of long-lived memory T cells (47). Despite their expression of the IL7R, it is not thought that signals through the IL7R drive T_{CM} cell development (32,51,52). Also, whether or not a $CD4^+$ T cell receives successive stimulation can have a direct impact on the degree of proliferation and the generation of memory (15,53,54) It is probable that a range of signal strengths exist with differential outcomes on individual $CD4^+$ T cells. Therefore, the overall extent of stimulation can impact the type of memory T cell generated and depends on the cumulative signals imparted on a given cell through successive interactions with DCs, costimulation, and the inflammatory milieu (6,40,55).

By using OTII cells to track the ag-specific $CD4^+$ T cell response, we found that most of the T_{CM} cells evident two weeks after *L. major* infection have ceased proliferating with many of them having divided only a few times. Why they fail to continue to proliferate is unknown. It is possible that T cells receive an inhibitory signal that actively inhibits proliferation (56). However, since continued TCR stimulation is required for $CD4^+$ T cells to continue to divide (57), the simplest explanation for the proliferative arrest may be a loss of TCR signaling. Thus, the proliferative arrest might result from the lack of a positive signal rather than the presence of a negative signal. For example, it has been proposed that weaker signals are the result of a stochastic event caused by migration away from the site of antigen presentation (49,58). Consistent with this idea are our findings that some of the T_{CM} cells are present in ndLNs soon after infection. These cells have the potential to migrate back through the dLN where they can be stimulated once again and give rise to Th1 cells as needed to control the parasite burden. Competition among responding T cells can also have a negative impact on the ag-specific T cell response as indicated in several studies using TCR transgenic cells (40,41). Similar to these studies, we found that altering the number of ag-specific T cells influenced the extent of proliferation. However, it did not change the percentage of T cells that exhibited a T_{CM} phenotype. Moreover, polyclonal responses—where a diverse repertoire of ag-specific T cells is maintained at physiologic frequencies—exhibited the same early development of T_{CM} cells. Thus, while competition with non-physiologic numbers of T cells can influence the magnitude of the proliferative response, it does not appear to be responsible for the generation of T_{CM} cells following *L. major*

infection. On the other hand, we found that when T cells arrived in the dLN did influence the nature of the T cells that responded such that only the cells recruited into the dLN developed a T_{CM} phenotype. Using a peptide immunization model with similar results, it was suggested that this was due to decreasing antigen over time (46). However, following *L. major* infection, the parasite burden continues to increase during the first several weeks of infection, although it has also been proposed that there are two waves of antigen presentation with the second wave commencing after two weeks (59,60). Thus, a more likely explanation for our findings might be the ability of *Leishmania* to impair antigen presentation by DCs resulting in an overall decrease in the extent of T cell activation (61-64). By two weeks pi, those cells that had fully diluted CFSE (>5 div) were a heterogeneous population containing both $CD62L^{high}$ and $CD62L^{low}$ cells.

Infection of C57BL/6 mice with *L. major* is associated with the development of a cutaneous lesion that resolves after several weeks due to the development of $CD4^+$ Th1 response. However, a small number of parasites persist in these mice. The control of these remaining parasites is dependent upon the maintenance of an effective Th1 response, such that treatment of mice that have resolved a primary *L. major* infection with anti-IFN-γ leads to the reactivation of the disease. How this Th1 immunity is maintained is not well understood. One possibility involves the continued recruitment of naïve T cells into the response, yet we have found that naïve *Leishmania*-specific T cells respond poorly in the presence of previously activated T cells (45). Alternatively, the T_{CM} cell population that we have described here may serve as an additional source of ag-experienced T cells that can recirculate through the dLN and receive additional signals to further differentiate into Th1 effector cells as needed to help control the infection. Certainly, if most highly activated Th1 cells eventually die, the maintenance of a population of T_{CM} cells might be essential to control the parasites that persist for the lifetime of the animal.

In summary, we have demonstrated that the early $CD4^+$ T cell response to *L. major* infection involves the generation of both effector T cells and T cells with the characteristics of T_{CM} cells. The heterogeneity of the response is characterized by differential expression of CD62L, CCR7, and the IL7R, as well as the ability to produce the effector cytokine IFN-γ. These T_{CM} cells are available as an expanded pool of ag-specific T cells able to be restimulated and differentiate into effector cells, a function that may be critical in controlling not only secondary infections but also the parasites that persist in *L. major* infections.

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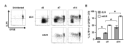

Figure 1. Dynamic IL7R expression on proliferating ag-specific CD4$^+$ T cells following *L. major* infection

(A) OTII cells were CFSE-labeled and transferred to Thy disparate recipients that were infected with Leish-OVA the following day. Draining (dLN) and non-draining (ndLN) lymph nodes were harvested on the indicated days pi, and the donor OTII cell population (CD4$^+$ Thy1.1$^+$) was analyzed for IL7R expression. Plots are representative of 5 mice in 2 separate experiments. Peripheral LNs from uninfected controls were harvested and pooled 15 days following OTII transfer (d14 pi). The percentage of donor cells with an IL7R^{high} phenotype within the CFSE^{dim} population is shown in B for both the dLN (grey bars) and ndLNs (white bars). A Tukey post test was used to compare differences between individual groups. * $p < 0.05$

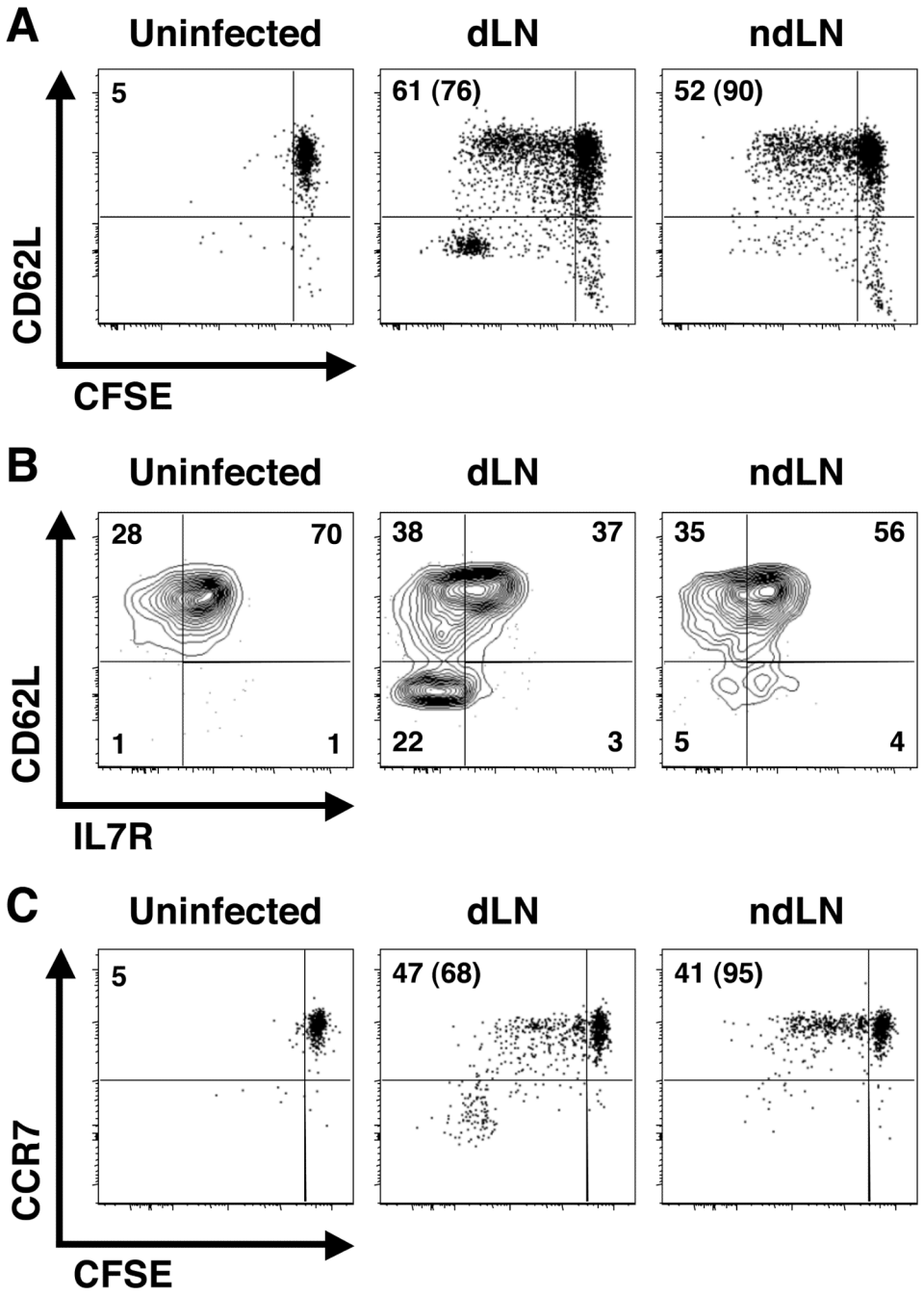

Figure 2. CD62L and CCR7 are expressed by a subset of *L. major*-specific CD4$^+$ T cells early following infection

(A-C) CFSE-labeled OTII cells were transferred to congenic recipients, which were infected with Leish-OVA the following day. The dLN and several ndLNs were harvested on day 10 pi and stained for surface expression of CD62L or CCR7. Plots have been gated on CD4$^+$ CD45.2$^+$ transgenic donor cells and are representative of 6-7 mice in 2-3 separate experiments. The phenotype of the donor cells in lymphoid tissue of uninfected mice is shown as a control. The numbers in the upper left of each plot (A and C) represent the percentage of donor cells that are CFSE^{dim} with the percentage of CFSE^{dim} cells exhibiting high levels of CD62L or CCR7 surface expression shown in parenthesis. In B, CD62L

versus IL7R expression is shown on the proliferated ($CFSE^{dim}$) cells from infected mice in A. Surface expression of these molecules is shown on naïve donor cells, which are $CFSE^{bri}$, from uninfected mice as a control. Numbers in B indicate the percentage of cells in each quadrant.

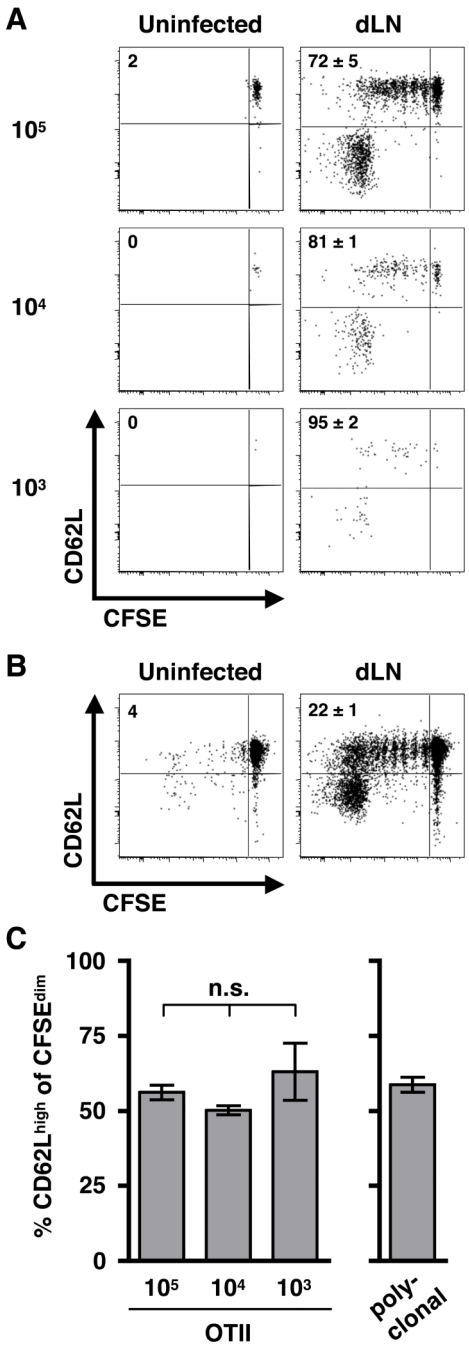

Figure 3. Phenotypic heterogeneity is maintained when varying the initial frequency of ag-specific cells

(A) $V\beta5^+$ $V\alpha2^+$ $CD4^+$ T cells were FACS purified from OTII mice and CFSE-labeled, and the indicated numbers of cells were transferred to naïve congenic recipients. The phenotype of the transferred cells ($CD45.2^+$ $CD45.1^-$) in the dLN on d10 is shown, and plots are representative of 3 mice per group in 2 separate experiments. To enhance detection of low frequencies of OTII cells, dLN tissue (infected mice) or pooled peripheral LNs (uninfected controls) were enriched for CD45.2 donor cells prior to flow cytometric analysis as described in the Materials & Methods. Numbers in each plot indicate the percentage of donor cells that are $CFSE^{dim}$ with the percentage of $CFSE^{dim}$ cells that are $CD62L^{high}$ in

parenthesis. (B) $CD4^+$ cells were enriched from the spleens and LNs of naïve C57BL/6 mice, and 5×10^6 cells were transferred to naïve CD45.1 recipients following CFSE-labeling. Recipient mice were infected with WT *L. major*, and the dLN was isolated after 2 weeks. Plots have been gated on $CD4^+$ $CD45.2^+$ cells and are representative of >12 mice in more than 4 separate experiments. (C) Bar graphs indicate the percentage of cells expressing high levels of CD62L for the OTII (A) and polyclonal (B) T cell transfers shown above. Using one-way ANOVA, we found that any differences between the groups were not significant (n.s.).

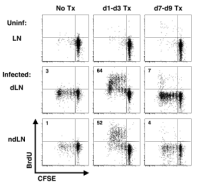

Figure 4. The CD62L^{high} population undergoes early proliferation followed by proliferative arrest

CFSE-labeled OTII cells were transferred to naïve congenic recipients which were infected with Leish-OVA the following day (d0). Groups of mice were given 6 injections of BrdU over a 3-day period (either d1-d3 or d7-d9). All mice were sacrificed on d10 at which time the incorporation of BrdU by the donor cells in the dLN was analyzed by flow cytometry. All plots have been gated on the $CD4^+$ $CD45.2^+$ $CD45.1^-$ donor cells and are representative of 3 mice per group in 2 separate experiments.

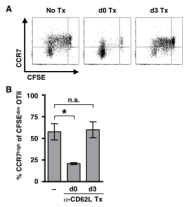

Figure 5. LN-homing can impact the early-emerging population of CCR7^{high} cells

(A) CFSE-labeled OTII cells were transferred to naïve congenic recipients one day prior to infection with Leish-OVA to allow for systemic distribution of naïve transgenic cells. Mice were then treated with MEL-14 (anti-CD62L) (250 μg) on either d0 (at the time of infection) or d3 to block LN-homing. All plots have been gated on the CD4^{+} CD45.2^{+} CD45.1^{-} donor cells and are representative of 3 mice per group in 2 separate experiments. (B) The bar graph indicates the percentage of CFSE^{dim} cells expressing high levels of CCR7 for each group of mice. Statistical significance was established using one-way ANOVA followed by a Dunnett post test. * $p < 0.05$; not significant (n.s.)

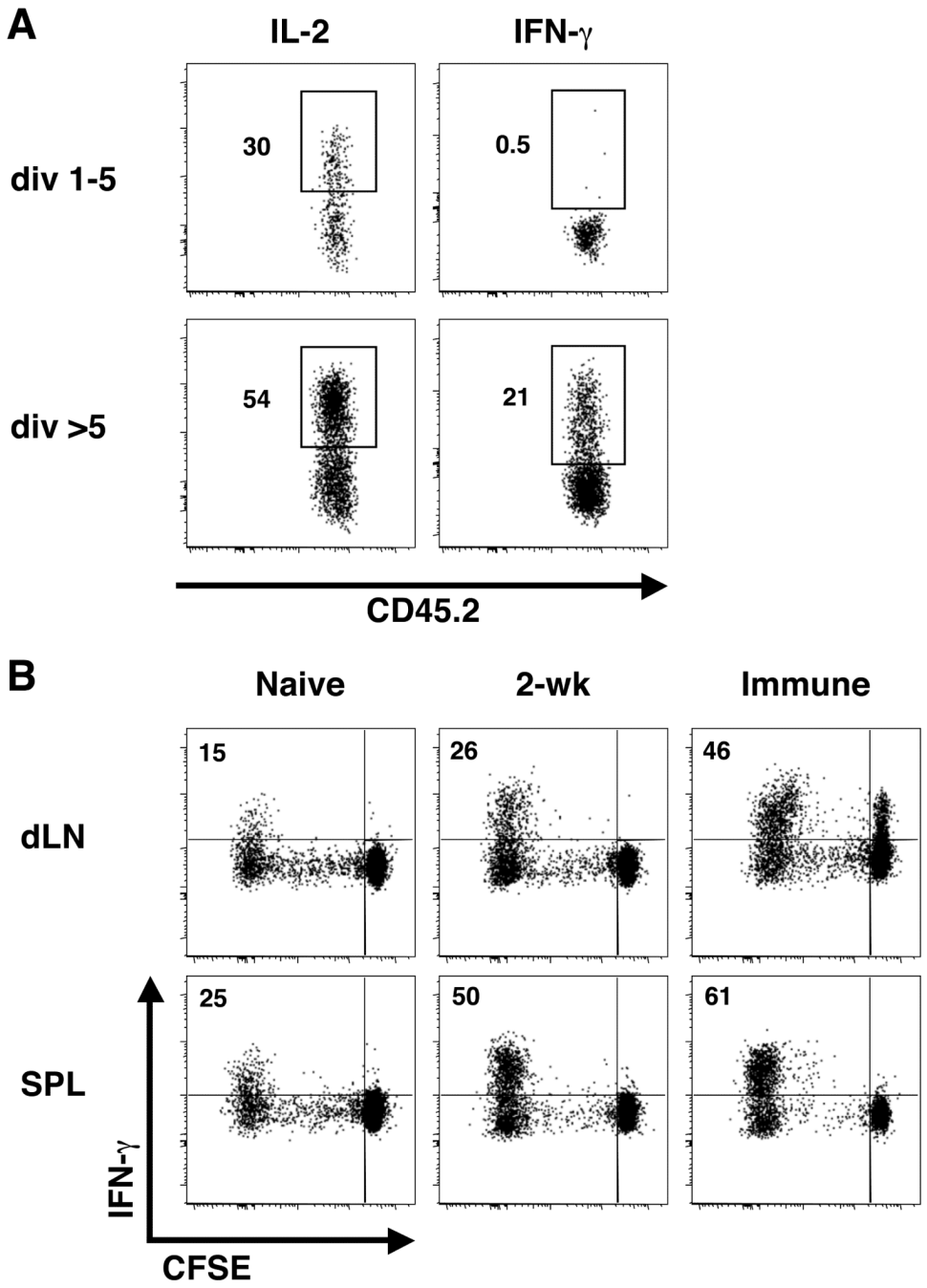

Figure 6. Cytokine production by T_{CM} cells

(A) Polyclonal $CD4^+$ T cells were enriched from naïve C57BL/6 mice and CFSE-labeled prior to transfer to naïve CD45.1 recipients. The recipient mice were infected with WT *L. major* the following day. After 2 weeks, the extent of CFSE dilution within the $CD4^+$ $CD45.2^+$ $CD45.1^-$ donor cells in the dLN was used to gate the plots shown in A which are representative of >12 mice in more than 4 separate experiments. Intracellular cytokine production was detected ex vivo following a 4hr *in vitro* stimulation. Numbers indicate the percent cytokine positive. (B) $CD4^+$ $CD62L^{high}$ cells from the spleens and LNs of naïve mice, mice that had been infected with 2×10^6 WT *L. major* 2 weeks prior, and immune mice (>12 weeks post *L. major* infection) were MACS purified, CFSE-labeled, and

transferred to congenic recipients that were challenged with WT *L. major* the following day. After 2 weeks, the dLN and spleen (SPL) were harvested, and cytokine production by the $CD4^+$ $CD45.2^+$ $CD45.1^-$ donor cells was determined as above. Plots are representative of 3-4 mice per group in 2 separate experiments. Numbers indicate the percentage of $CFSE^{dim}$ cells that are producing IFN-γ.