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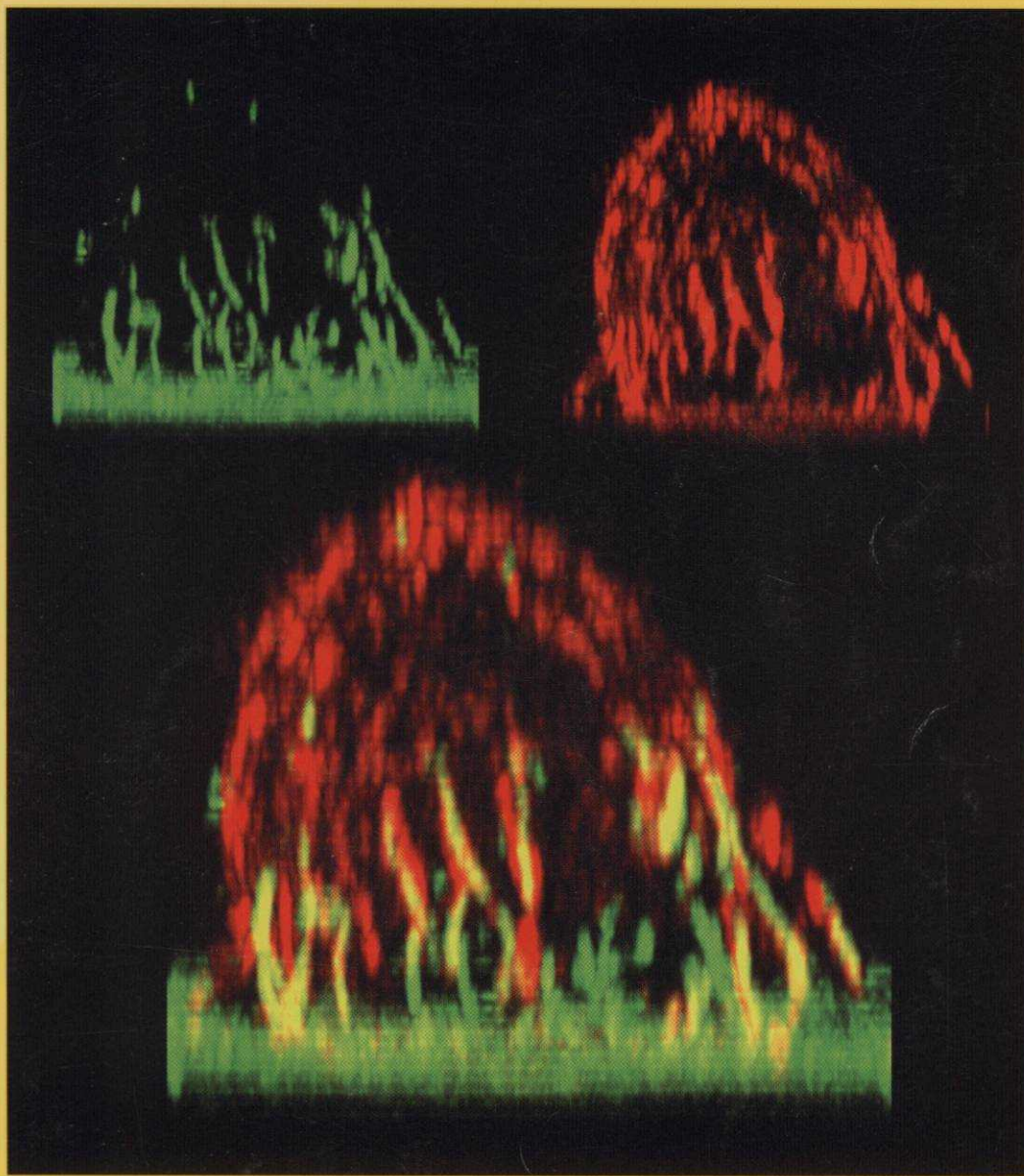
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On the cover: Side-view projection of leukocyte-endothelial interaction reconstructed from serial section confocal microscopy. Endothelial ICAM-1 (green) rapidly relocalized to newly formed microvilli-like membrane projections in response to binding LFA-1 (red) on leukocytes. These ICAM-1-enriched projections encircled the leukocytes extending up their sides, and clustered LFA-1 underneath into linear tracks. Colocalized ICAM-1 and LFA-1 appear yellow. Carman et al. Endothelial cells proactively form microvilli-like membrane projections upon intercellular adhesion molecule 1 engagement of leukocyte LFA-1. *J. Immunol.* 171:6135.

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IL-2 Is Not Required for the Initiation of CD8 T Cell Cycling but Sustains Expansion¹

Warren N. D'Souza and Leo Lefrançois²

Based primarily on in vitro data, IL-2 is believed to be the key cytokine for initiation of the cell cycle of activated T cells. However, the role of IL-2 remains unresolved for T cell responses in vivo. We examined whether the absence of IL-2-mediated signaling in CD8 T cells affected initiation of proliferation. Our results conclusively demonstrated that initial division of Ag-specific CD8 T cells following priming was IL-2 independent, regardless of the context in which Ag was presented. In contrast, the latter stage of the proliferative phase was IL-2-dependent, particularly in nonlymphoid tissues. Thus, activated CD8 T cells initially undergo IL-2-independent proliferation, but reach a critical juncture where the requirement for IL-2 as a growth factor gains prominence. *The Journal of Immunology*, 2003, 171: 5727–5735.

Upon stimulation with cognate Ag, naive T cells are activated and typically undergo a response that consists of three phases, namely, an expansion phase, a contraction phase, and a memory phase. An augmented expansion phase appears to lead to an increase in the size of the memory pool generated and, as a result, much research is aimed at potentiating the priming of T cells. Efficient priming is thought to be achieved by optimizing costimulation and providing the appropriate cytokine milieu to naive T cells during activation. The expansion phase is believed to be driven primarily by cytokines and IL-2 is one such prototypical cytokine that was originally identified based on its potent growth-promoting ability for T cells in vitro (1). IL-2 and its high-affinity receptor (IL-2R $\alpha\beta\gamma$) were shown to be expressed rapidly following activation in vitro and the absence of either IL-2 or IL-2R was thought to result in a "failure" of the immune response (2, 3). Also, one of the most important costimulatory molecules, CD28, is believed to function for the most part by increasing the production of IL-2 by T cells (4–7). Consequently, anergy or unresponsiveness that results from TCR occupancy in the absence of costimulatory signals is assumed to be due to the inability of the cells to produce IL-2 and the addition of exogenous IL-2 is capable of reversing the unresponsive phenotype (8, 9). It is well documented that the generation of certain CD8 T cell responses is dependent upon the presence of CD4 T cell help and one of the postulated mechanisms of help is the provision of IL-2 to the CD8 T cells (10). Also, recent data implicate IL-2 production by dendritic cells as an essential factor for the initiation of both CD4 and CD8 T cell responses (11). In addition, it is now apparent that regulatory T cells are key players in the immune system and these T_R cells are thought to suppress T cell proliferation by inhibiting IL-2 production by responding T cells (12). Thus, current dogma avers that the expression of IL-2 and the high-affinity IL-2R by

activated CD8 T cells is indispensable for the cell cycle progression of recently activated T cells.

In light of the central role that IL-2 is thought to play in governing T cell responses, it is quite intriguing that not only is there a lack of definitive data in support of IL-2 being the quintessential T cell growth factor in vivo, but evidence also exists opposing this notion. For example, mice with targeted disruptions of the IL-2 or IL-2R genes are not immunodeficient (as would have been predicted), but in fact display a severe lymphoproliferative disorder (13–16). Also, since the induction of anergy is thought to be due to a failure of T cells to produce IL-2, the demonstration that proliferation usually precedes anergy in vivo (17–19) argues that the production of IL-2 is not required for cell cycle initiation. Experimentation with mice that lack IL-2 or IL-2R components has yielded variable results, with some studies indicating no significant requirement for IL-2 in the expansion of CD8 T cells in vivo (20–22) while other studies point to an essential role for IL-2 (23, 24). We also observed a minimal requirement for IL-2 in mounting an antiviral CD8 T cell response within secondary lymphoid tissue (25).

Thus, although it is widely accepted that IL-2 plays a pivotal role in the early events following CD8 T cell activation, it is apparent that the requirement for IL-2 in the generation of CD8 T cell responses in vivo remains equivocal. More importantly, it is unclear whether the requirement for IL-2 observed in some model systems is due to an alteration in the entry and kinetics of CD8 T cell cycling (impaired priming) or due to lack of sustained proliferation. We sought to examine this issue directly by visualizing early cell division subsequent to activation in vivo and determining what effect the absence of IL-2 or IL-2R signaling had on the initiation and rate of CD8 T cell cycling. To this end, we tracked Ag-specific CD8 T cells throughout the proliferative phase in response to OVA when expressed either as a viral Ag, soluble Ag, tumor Ag, or self-Ag. Our results provided compelling evidence that the latter half of the proliferative phase was dependent on IL-2, but the early division of Ag-specific CD8 T cells subsequent to priming with cognate Ag was, even in the absence of overt inflammation, IL-2 independent.

Materials and Methods

Mice

C57BL/6J, C57BL/6-IL-2^{-/-}, and C57BL/6-CD25^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-Ly5.2 mice were obtained from Charles River Breeding Laboratories (Wilmington,

Department of Medicine, Division of Immunology, University of Connecticut Health Center, Farmington, CT 06030

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² Address correspondence and reprint requests to Dr. Leo Lefrançois, Division of Immunology, University of Connecticut Health Center, MC1319, Room L3072, 263 Farmington Avenue, Farmington, CT 06030-1319. E-mail address: llefranc@neuron.uconn.edu

MA) through the National Cancer Institute. The OT-I mouse line (26) was generously provided by W. R. Heath (Walter and Eliza Hall Institute, Parkville, Australia) and F. Carbone (Monash Medical School, Prahran, Victoria, Australia). We also generated OT-I-CD25^{-/-}RAG^{-/-} mice and S. Schoenberger (La Jolla Institute of Immunology, La Jolla, CA) kindly provided us with OT-I-IL-2^{-/-}RAG^{-/-} mice that were used in some experiments. Unless otherwise indicated, the cells used in the experiments were obtained from RAG^{+/+} OT-I mice (either IL-2^{-/-}, CD25^{-/-}, or normal). The presence of the OT-I transgene and the RAG mutation was detected by assessing the frequency of V α 2⁺V β 5⁺CD8⁺ cells and B220⁺ cells, respectively, in PBL. 232-4 mice expressing cytoplasmic OVA under control of the intestinal fatty acid-binding protein promoter have been previously described (27). OT-I mice were mated to IL-2^{-/-} mice. Offspring were screened for the IL-2 mutation by PCR. OT-I-IL-2^{-/-} mice were intercrossed to obtain OT-I-IL-2^{-/-} animals and OT-I-CD25^{-/-} animals were generated in a similar manner.

Isolation of lymphocyte populations

Intraepithelial lymphocytes (IEL)³ and lamina propria cells from the small intestine were isolated as previously described (28, 29). Spleens and lymph nodes (LN) were removed and single-cell suspensions were prepared using a tissue homogenizer. The resulting preparation was filtered through Nitex nylon mesh (Tetko, Kansas City, MO) and the filtrate was centrifuged to pellet the cells. To obtain lymphocytes from lungs, anesthetized mice were perfused with PBS containing 75 U/ml heparin until the tissue was cleared of blood, and the organs were removed and cells were isolated as previously described (30).

Flow cytometric analysis

Lymphocytes were resuspended in PBS, 0.2% BSA, and 0.1% NaN₃ (PBS, BSA, NaN₃) at a concentration of 1–10 × 10⁶ cells/ml followed by incubation of 100 μ l of cells at 4°C for 20 min with 100 μ l of properly diluted mAb. mAbs specific for the following Ags and coupled to the indicated fluorochromes were used: V β 5-FITC, V α 2-PE, CD25-PE, CD44-PE, CD8-PerCP, CD8-allophycocyanin, and CD4-PE (all from BD Pharmingen, San Diego, CA); CD8 α -(3.168)-FITC or biotin (31); and Ly5.1- or Ly5.2-FITC or -Cy5 (32). Streptavidin-FITC-Cy7 (Caltag Laboratories, Burlingame, CA) was used to detect biotinylated mAb. Relative fluorescence intensities were measured with a FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed using WinMDI software (J. Trotter, The Scripps Clinic, La Jolla, CA).

Adoptive transfer of cells, Ag challenge, and IL-2 administration

For adoptive transfer, each cell type was injected i.v. into B6 mice either separately or as a mixture containing an equal number of OT-I T cells and mutant OT-I cells from LN. In some experiments, the donor cells were labeled with the viable dye CFSE (0.01 mM; Molecular Probes, Eugene, OR) before transfer. Twenty-four hours later, mice were either left unchallenged or were infected i.v. with 1 × 10⁶ PFU of recombinant vesicular stomatitis virus (VSV)-OVA (33) or injected i.p. with soluble OVA (5 mg or 0.5 mg) or intradermally (i.d.) with 5 × 10⁶ live EL4 or E.G7 (34) cells. For the studies with the 232-4 animals, cells were transferred i.v. into these mice or B6 mice as controls. At various time points postinfection, cells were isolated for analysis. To determine the effect of exogenous IL-2, each mouse was injected i.p. with 5000 U of recombinant human IL-2 (generously provided by the National Cancer Institute Biological Resources Branch) at indicated time points.

Ex vivo intracellular IL-2 detection

Isolated cells were stained for cell surface Ags, fixed, and then permeabilized to detect intracellular cytokine according to the manufacturer's instructions (BD Pharmingen). The cells were stained immediately after isolation without in vitro culture. To determine whether the cells were capable of producing IL-2 after in vitro restimulation, cells were first cultured in the presence of GolgiStop (BD Pharmingen) for 5 h, with or without 1 μ g/ml SIINFEKL peptide. Abs used were anti-IL-2-PE or PE-conjugated, isotype-matched Ig as control.

Results

IL-2 and IL-2R α are expressed before cell division on activated antiviral CD8 T cells in vivo

T cells are known to express IL-2 and the high-affinity IL-2R (which includes IL-2R α) immediately following activation in vitro. However, since recent in vivo data suggest cell division may precede the expression of IL-2 and IL-2R α (35), we analyzed the kinetics of expression of IL-2 and the IL-2R α subunit (CD25) by CD8 T cells following a viral infection. We used an adoptive transfer system in which trackable OVA-specific Ly5.1 OT-I CD8 T cells (26) were CFSE-labeled and transferred to B6 (Ly5.2⁺) mice that were either left unimmunized or immunized a day later with a recombinant VSV- encoding OVA (VSV-OVA). Mice were sacrificed at early time points postinfection and splenic donor cells were analyzed for the expression of CFSE, cell surface CD25, and intracellular IL-2 directly ex vivo (without restimulation in vitro). At 12 h postinfection, OT-I cells had not yet divided but were already activated, as indicated by an increase in forward light scatter (Fig. 1B, inset). All of the OT-I cells also expressed high levels of CD25 (Fig. 1B, top panels) and approximately one-fifth of them produced IL-2 directly ex vivo (Fig. 1A). By as early as 58 h postinfection, we were unable to detect any ex vivo IL-2 production, but the cells were capable of producing the cytokine after a 5-h in vitro restimulation with peptide (data not shown). At 58 h, CD25 was still expressed at high levels on the dividing cells (Fig.

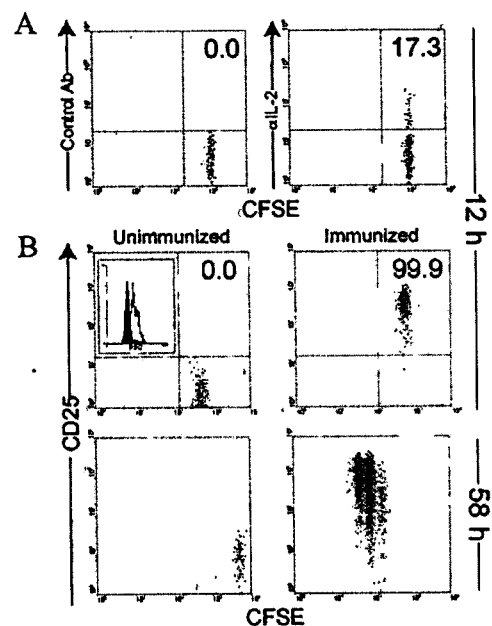


FIGURE 1. IL-2 and IL-2R α are immediately expressed upon activation of antiviral CD8 T cells in vivo. LN cells isolated from OT-I-RAG^{-/-} (Ly5.1/5.2⁺) mice were labeled with CFSE and adoptively transferred i.v. (1 × 10⁶ cells) into naive (Ly5.2⁺) B6 recipients. Mice were left unimmunized as controls or were immunized by i.v. injection with 1 × 10⁶ PFU VSV-OVA 1 day posttransfer. **A**, Splenocytes were isolated at 12 h postinfection and stained for intracellular IL-2 immediately following isolation, without in vitro restimulation. Dot plots are gated on donor cells and numbers indicated are the percentage of donor cells staining positive with the anti-IL-2 Ab or isotype control. **B**, Splenocytes were isolated at 12 h (top panels) and 58 h (bottom panels) postinfection and analyzed for the expression of CD25 on the donor cells. **B, inset**, Forward scatter histograms of donor cells within unimmunized (filled histogram) or immunized (open histogram) mice at 12 h postinfection. All plots shown are gated on donor cells. This experiment was performed twice with two mice per group at each time point.

³ Abbreviations used in this paper: IEL, intraepithelial lymphocyte; LN, lymph node; VSV, vesicular stomatitis virus; i.d., intradermal; PLN, peripheral LN; WT, wild type; γ_c , common γ -chain.

1B, bottom panels) and although the proliferative peak of the OT-I response to VSV-OVA occurs at around day 4 (96 h) postinfection, the expression of CD25 on the responding OT-I cells had dropped to levels slightly above background by 81 h postinfection (25). Thus, in our hands, antiviral CD8 T cells expressed IL-2 and CD25 immediately following activation and before cell division *in vivo* but the expression of both of these molecules was dramatically decreased before the peak of the proliferative phase.

Antiviral CD8 T cells undergo IL-2-independent cell cycling but are dependent on IL-2 for sustained expansion

The kinetics of expression of IL-2 and IL-2R α receptor presented a strong teleological argument in support of the notion that IL-2 functions immediately following activation. To investigate the requirement for IL-2 in the initiation of cell cycling *in vivo*, we introduced the OT-I TCR transgenes (V β 5 and V α 2) into IL-2-deficient (OT-I-IL2 $^{-/-}$) or IL-2R α -deficient (OT-I-CD25 $^{-/-}$) mice. In mice, IL-2-mediated signaling requires the presence of the high-affinity IL-2R (36, 37). IL-2-deficient and CD25-deficient mice share the same phenotype (13–15), confirming that under physiological conditions, the effects of IL-2 are conducted via signaling through the high-affinity IL-2R. Thus, *in vivo*, OT-I-IL2 $^{-/-}$ cells would be incapable of responding to autocrine IL-2, and OT-I-CD25 $^{-/-}$ cells would be unable to receive any IL-2-mediated signals (autocrine or paracrine). It should be noted that introduction of TCR transgenes into IL-2 $^{-/-}$ or CD25 $^{-/-}$ mice greatly delays development of lymphoproliferative disease and we used only young, healthy animals. We transferred a mixture of equal numbers of CFSE-labeled OT-I-RAG $^{-/-}$ and OT-I-CD25 $^{-/-}$ -RAG $^{-/-}$ cells into naive B6 recipients that were immunized a day later with VSV-OVA. All populations could be distinguished by their Ly5 phenotype. Mice were sacrificed at various time points following priming and the response of the transferred cells was analyzed in various lymphoid as well as nonlymphoid tissues. At an early time point postinfection (57 h), there was no obvious difference in the CFSE profile between the OT-I and the OT-I-CD25 $^{-/-}$ cells within the LN, spleen, and lung, thus revealing that the initiation of cell division proceeded normally in the absence of IL-2-mediated signaling (Fig. 2, top panels). The ratio of the OT-I:OT-I-CD25 $^{-/-}$ cells remained nearly equal in the LN and was ~40:60 in the spleen and 32:68 in the lungs. At 104 h postinfection (Fig. 2, bottom panels), it was not possible to make any conclusions about the division history of the cells since they had divided more than eight to nine times and diluted the dye to background levels. However, when the ratio of the two cell types at the peak of the response was analyzed, the ratio in the LN remained at ~50:50, whereas the ratio within the lungs was 71:29. The spleen consistently displayed a ratio (62:38) that was intermediate to that observed within the LN and nonlymphoid tissues. Thus, at 57 h postinfection, the number of OT-I-CD25 $^{-/-}$ cells in the lung was >2-fold higher than the number of wild-type (WT) OT-I cells. Yet, at 104 h the number of OT-I-CD25 $^{-/-}$ cells was only ~40% that of WT OT-I cells, supporting our hypothesis that priming of CD8 T cells was unaffected in the absence of IL-2 but that their continued expansion, particularly outside of LN, was dependent on IL-2-mediated signaling. The presence of increased numbers of OT-I-CD25 $^{-/-}$ cells in the lung at 57 h was in line with our previous observations that autocrine IL-2 had a down-regulatory role during the expansion phase in nonlymphoid tissues (25).

CD8 T cells are capable of IL-2-independent cell cycle initiation even in the absence of overt inflammation

The simplest explanation for the IL-2 independence displayed by antiviral CD8 T cells early during the response was that the myriad

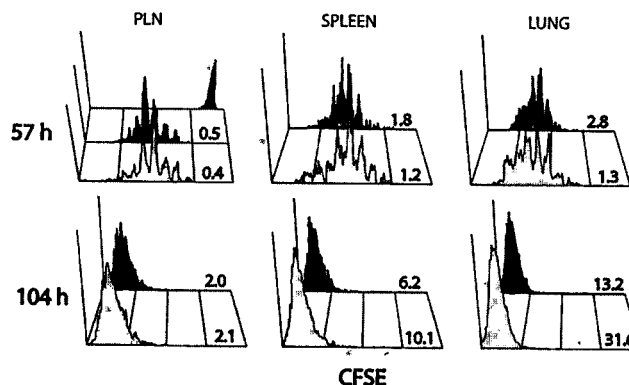


FIGURE 2. Antiviral CD8 T cells undergo IL-2-independent cell cycling but are dependent on IL-2 for sustained expansion. LN cells isolated from OT-I-RAG $^{-/-}$ (Ly5.1/5.2 $^{+}$) or OT-I-CD25 $^{-/-}$ -RAG $^{-/-}$ (Ly5.1 $^{+}$) mice were labeled with CFSE and adoptively transferred *i.v.* as a mixture (1×10^6 cells each) into naive (Ly5.2 $^{+}$) B6 recipients. The presence of a 1:1 ratio of the two cell types was confirmed before transfer. Mice were immunized by *i.v.* injection with 1×10^6 PFU VSV-OVA 1 day post-transfer. Fifty-seven hours (top panels) and 104 h (bottom panels) later, cells from the PLN, spleen, and lung were isolated and analyzed for the presence of donor cells by fluorescence flow cytometry. Histograms are gated on the indicated donor cells (gray histograms, OT-I-RAG $^{-/-}$ cells; black histograms, OT-I-CD25 $^{-/-}$ -RAG $^{-/-}$ cells) isolated from tissues of the same immunized animal. As a control, cells were also isolated from the LN of mice that were left unimmunized (PLN, 57 h, rear histogram). Numbers indicated are the percentage of CD8 $^{+}$ lymphocytes. Four mice were analyzed at 57 h and two mice were tested at 104 h. Similar results were obtained in a second experiment using OT-I-CD25 $^{-/-}$ and OT-I cells.

of inflammatory mediators generated during infection overcame the requirement for IL-2 as a growth factor. Hence, we questioned whether the initial proliferation of CD8 T cells in response to a nominal Ag might be IL-2 dependent. To this end, we conducted adoptive transfers of CFSE-labeled mixtures of OT-I and OT-I-CD25 $^{-/-}$ cells and challenged the recipient mice with soluble OVA. Cells were then isolated from various tissues at different time points during the proliferative phase of the response and analyzed by flow cytometry. The starting ratio of OT-I:OT-I-CD25 $^{-/-}$ cells in the mixture (data not shown) and within the mice that received the mixture of donor cells but were left unchallenged was ~40:60 in favor of the OT-I-CD25 $^{-/-}$ cells (Fig. 3). At 2 days postchallenge, the ratio of the OT-I cells:OT-I-CD25 $^{-/-}$ cells in the peripheral LN (PLN) and spleen remained unchanged (at ~40:60), and their CFSE profiles indicated identical division patterns even in the absence of IL-2-mediated signaling. On day 3, the ratio of OT-I:OT-I-CD25 $^{-/-}$ cells in the PLN and the spleen did not change significantly and their CFSE profiles were similar. By day 4, however, the ratio of OT-I cells:OT-I-CD25 $^{-/-}$ cells in the PLN was 53:47 and the ratio in the spleen stood at 58:42, in favor of the WT OT-I cells in both cases. Also, it was apparent from the CFSE profiles that by day 4 the OT-I-CD25 $^{-/-}$ cells in the PLN and spleen had begun to lag behind the WT OT-I cells and this reduced proliferation was the likely cause of the alteration in ratios (Fig. 3, A and B, bottom panels). This effect was even more dramatic in the lungs where the ~30:70 ratio of OT-I:OT-I-CD25 $^{-/-}$ cells on day 2 was now inverted to 70:30 at day 4 (Fig. 3C, top and bottom panels). Thus, even in the absence of an inflammatory milieu, IL-2 was dispensable for the initial expansion of Ag-specific CD8 T cells, whereas continued OT-I cell expansion was IL-2 dependent. Moreover, the role of IL-2 in late expansion was most evident in nonlymphoid sites. Hence, between days 3 and 4, while WT OT-I cells in spleen and lung continued expanding, the OT-I-CD25 $^{-/-}$

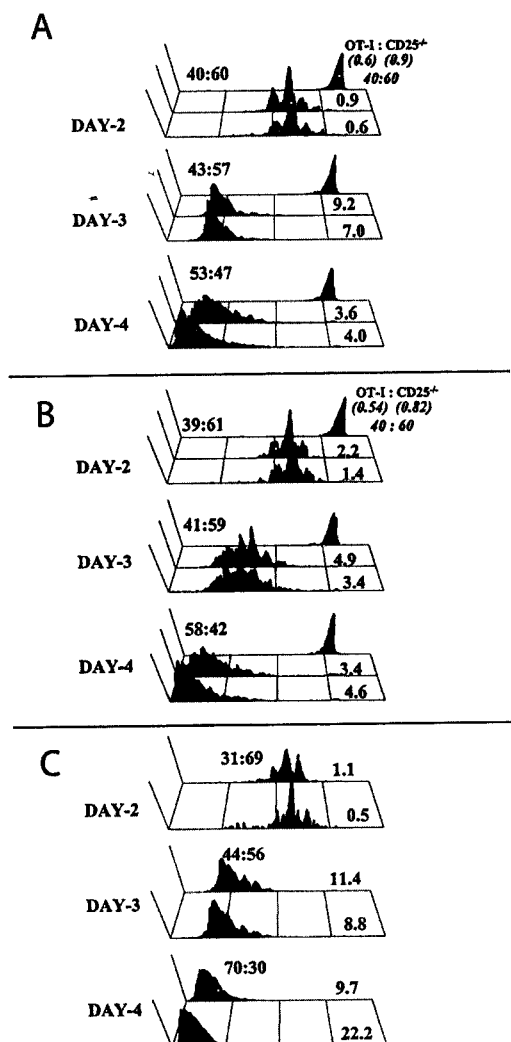


FIGURE 3. CD8 T cells are capable of IL-2-independent cell cycle initiation in the absence of overt inflammation. LN cells isolated from OT-I (Ly5.1/5.2⁺) or OT-I-CD25^{-/-} (Ly5.1⁺) mice were labeled with CFSE and adoptively transferred i.v. (1×10^6 cells each) as a mixture into naive (Ly5.2⁺) B6 recipients. Mice were immunized by i.p. injection with 5 mg soluble OVA 1 day posttransfer. At the later indicated times, cells from the PLN (A), spleen (B), and lung (C) were isolated and analyzed for the presence of donor cells by fluorescence flow cytometry. Histograms are gated on the indicated donor cells (gray front histograms, OT-I cells; black middle histograms, OT-I-CD25^{-/-} cells) isolated from tissues of the same animal that had been challenged. As a control, cells were also isolated from the LN of mice that were left unchallenged (rear histograms in B and C). Numbers indicated are the percentage of CD8⁺ lymphocytes and the ratios indicated are the ratios of OT-I cells:OT-I-CD25^{-/-} cells in the respective plots. This experiment was repeated twice with two or three mice per group at each time point.

population had already begun to contract (compare percentages of CD8s at the different time points). Similar results were obtained using a 10-fold lower concentration of OVA (data not shown).

The optimal expansion of autoreactive CD8 T cells, but not their initial proliferation, is dependent on IL-2

As mentioned earlier, mice that lack IL-2 or IL-2R components exhibit an uncontrolled expansion of autoreactive T cells, suggesting that self-reactive T cells may be capable of IL-2-independent expansion. However, the specificity of such cells is unknown,

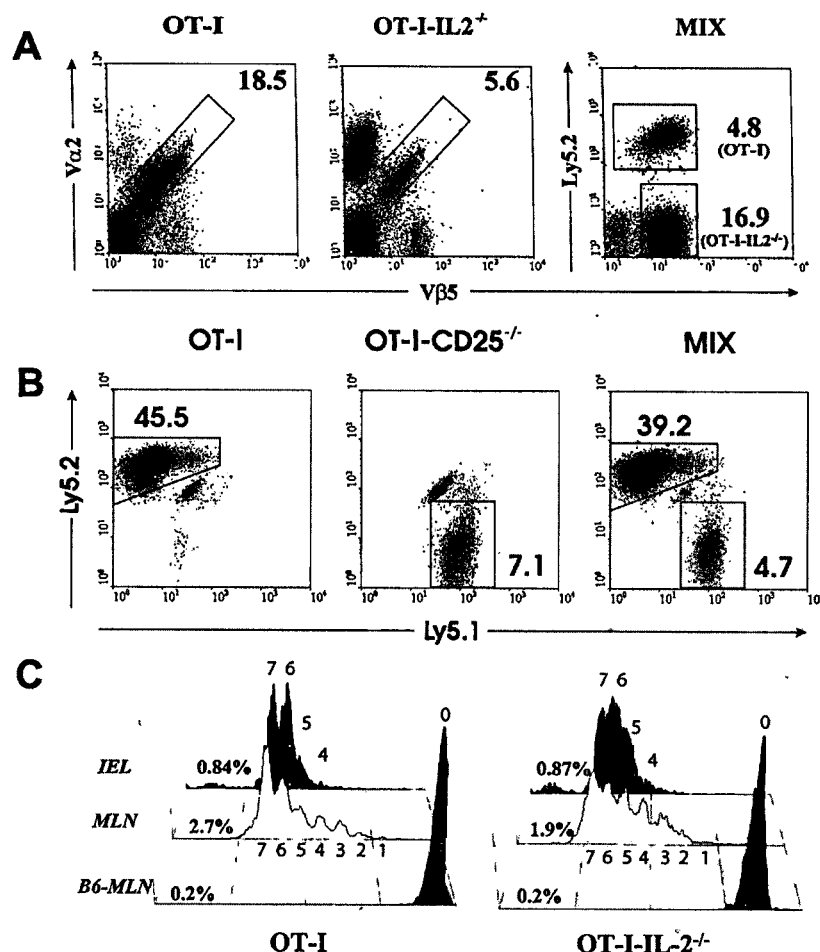
making analysis difficult. To track the response of autoreactive cells of known specificity and to determine the requirements for IL-2 in their expansion, we conducted adoptive transfers of OT-I, OT-I-IL2^{-/-}, or OT-I-CD25^{-/-} cells into the 232-4 line of transgenic mice. These mice express a cytoplasmic form of OVA specifically within intestinal epithelial cells and the transfer of OT-I cells into these animals attempts to mimic the scenario occurring when naive autoreactive T cells in the periphery encounter self-Ag in the absence of inflammation. Consistent with our earlier demonstrations that activated and not naive cells are capable of trafficking to the IEL compartment of the small intestine (33, 38), transfer of either OT-I, OT-I-IL2^{-/-}, or OT-I-CD25^{-/-} cells into WT B6 mice yielded barely detectable numbers of donor cells within this tissue (data not shown). Transfer of OT-I cells into 232-4 mice, however, leads to activation in the mesenteric LN and Peyer's patches and is followed by migration of these cells to the epithelium, which is the site of Ag expression (27). Thus, by day 6 posttransfer of OT-I cells into 232-4 mice, an impressive accumulation of donor cells within the epithelium had occurred (Fig. 4A, left panel). By comparison, the expansion of transferred OT-I-IL2^{-/-} cells in the IEL compartment was 4–5-fold lower than that of OT-I cells (Fig. 4A, left and middle panels). The results demonstrated that autoreactive CD8 T cells were capable of IL-2-independent expansion, although optimal expansion relied on the presence of IL-2. Interestingly, when a mixture of both cell types was transferred into 232-4 mice, the level of expansion of the OT-I-IL2^{-/-} cells in the IEL compartment was 2- to 4-fold higher than that of the OT-I cells (Fig. 4A, right panel). Again, this finding was in keeping with our previous results from analysis of an antiviral CD8 T cell response (25). Thus, when transferred as a mixture, IL-2 derived from WT OT-I cells was capable of mediating optimal expansion of OT-I-IL2^{-/-} cells while at the same time autocrine IL-2 limited the magnitude of the response within the IEL compartment.

To test the overall requirement for IL-2 in this system, we repeated the above experiments using OT-I-CD25^{-/-} cells. OT-I-CD25^{-/-} cells also underwent IL-2-independent expansion, although again it was markedly (6- to 8-fold) lower than that achieved by WT OT-I cells (Fig. 4B, left and middle panels). Likewise, even when transferred along with WT OT-I cells, OT-I-CD25^{-/-} cells proliferated to the same extent as when transferred separately because they were unable to receive IL-2 from the OT-I cells (Fig. 4B, right panel). Therefore, IL-2 was required for optimal overall expansion observed in the epithelium, but these studies did not distinguish early from late proliferation. To do so, we labeled OT-I-RAG^{-/-} and OT-I-IL2^{-/-}-RAG^{-/-} cells with CFSE and transferred them separately into 232-4 mice. We have shown that these mice are tolerant to OVA and hence upon adoptive transfer, the donor cells are the only cells that would be expected to produce IL-2 subsequent to activation. At day 3 post-transfer, we observed that the initial division of the cells in the mesenteric LN and the IEL compartment proceeded normally in the absence of IL-2 (Fig. 4C). Thus, autoreactive cells were also capable of initiating cell cycling in an IL-2-independent manner and only required IL-2 at the later stages of the expansion phase.

Tumor-specific CD8 T cells initiate cycling in an IL-2-independent manner

So far, we have been unable to demonstrate any role for IL-2 in initiating CD8 T cell proliferation regardless of the context in which Ag was presented. However, IL-2 has been implicated in the early and late expansion of OT-I cells responding to OVA when present as a tumor Ag (39). That study provided ample evidence that IL-2 was involved in supporting a prolonged T cell response,

FIGURE 4. The optimal expansion of autoreactive CD8 T cells, but not their initial proliferation is dependent on IL-2. **A**, LN cells isolated from OT-I or OT-I-IL-2^{-/-} mice were adoptively transferred i.v., either separately or as a mixture (MIX; 1×10^6 cells each whether singly or in a mixture), into 232-4 mice. IEL were isolated and analyzed by flow cytometry 6 days posttransfer. The OT-I mice used were Ly5.2⁺, while the 232-4 mice and the OT-I-IL-2^{-/-} mice were Ly5.1⁺ and hence OT-I-IL-2^{-/-} cells were identified as those that were V α 2⁺V β 5⁺CD8⁺ and Ly5.2⁻. Data shown in the transfer of separate cell populations was derived from gating on CD8⁺ cells and represents the entire V α 2⁺V β 5⁺CD8⁺ population which will include transferred and endogenous cells (the latter of which were always <0.4% of total lymphocytes in that compartment). Data shown in the transfer of mixtures is derived from gating on V α 2⁺CD8⁺ cells. The number indicated is the percentage of total lymphocytes. **B**, OT-I (Ly5.2⁺) and OT-I-CD25^{-/-} (Ly5.1⁺) LN cells were transferred either separately or as a mixture (MIX) into 232-4 (Ly5.1/5.2⁺) mice. IEL were isolated and analyzed by flow cytometry at 6 days posttransfer. Data shown are derived from gating on V α 2⁺CD8⁺ cells and numbers indicate percentage of total lymphocytes. **C**, OT-I-RAG^{-/-} (Ly5.2⁺) and OT-I-IL-2^{-/-}-RAG^{-/-} (Ly5.1⁺) LN cells were labeled with CFSE and transferred separately into 232-4 (Ly5.1/5.2⁺) mice or into B6 mice as controls. Mesenteric LN and IEL were isolated and analyzed by flow cytometry 3 days posttransfer. Histograms are gated on the donor cells and the percentages indicated are percentages of total lymphocytes. Also indicated on the plots is the number of cell divisions. Each experiment was repeated twice with two mice per group.



but early proliferation was not examined in this setting. To determine whether CD8 T cell responses directed against tumor-derived Ags relied on IL-2 for cell cycle initiation, we analyzed the effect of the absence of IL-2 on proliferation of OT-I cells within the draining LN in response to E.G7 tumor cells (EL4 thymoma transfected with the OVA gene). We conducted adoptive transfers of a mixture of CFSE-labeled OT-I cells (Ly5.1/5.2) and OT-I-CD25^{-/-} cells (Ly5.1) into naive B6 recipients (Ly5.2) which were then challenged i.d. 1 day later with E.G7 cells or the parental EL4 cells. The mice were sacrificed at day 7 postchallenge and the presence of donor cells in the LN (draining and nondraining) was examined by flow cytometry. In the experiment shown, the starting ratio of the two cell types was ~1:2 in favor of the OT-I-CD25^{-/-} cells. OT-I cells did not proliferate within the draining LN of control mice that were challenged with the parental tumor cell line (Fig. 5, Mixture + EL4) or within the nondraining LN of E.G7-challenged mice (data not shown). However, OT-I cells were activated and proliferated in the draining LN of mice that were challenged with the E.G7 cells (Fig. 5). The absence of IL-2-mediated signaling did not affect the proliferation of the cells within the draining LN since the CFSE profiles remained similar and the ratio of the two cell types was unchanged. Identical results were obtained when we transferred OT-I and OT-I-IL2^{-/-} cells (data not shown). Thus, IL-2 was not required during the initial activation and proliferation of tumor-reactive CD8 T cells within the local LN.

The administration of exogenous IL-2 does not alter early cell cycling but sustains the proliferative phase

We demonstrated that ex vivo IL-2 production and expression of CD25 were transient and occurred early in the response (Fig. 1).

Yet, we observed a more stringent requirement for IL-2 late in the proliferative phase. This suggested that the early burst of IL-2 production was dispensable for proliferation and that IL-2 produced thereafter drove the latter half of the expansion phase. Alternatively, it was possible that IL-2 was required during the initial priming, but its effects were only observed at a later stage. To determine whether sustained proliferation was dependent upon the presence of IL-2 during the initial activation, we conducted experiments using exogenous IL-2. Mice received OT-I cells and were then immunized with VSV-OVA. Some mice were also administered recombinant human IL-2 either during the priming phase or during the latter stages of the expansion phase and the kinetics of the OT-I response were followed. Groups of mice were treated daily with a relatively low dose of IL-2 (5000 IU/mouse) or PBS as a control either from days 0 to 2 (priming phase) or from day 3 onward (toward the end of the expansion phase). The administration of IL-2 during the priming phase had no effect on the kinetics of the OT-I response (Fig. 6A). In stark contrast, IL-2 administration during the latter half of the proliferative phase prolonged the expansion of the OT-I cells and the peak of the response was increased and delayed, occurring on day 5 instead of day 4 (Fig. 6A). OT-I cells from mice receiving IL-2 from days 0 to 6 showed response kinetics similar to those receiving IL-2 from days 3 to 6 (data not shown). These results argued that the sustenance of proliferation required the concurrent presence of IL-2. Although the early administration of IL-2 did not result in differential kinetics of the OT-I response, to determine its effects on the entry or rate of cell cycling, CFSE-labeled OT-I cells were transferred and their division profile was determined after IL-2 administration from days 0 to 2. The division profiles and numbers of proliferating

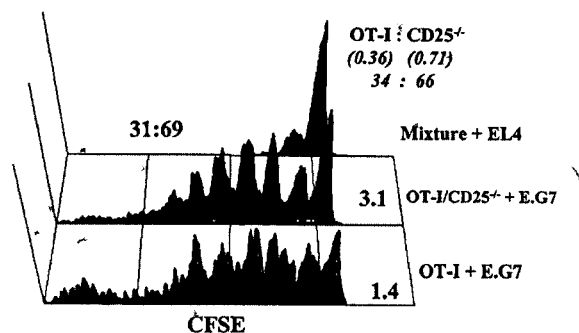


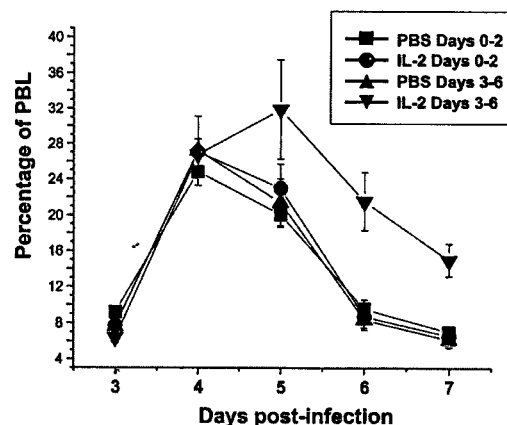
FIGURE 5. Tumor-specific CD8 T cells undergo IL-2-independent proliferation. CFSE-labeled mixtures of OT-I (Ly5.1/5.2⁺) and OT-I-CD25^{-/-} (Ly5.1⁺) cells were adoptively transferred into naive Ly5.2⁺ B6 mice. One day posttransfer, mice were challenged i.d. on the lower right flank with 5×10^6 E.G7 tumor cells or parental EL4 cells. Seven days later, cells from the draining or nondraining inguinal LN were isolated and analyzed for the presence of the donor cell types. Histograms are gated on the indicated donor cells isolated from the draining LN of mice that were challenged with the E.G7 cells or EL4 cells. Numbers indicated are the percentage of CD8⁺ lymphocytes and the ratios of OT-I cells:OT-I-CD25^{-/-} cells are indicated in the respective plots. This experiment was repeated twice with three to four mice per group.

OT-I cells at 58 h were unaltered even in the presence of exogenous IL-2 (Fig. 6B), confirming that exogenous IL-2 did not affect early CD8 T cell cycling.

Discussion

In the present study, we examined the necessity for the IL-2/IL-2R system during the expansion phase of CD8 T cells *in vivo*. The system used here offers several advantages to analyses performed within intact IL-2^{-/-} or IL-2R^{-/-} animals where the development of the lymphoproliferative disorder and autoimmunity confounds the interpretation of the results. The introduction of the OT-I transgene onto the IL-2^{-/-} or IL-2R^{-/-} background greatly delays the appearance of disease that is normally observed in these animals. Also, since adoptive transfers are conducted using phenotypically naïve lymphocytes from young (~4-wk old) mice into normal WT recipients before antigenic challenge, Ag-specific lymphocytes that are incapable of responding to IL-2 can be tracked before and immediately following activation in an otherwise normal environment. Also, the use of mixtures of adoptively transferred cells obviates the necessity to quantitate absolute cell numbers as comparisons between the two cell populations are made within the same recipient. We recently demonstrated that during a viral infection, IL-2 was not required for the expansion of Ag-specific CD8 T cells within peripheral LN, but was essential for optimal expansion within nonlymphoid tissues (25). These observations along with the results of other investigators led us to hypothesize that contrary to popular belief, the expression of IL-2 and its high-affinity receptor is not required to drive activated CD8 T cells into cycle. The current dogma that IL-2 initiates cell cycle entry rests on the assumption that, as observed *in vitro*, IL-2 and the high-affinity IL-2R are expressed immediately following activation and before cell division *in vivo*. This premise was called into question by Li et al. (35) who observed the expression of IL-2 and CD25 only by T cells that had undergone more than five divisions in response to alloantigen or superantigen *in vivo* (35). However, naïve T cells are known to activate IL-2 transcription before cell division and this has also been recently demonstrated *in vivo* upon activation with superantigen and LPS (40). In the system described here, both IL-2 and CD25 were expressed by anti-

A



B

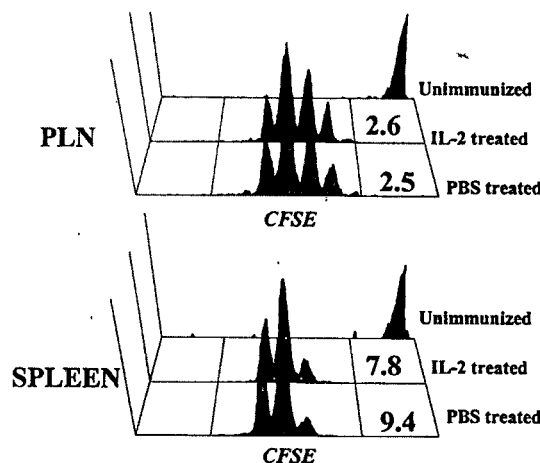


FIGURE 6. The administration of exogenous IL-2 does not alter early cell cycling but sustains the proliferative phase. As described in Fig. 1, OT-I-RAG^{-/-} cells (Ly5.1⁺) were adoptively transferred into Ly5.2⁺ B6 mice which were then immunized with VSV-OVA. Mice were divided into four groups: The first and second group received PBS or IL-2 from days 0 to 2, respectively, while the third and fourth group received PBS or IL-2 from days 3 to 6, respectively. For the groups that received IL-2, each mouse received daily i.p. injections of 5000 IU recombinant human IL-2 diluted in PBS. **A**, The presence of donor cells in the peripheral blood was determined at the indicated time points. **B**, As in **A**, except cells were labeled with CFSE before transfer and some mice were left unimmunized as controls. IL-2 or PBS was administered as in **A** from days 0 to 2. Mice were sacrificed at 58 h postinfection and analyzed for the presence of donor cells in the PLN and spleen. Plots shown are derived from gating on donor lymphocytes and numbers indicated are percentages of CD8⁺ lymphocytes. The experiment in **A** was performed twice with three mice in each group and in the experiment in **B**, two mice were analyzed.

viral CD8 T cells immediately after *in vivo* activation and before cell division, but we nonetheless observed a lack of requirement for the IL-2 pathway in early cell cycling (Figs. 1 and 2). One caveat that should be considered is that the frequency of Ag-specific cells in the adoptive transfer system could alter cytokine requirements. However, reducing the number of OT-I cells 10-fold did not affect the results (data not shown), and we previously reported similar results after infection of IL-2^{-/-} mice, although very early time points could not be examined. Therefore, our results from various *in vivo* models conclusively demonstrated that regardless of the context in which Ag was presented (viral/soluble/tumor/self), IL-2 was dispensable in the initiation of CD8 T cell

cycling. We have also observed that antiviral CD8 T cells exhibited uncompromised CTL activity even if they were generated in the absence of IL-2 (our unpublished results). Although it has not yet been analyzed whether IL-2 is needed for CD4 T lymphocytes to enter the cell cycle *in vivo*, the available literature suggest that IL-2 may not be required for cell cycle initiation of activated CD4 T cells (41, 42).

Our present studies also defined a crucial juncture at which the requirement for IL-2 as a growth factor gained prominence. Thus, although IL-2 was not required for cell cycle initiation, it was required for sustaining the proliferation of the responding cells. This conclusion was best illustrated during the CD8 T cell response to soluble OVA (Fig. 3) wherein a distinct pattern of cell division could be visualized even at day 4 postimmunization. The results convincingly demonstrated that although cell division began normally and initially proceeded at the same rate in the absence of IL-2 signaling, by the peak of the proliferative phase, less division was observed in the absence of IL-2-mediated signaling. This argued that the observed requirement for IL-2 was primarily due to sustained proliferation rather than increased survival, although it is difficult to conclusively distinguish between these two possibilities *in vivo*. This conclusion is also in agreement with other studies demonstrating that IL-2 can play a role in supporting the prolonged expansion of CD8 T cells (39, 43–46). Also, our studies with exogenous IL-2 (Fig. 5) strongly supported the conclusion that IL-2 was not important during early priming events, but helped sustain the proliferative phase. This result is in agreement with recent work by Blattman et al. (47) in which the lymphocytic choriomeningitis virus-specific CD8 response could be sustained by exogenous IL-2, although early priming events were not analyzed. The apparent paradox that exogenous IL-2 could act on T cells even after the peak of the response, a time point at which the expression of the high-affinity IL-2R is down-regulated, was resolved by the finding that OT-I cells within the mice that received IL-2 maintained higher levels of CD25 on their surface (data not shown). This again is consistent with the results of Blattman et al. (47) and early *in vitro* studies demonstrating that IL-2 is capable of up-regulating the expression of the IL-2R α chain (48).

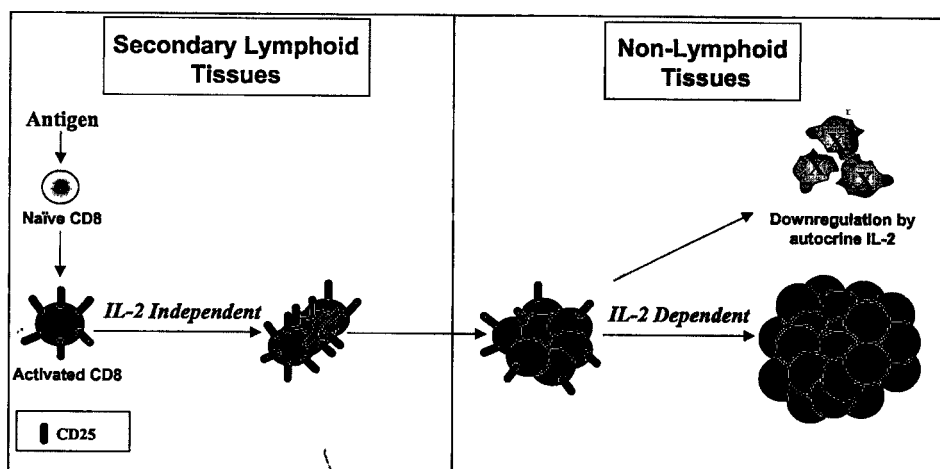
We consistently observed that IL-2 was more critical for sustaining expansion within nonlymphoid vs lymphoid tissues. These observed differences between the secondary lymphoid and nonlymphoid tissues could be due to several mutually nonexclusive factors. We and others have demonstrated that subsequent to activation of T cells within secondary lymphoid tissue, the cells migrate into the nonlymphoid sites (30, 49) where they may continue to proliferate. In this scenario, we would hypothesize that the cells

within nonlymphoid tissues might be expected to have undergone more divisions than their counterparts within the secondary lymphoid tissues, and this might result in their exhibition of an increased IL-2 dependence. A second factor might be the anatomy of the tissue within which the cells are localized, since the organization of lymphocytes within secondary lymphoid tissues differs greatly from that within the nonlymphoid sites. Secondary lymphoid organs are geared toward the promotion of cell-cell interactions unlike the nonlymphoid tissues which do not seem to display any obvious organizational scheme. Accordingly, one could speculate that apart from IL-2, other growth-promoting stimuli and/or cytokines might be more readily available within the secondary lymphoid tissues. Third, IL-2 can be bound by the extracellular matrix (50) and although it is not known whether there are differences in the display of IL-2 in different tissues, it could be another influencing factor. It is important to mention that the reduced number of OT-I-IL-2^{-/-} or OT-I-CD25^{-/-} cells in nonlymphoid tissues at the peak of the response was not likely due to impaired migration since at early time points postimmunization, both populations exhibited greater accumulation in these sites. The observation that the relative requirement for IL-2 in the splenic response lay in between that observed within LN and that within nonlymphoid tissues may be explained by the fact that the spleen, apart from being a site of primary activation, also contains circulating cells.

It has recently been demonstrated that once activated, T cells become committed to undergo a programmed expansion phase (51–53). In keeping with the current dogma, IL-2 was implicated in the initiation of this program within the committed cells (51, 52). However, this requirement was only demonstrated *in vitro*, where the indispensable growth-promoting ability of IL-2 is well established. These studies did not determine whether cell cycle initiation *in vivo* was IL-2 dependent and our results would support a model (Fig. 7) where following activation of CD8 T cells within the secondary lymphoid tissues, cell cycle progression is initiated in the absence of IL-2-mediated signals. These cells then continue to proliferate and migrate into the nonlymphoid tissues where IL-2 assumes an important role in supporting their prolonged expansion. Limiting the magnitude of the response may be achieved by the induction of apoptosis in a subpopulation of responding cells and autocrine IL-2 controls this event within nonlymphoid sites.

Our contention then that the initiation of the “division program” is IL-2 independent raises the question as to whether or not any extrinsic signals (cytokines) are required to trigger this proliferation, and, if so, what might these cytokines be? IL-2 is a member

FIGURE 7. Proposed model for the function of IL-2 in CD8 T cell responses. A naive T cell, upon encountering Ag within secondary lymphoid tissue, is activated and initiates a division program that is IL-2 independent. The activated cells continue proliferating and some migrate out into nonlymphoid tissues. The sustained expansion of the cells, particularly outside of secondary lymphoid tissues, is critically dependent upon the presence of IL-2 and signaling via the high-affinity IL-2R. Also, within the nonlymphoid tissues, autocrine IL-2 assumes the role of a negative regulator in a subset of cells and serves to control the overall magnitude of the response within these sites.



of the common γ_c -chain (γ_c)-dependent family of cytokines whose receptors share the γ_c . This family also includes the cytokines IL-4, IL-7, IL-9, IL-15, and IL-21, which are known to promote growth and survival of T cells in vitro and in vivo. The IL-15R utilizes the IL-2R β chain in addition to the γ_c and thus IL-15 shares several activities with IL-2 (54, 55). Recent data suggest that IL-15 may drive the early expansion of superantigen-reactive or -alloreactive CD8 T cells in vivo (35), although other data generated using IL-15-deficient or IL-15R α -deficient mice indicate that antiviral CD8 T cell responses can be generated in the absence of IL-15 (56, 57). It has also been demonstrated that T cells that are unable to utilize either IL-7 (58), or both IL-2 and IL-4 (59), or IL-2 and IL-15 (60) are all capable of mounting T cell responses (although impaired to varying degrees in some instances). Studies performed using mice that lack the γ_c indicate that CD4 T cells are capable of γ_c -independent proliferation (61, 62); however, considering that some members of the γ_c -dependent cytokine family (IL-7 in particular) are essential for the normal development of lymphocytes (63), the definitive experiment would involve the assessment of T cell responses within mice that lack γ_c specifically within mature T cells. Although these studies would suggest a lack of requirement for these cytokines in initiating cell cycling, none of these studies analyzed the rate and kinetics of early cell division. It also remains possible that other known or unknown cytokines drive early T cell proliferation or, conversely, T cells may be capable of initiating programmed division in the absence of soluble extrinsic factors.

In conclusion, although IL-2 is often still deemed essential for the initiation of cell division within T cells, our results demonstrated convincingly that early CD8 T cell proliferation subsequent to activation was IL-2 independent, whereas continued expansion was IL-2 dependent. These findings held true in several different experimental settings and thus call into question the "textbook paradigm" which cultivates the sentiment that IL-2 is crucial for driving proliferation immediately following CD8 T cell activation. Apart from providing a better framework for the understanding of CD8 T cell responses in vivo, this study also has important implications for the development of vaccination and therapeutic strategies that involve manipulation of the IL-2 cytokine system.

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