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Selective Depletion of Nonspecific T Cells During the Early Stage of Immune Responses to Infection

Jiu Jiang, Lisa L. Lau, and Hao Shen

Transient T cell depletion occurs before the development of an effective immune response to infection. In this study we show that most T cells, regardless of specificity, are induced to express early activation markers soon after infection with Listeria monocytogenes or lymphocytic choriomeningitis virus. Ag-specific T cells are further activated to display late activation markers and undergo extensive proliferation. As Ag-specific T cells begin to expand, nonspecific T cells are depleted en masse and exhibit no sign of further activation or proliferation before their depletion. This selective depletion of nonspecific T cells is due to in situ death via apoptosis, as visualized by confocal microscopy. Thus, early activation and subsequent depletion of nonspecific T cells are integral parts of the immune response to proinflammatory infections. These results have important implications for our understanding of early events in the development of a robust T cell response.


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Abbreviations used in this paper: LM, Listeria monocytogenes; AICD, activation-induced cell death; LCMV, lymphocytic choriomeningitis virus; rLM, recombinant LM; wtLM, wild-type LM strain.
Adoptive transfers

Splenocytes were isolated from P14 or OT-I mice and labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) in PBS, then quenched with 100% FCS. Cells were then washed with RPMI 1640/10% FCS and resuspended in PBS. P14 and OT-I T cells were purified by MACS using CD8a Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were transferred i.v. into recipient mice (2–5 × 10^6 cells/mouse, or 1–2 × 10^7 cells/mouse for confocal microscopy). For cotransfer experiments, equal numbers of purified CFSE-labeled P14 and OT-I cells were mixed and transferred. One day after transfer, recipients were infected with LM or LCMV.

Flow cytometry

Single-cell suspensions of splenocytes were resuspended in PBS/1% BSA/0.1% NaN₃ at a concentration of 1–5 × 10⁷ cells/ml, stained for cell surface markers using mAbs from BD PharMingen (San Diego, CA), and fixed with 2% paraformaldehyde/PBS. Flow cytometry was performed with a FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

In situ detection of apoptotic cells in spleens using TUNEL

Frozen 5-μm-thick spleen sections were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. TUNEL-positive cells were detected using the In Situ Cell Death Detection Kit/TMR Red (Roche, Indianapolis, IN). Sections were viewed and photographed using a laser-scanning confocal fluorescence microscope system (1024-ES; Bio-Rad, Hercules, CA).

Results

Kinetics of T cell depletion and expansion following LM infection

To examine the early events of T cell response, we infected C57BL/6 mice with LM and followed the kinetics of activation

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**FIGURE 1.** Kinetics of T cell depletion and expansion during the early stage of LM infection. C57BL/6 mice were infected with LM, and on days 0, 3, 5, and 7 splenocytes were stained for CD4, CD8, and CD44 and analyzed by FACS. A, Percentages of splenocytes that are CD4⁺ or CD8⁺. B, Percentages of CD8⁺ cells that are CD44high or CD44low. C, The number of CD4 and CD8 T cells per spleen. FACS plots in A and B are from representative mice in each group, and data in C are the mean ± SD of three to five mice per group. Similar results were observed in two independent experiments.

**FIGURE 2.** Proliferation of Ag-specific T cells is accompanied by depletion of nonresponding T cells during the early stage of LM infection. A, CFSE-labeled gp33-specific P14 T cells (Thy1.2) were adoptively transferred into congenic Thy1.1 C57BL/6 mice. Recipient mice (three to five per group) were infected with wtLM or rLM-gp33. On day 3 postinfection, transferred P14 cells and host CD8 T cells in the spleen were identified by CD8⁺/Thy1.2⁻ and CD8⁺/Thy1.2⁺ staining, respectively (upper panel; numbers indicate the percentages of total splenocytes). Proliferation and activation of transferred P14 cells were visualized by loss of CFSE and down-regulation of CD62L (lower panel; gated on Thy1.2⁻/CD8⁺ cells). Absolute numbers of transferred P14 cells in spleens were determined (bar graph). Similar results were obtained in five separate experiments. B, Equal numbers of CFSE-labeled, MACS-enriched OT-I cells (Thy1.1⁺/Vβ5⁺) and P14 cells (Thy1.1⁻/Vβ8⁺) were cotransferred into congenic Thy1.2 C57BL/6 mice. Recipient mice (three per group) were infected with rLM-gp33 or rLM-OVA. Proliferation of transferred OT-I and P14 cells in spleens was examined 3 days postinfection (dot plots are gated on Thy1.1⁻ cells, and OT-I cells are distinguished from P14 cells by Vβ5⁻ staining). Total numbers of OT-I and P14 cells in spleens were determined (bar graph). Similar results were obtained in three independent experiments.
Selective depletion of nonspecific T cells during LM infection

To determine whether most of the activated T cells are specific to LM Ags or there is a substantial bystander activation of nonspecific T cells, we employed an adoptive transfer model using P14 TCR transgenic cells that are specific to the H-2D\textsuperscript{b}-restricted gp33–41 epitope of LCMV. Before transfer, P14 cells were labeled with CFSE, a vital fluorescent dye that is equally partitioned into daughter cells, thus allowing visualization of cell division. Recipient mice were infected with a recombinant LM strain (rLM-gp33) that expresses the gp33–41 epitope or, as a control, the parental wild-type strain of LM (wtLM). On day 3 postinfection we examined the proliferation of P14 cells by analyzing CFSE profiles and determined their activation status by staining for CD62L, a surface marker whose expression is down-regulated in activated T cells. As expected, the transferred P14 cells became activated and proliferated rapidly in response to rLM-gp33 infection, while few, if any, P14 cells divided or displayed an activation phenotype in PBS-treated or wtLM-infected mice (Fig. 2A). Surprisingly, however, a substantial loss of P14 cells was noted in the wtLM-infected mice. Transferred P14 cells constituted 0.22% of the total splenocytes, which dropped to only 0.03% in wtLM-injected mice by day 3 postinfection. The total numbers of P14 cells per spleen were significantly lower in wtLM-infected (\(\sim 5 \times 10^3\)) compared with PBS-treated (\(\sim 1 \times 10^5\)) mice. Thus, >90% of transferred P14 cells were depleted in the spleens of wtLM-infected mice. In contrast, the P14 cells expanded >10-fold, to \(2 \times 10^6\) per spleen by day 3 in rLM-gp33 infected mice. These results indicate that gp33-specific P14 cells proliferated rapidly in response to rLM-gp33 infection, but were depleted in the spleen of wtLM-infected mice.

Depletion of gp33-specific P14 cells in wtLM-infected, but not rLM-gp33-infected, mice could be due to 1) genetic alterations in rLM-gp33 that render this strain less virulent and/or unable to cause T cell depletion, or 2) expression of the gp33 epitope in rLM-gp33 that provides antigenic stimulation and rescues P14 cells from depletion. We examined the first possibility by assessing the virulence of rLM-gp33 and its ability to induce depletion of polyclonal T cells. The 50% lethal dose and in vivo growth kinetics were similar between rLM-gp33 and wtLM, and infection with rLM-gp33 and wtLM induced similar levels of depletion of polyclonal T cells in normal mice (data not shown). In the adoptive transfer mice (Fig. 2A), the host CD8 T cells (CD8\(^{+/}\)/Thy1.2\(^{-}\)) decreased from 8.26% in PBS-treated to 3.30 and 3.91% in wtLM- and rLM-gp33 infected mice, respectively. These results demonstrate that genetic alterations in rLM-gp33 did not render this strain less virulent or unable to cause T cell depletion. We then considered the second possibility, that T cell depletion during the early stage of LM infection is limited to nonspecific T cells, while Ag-specific cells are activated to expand rapidly. To investigate this possibility, we transferred a mix of two populations of CD8 T cells with defined specificities: P14 cells specific to the gp33 epitope and OT-I cells specific to OVA. Recipient mice were then infected with isogenic strains of rLM-gp33 or rLM-OVA (16). By day 3 postinfection OVA-specific OT-I cells were depleted, while gp33-specific P14 cells proliferated in mice infected with rLM-gp33 (Fig. 2B). Conversely, OT-I cells proliferated and increased in number, while P14 cells were depleted in rLM-OVA-infected mice. These results provide internal controls within the same mice and clearly demonstrate that nonspecific CD8 T cells in the spleen are depleted while Ag-specific CD8 T cells are activated to expand during LM infection.

It is possible that depletion of Ag-specific cells also occurs but is accompanied or followed by Ag-driven expansion, resulting in...
an increase in total P14 cells despite their depletion. We thus took a quantitative approach to assess possible depletion of P14 cells in rLM-gp33 infected mice. We closely followed the proliferation of transferred, CFSE-labeled P14 cells at different time points after infection (Fig. 3). In wtLM-infected mice, there was no sign of P14 cells dividing before their depletion, which occurred between 36–48 h, resulting in >90% reduction of P14 cells by 48 h (Fig. 3, A and B). In rLM-gp33 infected mice there was no loss of P14 cells before their recruitment into division (0–36 h). By 48 h more P14 cells were present as a result of their proliferation, which gave rise to many daughter cells in successive divisions, as evident by their distinct peaks of CFSE fluorescence (Fig. 3A). To determine whether there was loss of P14 cells during proliferation, we deduced the number of original P14 cells (precursors) required to generate these daughter cells as previously described (19). If some P14 cells were lost during proliferation, the sum of the deduced number of precursors should be fewer than the number of P14 cells in PBS-treated control mice. However, our calculations revealed that the number of deduced P14 precursors in rLM-gp33 infected mice was not significantly different from that in PBS-treated control mice (Fig. 3C). Therefore, there was marginal loss of gp33-specific CD8 T cells in rLM-gp33-infected mice before and during the early phase of their expansion.

**Depletion of nonspecific T cells by apoptosis**

Early T cell depletion in the spleen of LM-infected mice may be explained by two possible mechanisms: altered trafficking or cell death. Previous studies have examined the possibility of altered trafficking as a potential mechanism, but found no supporting evidence (1, 6, 7). Lymphocyte depletion during the early stage of LM infection was observed in many organs (1, 7), and no redistribution of radiolabeled T cells from spleens into other organs was evident following LM infection (4). In light of our new findings, it is possible that Ag-specific T cells might be selectively retained in the spleen to respond to antigenic stimulation, while nonspecific T cells migrate out of spleen into other organs. We examined total T cell numbers and the distribution of Ag-specific and nonspecific T cells in different compartments following LM infection. Consistent with the reported data (4, 7), we found that LM infection resulted in depletion of total T cells not only in the spleen, but also in other compartments, including blood, liver, bone marrow, and lymph nodes (data not shown). In all organs examined, the P14 population expanded while the numbers of OT-I cells decreased following rLM-gp33 infection. Conversely, the OT-I population increased while the number of P14 cells declined in rLM-OVA-infected mice. Thus, depletion of nonspecific cells following LM infection occurs, although to varying degrees, in all compartments examined. Furthermore, we did not observe any increase in total or nonspecific T cells in the blood preceding their depletion, which otherwise would indicate increased trafficking of these cells to other organs, nor did we observe the return of nonspecific T cells by day 7 when the spleen was repopulated (data not shown). Non-specific T cells do not become fully activated during LM infection (Figs. 2 and 5), and the increase in T cell number from days 3–7
is mostly by accumulation of activated (CD44 high) T cells, which make up the majority (>80%) of the T cells by day 7 (Fig. 1). Together these results indicate that the repopulation of T cell compartments is mostly by the proliferation of Ag-specific T cells. Although it is difficult to completely rule out the possibility that some T cells may have trafficked to unusual places that we have not examined, our results together with previous studies by several groups indicate that depletion of nonspecific T cells is unlikely to be due to redistribution of these cells into other organs.

The foci of LM infection are characterized by massive lymphocyte apoptosis, and this in situ cell death has been shown to be responsible for T cell depletion (7). We considered the possibility that apoptotic lymphocytes seen during the early stage of LM infection are activated to display early activation markers (CD69, CD44, CD62L, and CD11a) on CD8 T cells was examined (gated on CD8+ cells). OT-I and P14 cells were cotransferred into C57BL/6 mice, which were then infected with rLM-OVA or rLM-gp33 as described in Fig. 1B. The expression of activation markers (CD69, CD44, CD62L, and CD11a) on OT-I and P14 cells was examined on day 3 after infection. Solid line, OT-I cells; filled gray area, P14 cells.

Partial activation of nonspecific T cells before depletion

Resting and activated lymphocytes differ in their sensitivities to various death signals and have dissimilar requirements for survival (20). We examined the activation phenotypes of nonspecific T cells before their depletion (Fig. 5). Within 24 h following LM infection, most T cells in the spleen up-regulated their surface expression of the early activation marker CD69 (Fig. 5A), and this included both Ag-specific and nonspecific T cells (Fig. 5B). Ag-specific T cells (P14 in rLM-gp33-infected mice and OT-I in rLM-OVA-infected mice) were further activated to divide and display a full spectrum of late activation phenotypes (CD44 high, CD62L low, and CD25 high). On the other hand, nonspecific T cells (P14 cells in rLM-OVA-infected mice and OT-I in rLM-gp33-infected mice) did not divide or display any late activation markers. These results indicate that nonspecific T cells are activated in the early stage of LM infection, but only to a state of partial activation before their depletion. T cells receiving incomplete and inadequate activation signals do not progress through the cell cycle, but instead undergo apoptosis (20). Thus, it is possible that partial activation may lead to apoptosis of nonspecific T cells during the early stage of LM infection. Ag-specific T cells, on the other hand, are rescued from apoptosis by TCR engagement, which stimulates them to become fully activated, divide, and differentiate into effector cells to participate in the control of infection.

Depletion of nonspecific T cells during LCMV infection

IFN-α/IFN-β are strongly induced in many viral and bacterial infections (21, 22) and are known to stimulate naive T cells to a state of partial activation with up-regulation of CD69 surface expression (23, 24). Products of viral and bacterial pathogens, such as poly L-C, LPS, and undermethylated CpG DNA, induce IFN-α/IFN-β-mediated partial activation of naive T cells (23, 24). Thus, we asked whether partial activation and selective depletion of nonspecific T cells also occur in the early stage of other infections. A previous study reported depletion of T cells during LCMV infection, but did not demonstrate whether the depletion is limited to nonspecific T cells (3). We cotransferred P14 and OT-I cells into recipient mice and examined these two populations on day 3 following LCMV infection (Fig. 6). LCMV infection resulted in depletion of OT-I and the host polyclonal CD8 T cell population, but not P14 cells (Fig. 6A). Most T cells, including both P14 and OT-I cells, expressed the early activation marker (CD69), while only P14 cells specific to the LCMV gp33 epitope displayed late activation markers (e.g., CD62L low; Fig. 6B). Confocal microscopy showed that a substantial number of OT-I cells (~40% with ~200 cells counted), but few P14 cells (<2% with ~200 cells counted), were TUNEL-positive in LCMV-infected mice (Fig. 6C). These results demonstrate that LCMV infection also induces partial activation of most T cells, which is followed by selective depletion of nonresponding T cells. Together, our results indicate that selective depletion of nonresponding T cells is probably a general phenomenon and an integral part of the T cell response to many infections.

Discussion

Our results suggest a model in which most T cells, regardless of their specificity, are activated to display early activation markers soon after infection (Fig. 7). This is followed by depletion via apoptosis of the vast majority of peripheral T cells that are not specific to Ags of the infecting agents, while a very small percentage of T cells that recognize the infecting agents are rescued from
apoptosis by antigenic stimulation and are further activated to proliferate and mount a protective immune response. At the end of the immune response when the Ag-specific T cell population contracts, it is likely that the peripheral naive T cell repertoire is then reconstituted by thymic output and/or homeostatic proliferation.

How do most T cells become activated regardless of their specificity soon after infection? This presumably occurs during proinflammatory infections where IFN-α/IFN-β are strongly induced, and IFN-α/IFN-β are known to activate naive T cells to up-regulate early activation markers without division (24, 25). Early activation of T cells by cytokines may produce a state of readiness that allows T cells to respond quickly upon encountering the cognate Ag. Indeed, IFN-α/IFN-β enhance the proliferation of naive T cells when they are stimulated by viable APC with Ag and appropriate costimulatory signals (24, 25). On the other hand, IFN-α/IFN-β have inhibitory effects when T cells do not receive appropriate stimulatory signals (24, 25). This is consistent with our finding that nonspecific T cells are induced to undergo apoptosis during the early stage of infection. Thus, early partial activation of T cells by cytokines may sensitize them to respond to Ags quickly or to die if they are not stimulated by appropriate antigenic and costimulatory signals.

Why are nonspecific T cells selectively depleted before the development of an effective Ag-specific response? At the beginning of any given infection, Ag-specific cells are very few, while nonspecific T cells are numerous in number and diverse in their TCR repertoire, some of which may become autoreactive as a result of their activation. Selective depletion of nonspecific T cells may serve to remove these activated T cells that are irrelevant in combatting the infection. It is also possible that depletion of numerous nonspecific T cells may allow for maximal expansion of the Ag-specific T cell population by providing favorable space with more access to APC, cytokines, and growth factors. Indeed, depletion of nonspecific T cells is followed by massive proliferation of Ag specific T cells (~1000-fold expansion) (26–28). Our results indicate that early activation and subsequent depletion of nonspecific T cells are integral elements of the immune response to proinflammatory infections and may serve an immunological function. Depletion of nonspecific T cells involves both naive and resting memory T cells (data not shown). Thus, attrition of bystander memory
T cells may be part of a more global depletion of nonspecific T cells that occurs following certain heterologous infections.

What is the mechanism for selective depletion of nonspecific T cells? Activated T cells are prone to apoptosis, and their survival depends upon appropriate stimulation of TCR by antigenic peptides and costimulation (20). It is possible that death of nonspecific T cells is a default pathway subsequent to their partial activation by IFN-α/IFN-β. Signaling through the IFN-α/IFN-β receptor is required for initial T cell activation (24) and for depletion of T cells at the early stage of LCMV infection (3). A major pathway of intracellular signaling following IFN-α/IFN-β receptor engagement involves STAT1. However, our preliminary results show that T cells deficient in STAT1 are slightly more sensitive to apoptosis induced during the early stage of LM infection (unpublished observations). Recent studies have shown that IFN-α/IFN-β can inhibit and promote IFN-γ production by T cells by activating STAT1 and STAT4, respectively (21, 22, 29). Thus, IFN-α/IFN-β can signal through STAT4 in addition to STAT1, and it is possible that early T cell activation by IFN-α/IFN-β may involve the STAT4 signaling pathway. The survival and death of these activated T lymphocytes is probably further controlled by pro- and antiapoptotic factors. The expression of the antiapoptotic protein, Bcl-xL, is known to increase the resistance of T cells to Fas-induced apoptosis (30). However, Bcl-xL-transgenic T cells are equally sensitive to apoptosis induced during the early stage of LM infection (J. Jiang and H. Shen, unpublished observations). This result is consistent with previous findings that early T cell depletion during infection is independent of signaling through Fas and TNF receptors (7). We have preliminary results indicating that TNF-related apoptosis-inducing ligand (TRAIL) may be involved. We observed that T cell apoptosis during the early stage of LM infection is reduced in TRAIL knockout mice and by treatment with soluble death receptor 5 that blocks binding of TRAIL to its receptors (J. Jiang and H. Shen, unpublished observations). Elucidation of these underlying mechanisms may allow us to develop new avenues for modulation of the immune response.

In conclusion, our results uncover several important early events in the T cell response to infection and provide a foundation for understanding molecular and cellular factors involved in the initiation of effective T cell responses to infection.