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Opposing Effects of TGF-β and IL-15 Cytokines Control the Number of Short-Lived Effector CD8+ T Cells

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SUMMARY

An effective immune response against infectious agents involves massive expansion of CD8+ T cells. Once the infection is cleared, the majority of these effector cells die through unknown mechanisms. How is expansion controlled to maximize pathogen clearance and minimize immunopathology? We found, after *Listeria* infection, plasma transforming growth factor β (TGF-β) titers increased concomitant with the expansion of effector CD8+ T cells. Blocking TGF-β signaling did not affect effector function of CD8+ T cells. However, TGF-β controlled effector cell number by lowering Bcl-2 amounts and selectively promoting the apoptosis of short-lived effector cells. TGF-β-mediated apoptosis of this effector subpopulation occurred during clonal expansion and contraction, whereas interleukin-15 (IL-15) promoted their survival only during contraction. We demonstrate that the number of effector CD8+ T cells is tightly controlled by multiple extrinsic signals throughout effector differentiation; this plasticity should be exploited during vaccine design and immunotherapy against tumors and autoimmune diseases.

INTRODUCTION

Encounter with foreign antigen induces naive T cells to expand massively, a process called clonal expansion, and differentiate into effector T cells. The high proliferative capacity and cytotoxic activity of antigen-activated CD8+ T cells requires an effective control of their life span in order to maintain lymphocyte homeostasis and to avoid immunopathology. A contraction period follows clonal expansion, during which time the majority of effector cells die rapidly by apoptosis (Hand and Kaech, 2008; Marrack and Kappler, 2004). A small proportion of effector cells survives and further differentiates into long-lived memory cells (Kaech and Wherry, 2007). Increasing the quantity and quality of memory CD8+ T cells is crucial to improving the efficacy of most vaccines (Seder et al., 2008). Another important focus in the vaccine field is to generate enhanced expansion of effector cells because of the direct relationship between the number of effector cells at the peak of expansion and the final number of memory cells that are formed (Hou et al., 1994; Murali-Krishna et al., 1998). However, very little is known about the signals that influence the pool size of the effector cells.

Recent elegant work has described multiple subpopulations of effector CD8+ T cells that exist during clonal expansion in response to some viral and bacterial infections (Joshi et al., 2007; Sarkar et al., 2008). The subset of effector CD8+ T cells that survive and become memory cells are sometimes referred to as memory precursor effector cells (MPECs), whereas the population that dies during contraction is referred to as short-lived effector cells (SLECs) (Joshi et al., 2007; Joshi and Kaech, 2008). Both of these populations have been shown to have very similar functional ability at the peak of the immune response; however, they greatly differ in their memory potential and survivability (Joshi et al., 2007; Sarkar et al., 2008). A major challenge that remains is to identify extrinsic and intrinsic signals that make SLECs susceptible to apoptosis and the signals that promote the survival and maintenance of the MPECs (Hand and Kaech, 2008).

The common gamma chain cytokines interleukin-2, -7, and -15 (IL-2, IL-7, and IL-15) are critical regulators of activated T cell proliferation, survival, and memory T cell formation (Boyman et al., 2007; Ma et al., 2006; Malek, 2008). IL-2 is a secreted cytokine that binds trimeric receptors made up of IL-2Rα (CD25), the β receptor (CD122), and the common γ-chain (cγc) receptor on the surface of target cells. IL-2 plays an important role in the differentiation and early expansion of a functional effector population and is required for secondary expansion of memory CD8+ T cells, but does not seem to cause the contraction of effector T cells (Blattman et al., 2003; D'Souza and Lefrancois, 2003; Williams et al., 2006). IL-7 is also a secreted cytokine that binds to the dimeric IL-7Rα (CD127) and the cγc receptor on the surface of target cells (Ma et al., 2006). Effector T cells with high expression of the IL-7Rα during acute infections are considered memory precursor cells (Joshi and Kaech, 2008; Kaech and Wherry, 2007). However, transgenic overexpression of IL-7Rα had no effect on effector or memory T cell survival, demonstrating that the IL-7Rα expression does not instruct the formation of memory T cells by allowing them to better compete for IL-7 (Hand et al., 2007; Haring et al., 2008). Thus, the downregulation of IL-7Rα appears to be an indicator of reduced memory CD8+ T cell potential but is not the major cause of effector T cell death after acute infections such as LCMV and *Listeria* (Hand and Kaech, 2008). IL-15 is a membrane-bound cytokine that is presented in *trans*

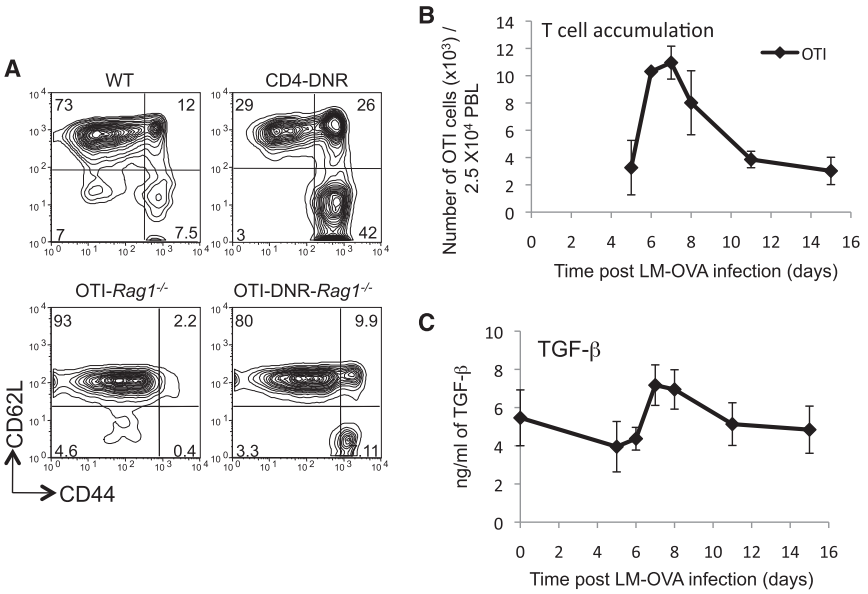

Figure 1. TGF-β Signaling May Be Important for Controlling the Number and Function of Effector CD8+ T Cells

(A) Splenocytes from 6-week-old mice under steady-state conditions were gated on CD8+ T cells. Data shown are representative of at least 10 different sets of mice with similar profiles.

(B) 5 × 10^4 naive OTI-*Rag1*$^{-/-}$ cells were adoptively transferred into wild-type B6 hosts, and OTI cell expansion in response to LM-OVA infection was measured in a longitudinal experiment. Data are plotted as the fraction of OTI cells among 25,000 total peripheral blood lymphocytes (PBLs). Average and SD of 4 mice is shown.

(C) Total TGF-β levels detected with ELISA after acidification of plasma collected from infected mice from part (B).

on the surface of monocytes or dendritic cells by the IL-15Rα (Ma et al., 2006). Cells that express the β receptor CD122 and the cγc receptor can respond to *trans*-presented IL-15 and transduce IL-15 signaling through this complex (Burkett et al., 2003; Dubois et al., 2002). More recent work indicates that in response to some acute infections, the survival of MPECs depends on both IL-7 and IL-15, whereas the survival of SLECs depends on IL-15 because these cells lack IL-7Rα (Joshi et al., 2007; Rubinstein et al., 2008; Yajima et al., 2006). In addition, exogenous administration of IL-2 or IL-15 greatly improves the survival of SLECs during contraction without inducing their proliferation (Rubinstein et al., 2008). In line with these observations, it is thought that SLECs die for reasons other than sole deprivation from these cytokines, although the extrinsic signals that further promote the apoptosis of SLECs have not yet been identified (Joshi and Kaech, 2008).

Transforming growth factor β (TGF-β) is a pleiotropic cytokine with potent regulatory activity (Li and Flavell, 2008a, 2008b; Li et al., 2006b). Monomeric TGF-β binds to TGF-β receptor II (TGF-βRII), triggering the kinase activity of the cytoplasmic domain that in turn activates TGF-βRI. The activated receptor complex leads to nuclear translocation of Smad molecules and transcription of target genes (Li et al., 2006b). To understand the role of TGF-β signaling in T cell biology, we developed a mouse model where the expression of a dominant-negative form of TGF-βRII (DNR), lacking the kinase domain, inhibits TGF-β signaling in both CD4+ and CD8+ T cells (Gorelik and Flavell, 2000). T cells in this model expand at a higher rate, acquire an activated phenotype, and produce effector cytokines, and the mice eventually succumb to autoimmunity (Gorelik and Flavell, 2000). Via this and other mouse models, the pivotal function of TGF-β under steady-state conditions has been identified to maintain tolerance through the regulation of lymphocyte proliferation, differentiation, and survival (Gorelik and Flavell, 2000; Li and Flavell, 2008a, 2008b). It is known that the regulatory activity of TGF-β is modulated based on the cell type, its differentiation state, expression of costimulatory molecules, and the presence of other cytokines (Li and Flavell, 2008a, 2008b; Li et al., 2006b). However, the role of TGF-β in controlling CD8+ T cell activation, proliferation, differentiation, and survival has not been addressed under infectious conditions.

We show here that blockade of TGF-β signaling during effector T cell differentiation did not alter ex vivo cytokine production and in vivo CTL activity of CD8+ effector T cells. Surprisingly, we found that clonally expanding CD8+ T cells underwent massive apoptosis even before reaching the "contraction" phase. However, blockade of TGF-β signaling on effector CD8+ T cells caused an increase in their total number, which was a result of reduced apoptosis of short-lived effector cells during clonal expansion. We further demonstrate that TGF-β provides an extrinsic signal to further downregulate Bcl-2 expression in effector cells and selectively promote the apoptosis of SLECs. Finally, we present evidence for a model in which TGF-β and IL-15 provide opposing extrinsic signals to control the number of SLECs during clonal expansion and contraction. These findings identify a unique role for TGF-β in controlling the number of clonally expanding effector CD8+ T cells under acute inflammatory conditions. The relevance and implications of these findings for vaccine design and potential immunotherapy against tumors and certain autoimmune diseases are further discussed.

RESULTS

Systemic Increase in TGF-β Expression at the Peak of T Cell Proliferation during *Listeria* Infection

Overexpression of a dominant-negative form of TGF-β receptor II specifically in T cells under the control of the CD4 promoter (CD4-DNR), lacking the CD8 silencer, results in the accumulation of CD44hi effector-memory T cells (Figure 1A, top; Gorelik and Flavell, 2000). These observations, and numerous other studies, underline the importance of TGF-β in maintaining peripheral tolerance under steady-state conditions (Li and Flavell, 2008a, 2008b; Li et al., 2006b). However, the biological role of TGF-β during an acute infection is still poorly understood. To assess

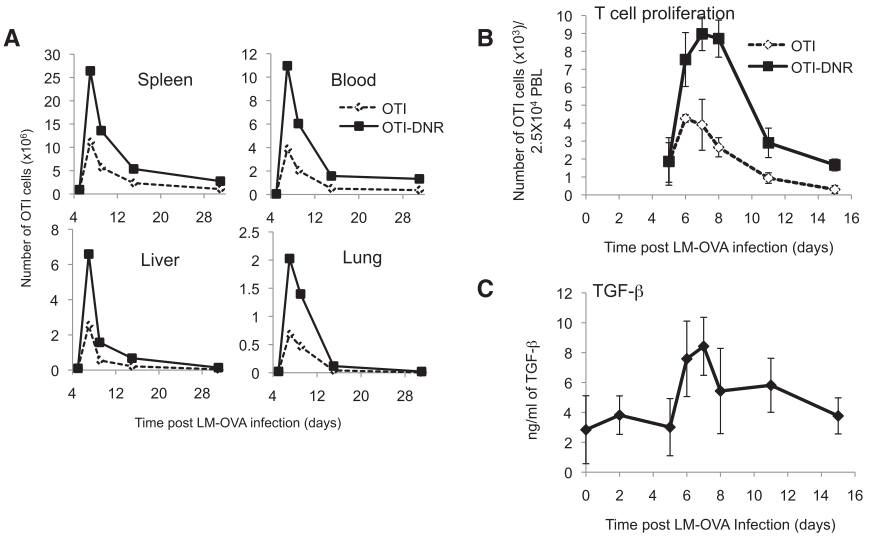

Figure 2. An Increase in the Number of OTI-DNR Compared to OTI Cells at the Peak of CD8+ T Cell Clonal Expansion in Response to LM-OVA

(A) 2.5 × 10^4 each of naive OTI-*Rag1*$^{-/-}$CD45.1.1 and OTI-DNR-*Rag1*$^{-/-}$CD45.1.2 cells were adoptively cotransferred into naive hosts, followed by infection with LM-OVA 1 day later. The absolute number of each OTI and OTI-DNR population from each organ is plotted over time. For the blood measurements, the number of OTI cells per 1 ml of blood is plotted. Data are representative of two independent experiments with similar results.

(B and C) Cotransfer and infection was performed as in (A), except the same group of mice were bled for a longitudinal experiment to compare the kinetics of OTI and OTI-DNR T cell accumulation among PBLs while measuring plasma TGF-β levels in the same infected mice. Average and SD is shown. Data are representative of two independent experiments with similar results and a total of 10–15 mice in each experiment.

whether TGF-β plays a role in CD8+ T cell biology during an acute infection, we adoptively transferred 5 × 10^4 naive OTI transgenic T cells, expressing $OVA_{257-264}$ K^b-specific TCR, into wild-type mice and infected them 1 day later with recombinant *Listeria monocytogenes*-expressing OVA (LM-OVA). OTI T cell expansion was measured among the peripheral blood lymphocytes (Figure 1B), and total TGF-β was measured from blood plasma (Figure 1C). The observation that plasma TGF-β rise during an acute infection with similar kinetics to the accumulation of CD8+ T cells prompted us to further investigate the role of TGF-β in effector T cell development and function.

Blockade of TGF-β Signaling in CD8+ T Cells Results in Enhanced Accumulation of Effector Cells

To obtain large numbers of antigen-specific naive CD8+ T cells that express the TGF-βRII DNR, we crossed the CD4-DNR mice onto OTI transgenic mice and further crossed them onto a *Rag1*$^{-/-}$ and CD45.1 congenic marker (Figure 1A, bottom). Unlike the original CD4-DNR mice (Figure 1A, top; Gorelik and Flavell, 2000), the OTI-DNR-*Rag1*$^{-/-}$ (OTI-DNR) mice do not develop autoimmunity, and large numbers of antigen-specific naive ($CD62L^{hi}$-$CD44^{lo}$) T cells can be isolated from young mice (Figure 1A, bottom). To address the role of TGF-β signaling during CD8+ T cell differentiation and effector generation in response to an acute infection, we wanted to perform competitive adoptive cotransfer of OTI and OTI-DNR naive cells into the same host followed by in vivo activation via LM-OVA. Further, we employed low numbers (around 25,000 sorted naive CD8+ T cells of each type) to avoid the artifacts that can accompany transfers of too many cells (Marzo et al., 2005).

DNR-bearing T cells cannot be adoptively transferred into C57Bl/6 mice, because they are immunologically rejected because they contain human rather than mouse DNR sequence (Figure S1 available online). Instead, we used our CD11c-DNR mice as recipients of adoptive transfers (Laouar et al., 2005). These mice express the same human TGF-βRII DNR transgene in dendritic cells and natural killer cells under the control of the CD11c promoter and therefore are immunologically tolerant to the human DNR sequence. These mice have been well characterized and no observable defect has been detected in any of the T cell compartments (Laouar et al., 2005). Nevertheless, to control for any untoward effects of the CD11c-DNR transgene on the data, all of the experiments involving adoptive transfers were performed side by side with transfers of wild-type OTI cells into both CD11c-DNR transgene-positive and -negative mice. For the remainder of this study, the main figures contain the comparison of OTI and OTI-DNR cells transferred into the CD11c-DNR hosts (labeled as "host"), whereas the accompanying supplemental figures contain the same analysis of the OTI cells transferred into transgene-negative wild-type littermates. It is noteworthy that no substantial difference was observed in our studies between the results obtained when OTI cells were transferred into either host.

When OTI and OTI-DNR cells were cotransferred and activated with LM-OVA, a massive (3-fold) increase in the number of OTI-DNR effector cells was observed in blood and both lymphoid and nonlymphoid organs (Figures 2A and 2B). To assure that this difference was not somehow related to a substantial reduction in the amount of antigen present during priming in the CD11c-DNR mice, we measured bacterial burden 3 days after infection. We detected similar numbers of bacteria in the spleen and liver of wild-type and CD11c-DNR mice that had no transgenic T cells or had been adoptively transferred with either 5 × 10^4 OTI or OTI-DNR cells (data not shown). Notably, the difference between OTI and OTI-DNR cell numbers became apparent only after day 5 postinfection (p.i.) (Figure 2B). This also suggests that the difference in OTI-DNR accumulation is not related to a difference in priming and activation of the transgenic T cells.

Similar to infection in wild-type hosts (Figure 1C), a 3-fold increase in the amount of plasma TGF-β was also detected in the CD11c-DNR recipients during the peak of T cell proliferation (Figure 2C). Remarkably, the time at which the difference in the expansion between OTI and OTI-DNR cells first becomes apparent (around day 6 postinfection) directly correlated with the time at which plasma TGF-β also peaked during the course of infection, suggesting a direct effect of TGF-β on the number

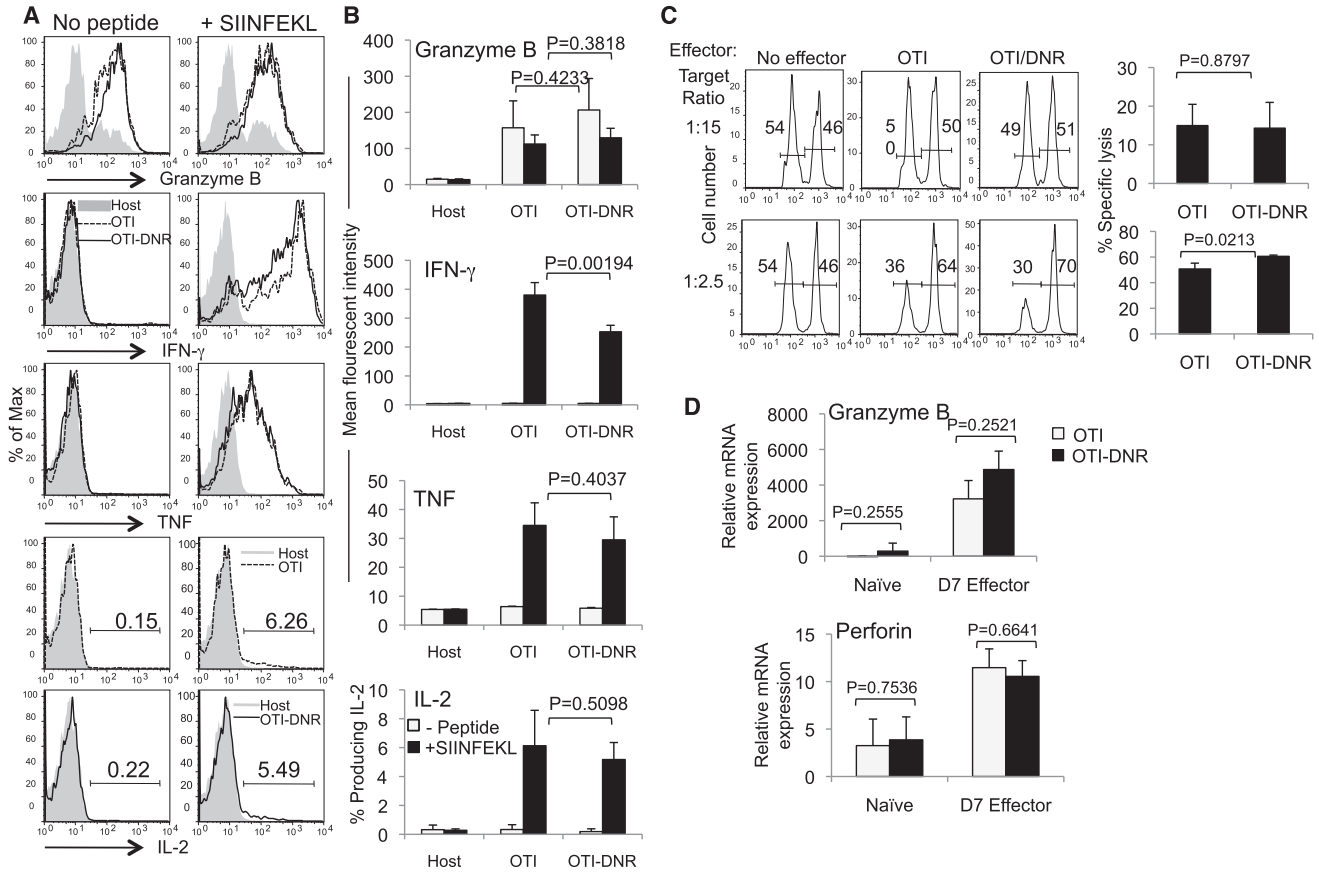

Figure 3. Blockade of TGF-β Signaling Does Not Alter Cytokine Production and CTL Activity of Effector CD8+ T Cells

(A) Adoptive cotransfer and LM-OVA infection was performed as described in Figure 2A. Intracellular cytokine staining of spleen cells isolated 7 days p.i. Host (CD45.2.2), OTI (CD45.1.1), and OTI-DNR (CD45.1.2) cells were identified based on staining with CD8 and the corresponding congenic markers. Splenocytes were stimulated with or without SIINFEKL peptide. One representative histogram is shown.

(B) Average and SD of four different cotransfers. Data are representative of three independent experiments with similar results.

(C) In vivo CTL assay performed with effector OTI and OTI-DNR cells isolated from the spleen of infected mice 7 days p.i. Representative raw data are shown on the left. Summary of two independent experiments is shown on the right, with average and SD of 4 recipients of each population.

(D) Relative mRNA expression of cytolytic molecules from day 7 effector cells. Samples were first normalized to HPRT. Relative fold increase over naive OTI cells is shown. Data are average and SD of three independent experiments.

of effector CD8+ T cells. TGF-β amounts in the plasma dropped by the middle of the contraction phase and returned to baseline by 2 weeks postinfection. Collectively, these data suggest that TGF-β does not play a major role during the early phases of CD8+ T cell priming and activation, but rather, that it plays a key role in determining the number of effector T cells at the peak of clonal expansion.

Blockade of TGF-β Signaling Does Not Alter Effector Function of CD8+ T Cells upon *Listeria* Infection

Many studies have suggested that under steady-state conditions or in the presence of tumors, TGF-β inhibits cytokine production and CTL activity of CD8+ T cells (Ahmadzadeh and Rosenberg, 2005; Li et al., 2006a; Marie et al., 2006; Thomas and Massague, 2005; Trapani, 2005). To address whether TGF-β plays a similar role during effector T cell differentiation in response to an acute infection, we compared ex vivo cytokine production and in vivo CTL activity of effector OTI and OTI-DNR cells that were generated in the same host (Figure 3). No significant difference in granzyme B, TNF, or IL-2 production was observed between OTI and OTI-DNR day 7 effector T cells when restimulated ex vivo with SIINFEKL peptide (Figures 3A and 3B; Figure S2A). OTI-DNR cells contained a larger population of the low-IFN-γ producing effector cells, which resulted in slightly lower average mean fluorescent intensity of the whole population (Figures 3A and 3B, compare histogram to bar graphs). To compare CTL activity of OTI and OTI-DNR effector T cells, day 7 effectors were isolated by cell sorting and the same number of each effector population was retransferred into naive hosts along with different ratios of target cells, and specific lysis was measured in vivo (Figure 3C). Once again, no difference was observed in the cytolytic activity of OTI and OTI-DNR effector cells (Figure 3C; Figure S2B). These findings were consistent with similar mRNA expression of both granzyme B and perforin in the OTI and OTI-DNR effector cells (Figure 3D). Collectively, these data suggest that TGF-β does not play a major role in inhibiting the effector function of CD8+ T cells under acute *Listeria* infection conditions.

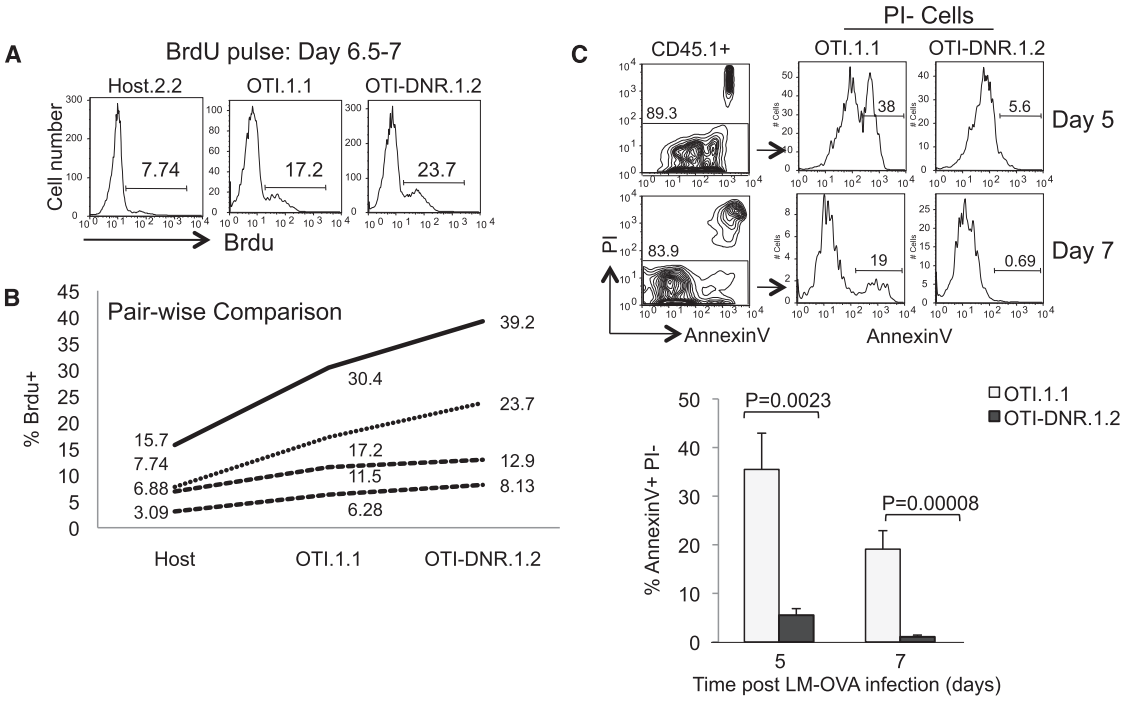

Figure 4. TGF-β Promotes Apoptosis of Effector CD8+ T Cells during Clonal Expansion

(A) Histograms showing BrdU incorporation of clonally expanding OTI and OTI-DNR cells from cotransfer experiments. Splenocytes were first gated on CD8+ T cells, and host, OTI, and OTI-DNR subpopulations were separated based on congenic markers.

(B) Pair-wise comparison of each subpopulation in four different animals with varying degrees of BrdU incorporation. Data are representative of three independent experiments with similar results.

(C) Dead versus apoptotic cells from day 5 (PBL) and day 7 (spleen) of infected mice were identified by AnnexinV and PI staining. OTI and OTI-DNR cells were then separated based on congenic markers among the PI− population. Average and SD of 3 (day 5) and 4 (day 7) mice per group is shown.

TGF-β Promotes the Apoptosis of Effector CD8+ T Cells

When TGF-β signaling was blocked in CD8+ effector T cells, a 3-fold higher number of effector T cells accumulated at the peak of clonal expansion in vivo (Figure 2). T cell accumulation is a result of the combination of T cell death and proliferation, and it has been suggested that TGF-β can inhibit T cell proliferation and induce apoptosis of activated T cells under a variety of in vitro conditions (Marrack and Kappler, 2004; Sillett et al., 2001; Wolfraim et al., 2004). Therefore, we asked whether blockade of TGF-β signaling in vivo would enhance the proliferative ability of effector T cells, promote their better survival, or both. The proliferation of OTI versus OTI-DNR cells was measured by the incorporation of BrdU near the peak of clonal expansion in adoptive cotransfer experiments (Figure 4A; Figure S3A). Although the range in BrdU incorporation differed in each individual mouse, we observed a consistent increase in BrdU incorporation in OTI-DNR compared to OTI cells (Figure 4B). It is generally thought that T cells proliferate during clonal expansion and die only during the contraction phase (Marrack and Kappler, 2004; Williams and Bevan, 2007). Contrary to this, we observed massive apoptosis of OTI cells on day 5 postinfection (Figure 4C; Figure S3B) at the time when T cells are still clonally expanding. However, the OTI-DNR effector cells, in the same host, were mostly protected from the apoptosis that occurred during (day 5) and at the peak of (day 7) clonal expansion (Figure 4C). These data suggest that blockade of TGF-β signaling allows better proliferation of effector CD8+ T cells, but most importantly, it protects OTI-DNR cells from the massive apoptosis that occurs during clonal expansion.

Selective Accumulation of SLECs at the Peak of Clonal Expansion in the Absence of TGF-β Signaling

Survival of effector CD8+ T cells is highly dependent on many cell-intrinsic and -extrinsic factors including signaling from the receptors of the cγc cytokines IL-2, IL-7, and IL-15. As a result, many changes occur in the surface expression of the receptors for these cytokines during effector CD8+ T cell differentiation (Boyman et al., 2007; Kaech and Wherry, 2007; Masopust et al., 2004; Williams and Bevan, 2007). Because we have shown previously that TGF-β can modulate the expression of CD122, via regulating T-bet in CD4+ Th1 cells (Li et al., 2006a), we wanted to see whether the receptor expression for the cγc cytokines was altered on effector CD8+ OTI-DNR compared to OTI cells, resulting in their better survival. Similar to the expansion phenotype, we did not find any difference in surface expression of any of the molecules we analyzed on days 4 or 5 postinfection (Figure 5A; Figure S4A), including complete downregulation of CD25 on both OTI and OTI-DNR cells by day 5 postinfection (data not shown). However, a difference in the upregulation of CD127, the IL-7Rα chain, was observed starting at day 7 postinfection that also persisted into the early memory phase (Figure 5B). In contrast, no difference was detected in the

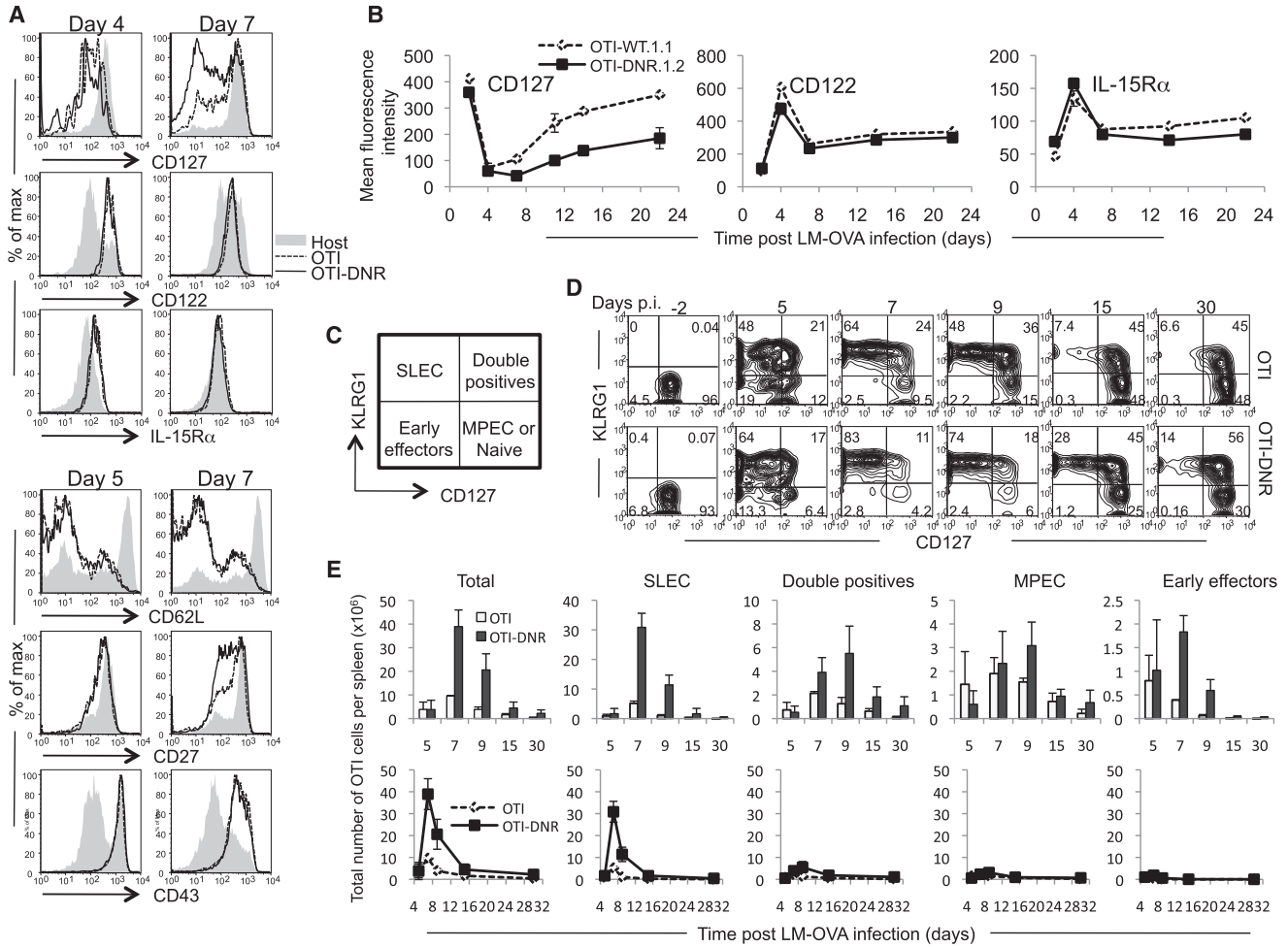

Figure 5. Selective Apoptosis of SLECs by TGF-β

(A) Phenotypic comparisons between OTI and OTI-DNR effector CD8+ T cells. Histograms comparing the expression of each surface molecule on host CD8+ (solid gray), OTI (dashed line), and OTI-DNR (black line) T cells from cotransfer experiments. Data are representative of more than four independent experiments with similar results.

(B) Expression pattern of indicated surface receptors was analyzed on OTI and OTI-DNR CD8+ T cells isolated from peripheral blood of LM-OVA-infected mice at the indicated time points. Data are average and SD of 3–5 individual cotransfers and represents 1 out of 3 independent experiments with similar results.

(C) Representative diagram of each effector subpopulation separated based on CD127 and KLRG1 expression and the terminology used to describe each subset.

(D) Representation of the difference seen in the percentages of OTI and OTI-DNR SLECs and MPECs isolated from the spleen of infected mice at the indicated time points. Time point −2 days p.i. refers to naive cells prior to adoptive transfer.

(E) Bar graphs (top) comparing the absolute number of total and each OTI and OTI-DNR subpopulation from the spleen of infected mice at each indicated time point. Longitudinal graphs (bottom) comparing the contribution of each subpopulation, on the same scale, to the overall increase in the number of OTI-DNR effector cells. Data are average and SD of 2–3 mice per time point and represents 1 out of 2 independent experiments with similar results.

expression of CD122 and IL-15Rα between OTI and OTI-DNR cells throughout effector T cell differentiation (Figure 5B). Similar to CD127, we also observed a difference in the upregulation of CD27 starting at day 7 postinfection, whereas the expression of CD62L and CD43 remained the same between the OTI and OTI-DNR cells (Figure 5A, bottom; Figure S4A).

It has recently been shown that CD127 and killer cell lectin-like receptor subfamily G1 (KLRG1) can be used to differentiate between two types or subsets of differentiating effector CD8+ T cells. Memory precursor effector cells (MPECs) are enriched in the KLRG1^{lo}-CD127^{hi} population, and short-lived effector cells (SLECs) are enriched in the KLRG1^{hi}-CD127^{lo} and CD27^{lo} population (Joshi et al., 2007; Sarkar et al., 2008). Because we observed a larger fraction of CD127^{lo} and CD27^{lo} cells among the OTI-DNR cells at the peak of clonal expansion (Figures 5A and 5B), we hypothesized that blockade of TGF-β signaling may be altering the differentiation and/or the survival of MPECs and SLECs. To test this hypothesis, we determined the absolute numbers of each OTI and OTI-DNR effector subpopulation, based on CD127 and KLRG1 expression, throughout effector differentiation (Figures 5C–5E; Figures S4B and S4C). OTI-DNR cells had a higher percentage of SLECs (KLRG1^{hi}-CD127^{lo}) and a lower percentage of MPECs (KLRG1^{lo}-CD127^{hi}) compared to OTI cells (Figure 5D). However, when these percentages were

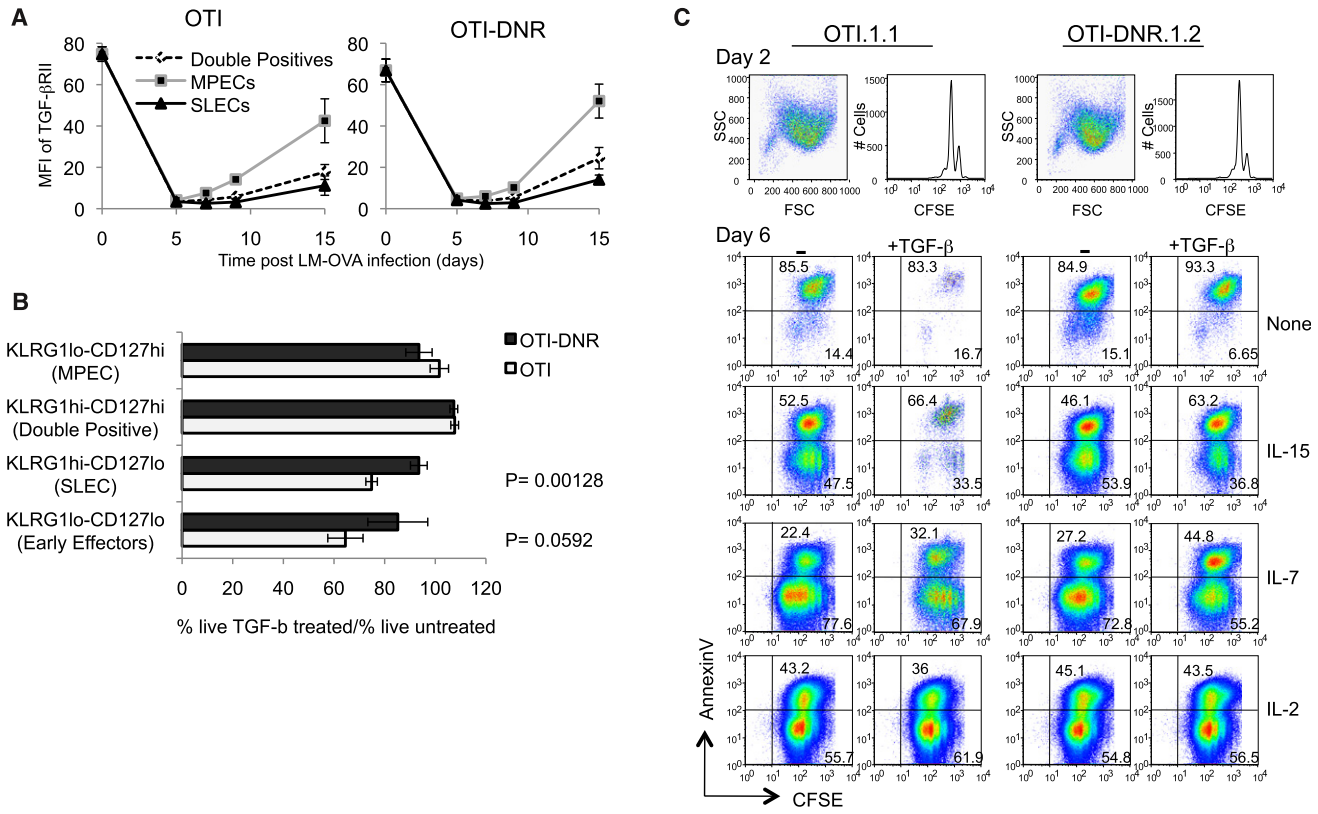

Figure 6. TGF-β Promotes the Apoptosis of Effector CD8+ T Cells both Ex Vivo and In Vitro

(A) TGF-βRII expression on naive and effector OTI and OTI-DNR subpopulations. After adoptive cotransfer and LM-OVA infection, OTI and OTI-DNR cells isolated from the spleen of infected mice were stained with CD127 and KLRG1 and either TGF-βRII or the isotype control antibodies. MFI of TGF-βRII was determined by subtracting the MFI of background staining observed with isotype control, which varied from 3 to 6, from the MFI of TGF-βRII staining on the same subpopulation of effector cells.

(B) After adoptive cotransfer and LM-OVA infection, day 7 spleens were isolated and a fraction of the splenocytes was cultured in media plus 2.5 ng/ml of TGF-β, while a similar fraction was just cultured in media alone for 16 hr. OTI and OTI-DNR cells were then stained with antibodies to congenic markers and CD127-KLRG1. The percent of live cells was determined in each fraction and the ratio of the percent live cells from TGF-β-treated cells is plotted over percent live cells of untreated cells for each subpopulation. Average and SD of four experimental wells is shown. Data are representative of 1 out of 2 independent experiments.

(C) Forward and side scatter plots and CFSE dilution of OTI and OTI-DNR cells after being stimulated for 48 hr with SIINFEKL-loaded APCs (top). Cells were washed and cultured in the absence or presence of 5 ng/ml of TGF-β and 20 ng/ml of IL-15, IL-7, or IL-2 for another 4 days. FACS plots represent total cell recovery and show all the events collected within 1 min. AnnexinV+ (dead or apoptotic) and AnnexinV− (live) and the CFSE dilution of each fraction is shown. Data are representative of 1 out of 3 independent experiments with similar results.

converted to absolute numbers for each subpopulation in the spleen of infected mice, it became evident that the number of MPECs remained similar between OTI and OTI-DNR cells, whereas there were almost six times more OTI-DNR SLECs compared to OTI SLECs at the peak of clonal expansion (Figure 5E, top). The contribution of the OTI-DNR SLEC subpopulation to the overall increase of the total number of OTI-DNR cells is most evident when the subpopulations are plotted on the same scale (Figure 5E, bottom). These data suggest that TGF-β signaling on effector CD8+ T cells selectively promotes the apoptosis of SLECs.

Despite a Low Degree of TGF-βRII Expression, TGF-β Can Promote the Apoptosis of SLECs Ex Vivo

We next wanted to explore the mechanism by which TGF-β exerts its apoptotic effects selectively on SLECs and not on MPECs. One obvious explanation for this selective mechanism may lie within differential expression of TGF-β receptor on the different subpopulations. Therefore, we measured and compared TGF-βRII surface expression on naive and effector subpopulations of OTI and OTI-DNR cells and found very high expression of TGF-βRII on naive compared to effector T cells (Figure 6A; Figure S5A). In fact, on day 5 postinfection, the expression of TGF-βRII was almost indistinguishable from the isotype control. However, by about day 7 p.i., the effector cells began upregulating the surface expression of TGF-βRII, and by day 15 postinfection, a clear difference was observed between the amount of TGF-βRII expression on the different subpopulations. The MPECs expressed the highest amount followed by the double-positive and SLEC subpopulations (Figure 6A; Figure S5A). Importantly, the expression pattern of TGF-βRII did not provide a clear explanation for the selective apoptotic effect of TGF-β on SLECs during clonal expansion; however, it raised the question as to whether the SLECs are even capable

of responding to TGF-β around day 7 postinfection. To address this, we generated in vivo day 7 effector cells (in cotransfer experiments), recovered these cells, and treated ex vivo half the recovered splenocytes with recombinant TGF-β, whereas the other half was just incubated in media without TGF-β for 16 hr (Figure S5B). Apoptotic effect of TGF-β on each effector subpopulation was determined by measuring the percent of live cells in TGF-β-treated compared to untreated cells. Interestingly, TGF-β had its greatest apoptotic impact on the OTI CD127^{lo} subpopulations, whereas it did not impact the OTI CD127^{hi} subpopulations or the OTI-DNR cells (Figure 6B; Figure S5C). These data suggest that SLECs can indeed respond to TGF-β despite the low expression of TGF-βRII on their surface, and that TGF-β has the same selective apoptotic effect on the OTI SLEC subpopulation ex vivo as it does in vivo.

TGF-β Antagonizes the Survival Effects of IL-15 on Clonally Expanding T Cells In Vitro

As inflammation subsides around the peak of clonal expansion and both IL-2 levels and CD25 expression on effector T cells becomes limiting, the MPECs continue to receive survival signals through both IL-7 and IL-15, whereas the survival of SLECs becomes mostly dependent on IL-15, because this population lacks the expression of IL-7Rα (D'Cruz et al., 2009; Hand et al., 2007; Joshi et al., 2007). Therefore, we hypothesized that OTI-DNR SLECs survive better than OTI SLECs, because they can respond better to IL-15, implying that TGF-β may either somehow modulate or override the survival signals provided by IL-15. To test this hypothesis, we wanted to compare the combinatorial effect of TGF-β and various different cγc cytokines on the survival of effector CD8$^+$ T cells; however, treatment of in vivo generated effector cells with these cytokines results in modulation of the perspective receptors (i.e., IL-7 downregulates IL-7Rα), compromising the analysis on the different subpopulations based on CD127 and KLRG1 expression. Instead, we set up an in vitro culture assay system to specifically address the extrinsic effects of TGF-β alone and in combination with the various cγc cytokines on clonally expanding CD8$^+$ T cells. Naive CFSE-labeled OTI and OTI-DNR cells were first cocultured with SIINFEKL peptide-loaded antigen-presenting cells for 48 hr, at which point OTI and OTI-DNR cells showed similar growth and cell division (Figure 6C, top). The cells were washed and recultured with either TGF-β alone or with TGF-β in combination with IL-15, IL-7, or IL-2 for another 4 days (Figure 6C, bottom). When clonally expanding effector cells were cultured in the presence of TGF-β, most of the cells in the culture underwent apoptosis and only about 40% of the cells were recovered compared to the No TGF-β control (Figure 6C, row labeled None). As expected, addition of the cγc cytokines greatly enhanced the recovery of all the cells, and the inclusion of TGF-β with the OTI-DNR cells had only a modest effect on all conditions (Figure 6C, right). Most notably, inclusion of TGF-β with wild-type OTI cells that were cultured in IL-15, compared to those that were cultured in IL-15 alone, resulted in a similar low magnitude of cell recovery (about 40%) as OTI cells cultured in TGF-β alone compared to no TGF-β. However, when cells were cultured in the presence of TGF-β and IL-7, or TGF-β and IL-2, about 80% and 100% of the cells, respectively, could still be recovered compared to the controls (Figure 6C). Thus, TGF-β has a potent apoptotic effect on clonally expanding T cells; however, the presence of IL-2 and IL-7 can fully and partially mask the effects of TGF-β, respectively. Remarkably, under similar conditions, IL-15 is not able to overcome the apoptotic effects of TGF-β. Thus, the magnitude of cell death was similar when cells were cultured with TGF-β alone or with the combination of IL-15 and TGF-β, whereas IL-7 was able to protect the clonally expanding T cells from the apoptotic effects of TGF-β. These observations provide evidence for our hypothesis that the mechanism by which TGF-β selectively promotes apoptosis of SLECs is because these cells respond to IL-15 and not IL-7, whereas MPECs can respond to both IL-15 and IL-7.

TGF-β and IL-15 Exert Opposing Effects on SLECs during an Immune Response to *Listeria* Infection

The cγc cytokines IL-2, IL-7, and IL-15 mediate life and death signals by activating the Jak-Stat and PI3K survival pathways. One of the most important outcomes of these signaling pathways is regulation of factors such as Bcl-2 and Bim, which determine survival or death of effector cells (D'Cruz et al., 2009). The observation that TGF-β can override survival signals provided by IL-15 but not those provided by IL-7 or IL-2 (Figure 6C) prompted us to determine whether TGF-β acted on IL-15 signaling, or whether it promoted the apoptosis of SLECs independent of this cytokine. To begin addressing this, we first asked whether OTI-DNR cells biologically respond better to IL-15. In the absence of exogenous TGF-β, both OTI and OTI-DNR cells responded similarly to a titration of doses of IL-15 (data not shown), suggesting that blockade of TGF-β signaling does not change the sensitivity of their responsiveness to IL-15. In support of this, we also did not see a difference in the kinetics of phosphorylation and dephosphorylation of Stat5 upon stimulation with different concentrations of IL-15 and in the presence and absence of TGF-β (data not shown). Subsequently, we also measured the extent of Akt phosphorylation in response to IL-15 in OTI and OTI-DNR cells in the presence or absence of TGF-β, and again did not observe any difference between the two cell types (data not shown). We concluded from these studies that TGF-β most likely was not overriding IL-15 signaling by inhibiting or dampening the downstream signaling pathways. Finally, we asked whether TGF-β had an impact on the final outcome of these signaling events, namely the expression of Bcl-2 and Bim, whose balance determines the life and death of short-lived effector T cells (Hildeman et al., 2007; Marrack and Kappler, 2004; Wojciechowski et al., 2006). To begin, we isolated mRNA from naive and day 7 effector OTI and OTI-DNR SLECs and compared the expression of several genes involved in function, differentiation, and survival of effector CD8$^+$ T cells (Figure S6). This analysis further validated the absence of a role for TGF-β in regulating the expression of genes involved in effector function and differentiation of CD8$^+$ T cells under these inflammatory conditions. However, we consistently found a 2-fold increase in the expression of Bcl-2 in OTI-DNR compared to OTI effector cells, whereas other molecules such as T-bet remained comparable at both RNA and protein level (Figures 7A and 7B; Figure S7A). These data suggested that TGF-β is negatively influencing the expression of Bcl-2 in effector T cells. Because signaling by the cγc cytokines is the

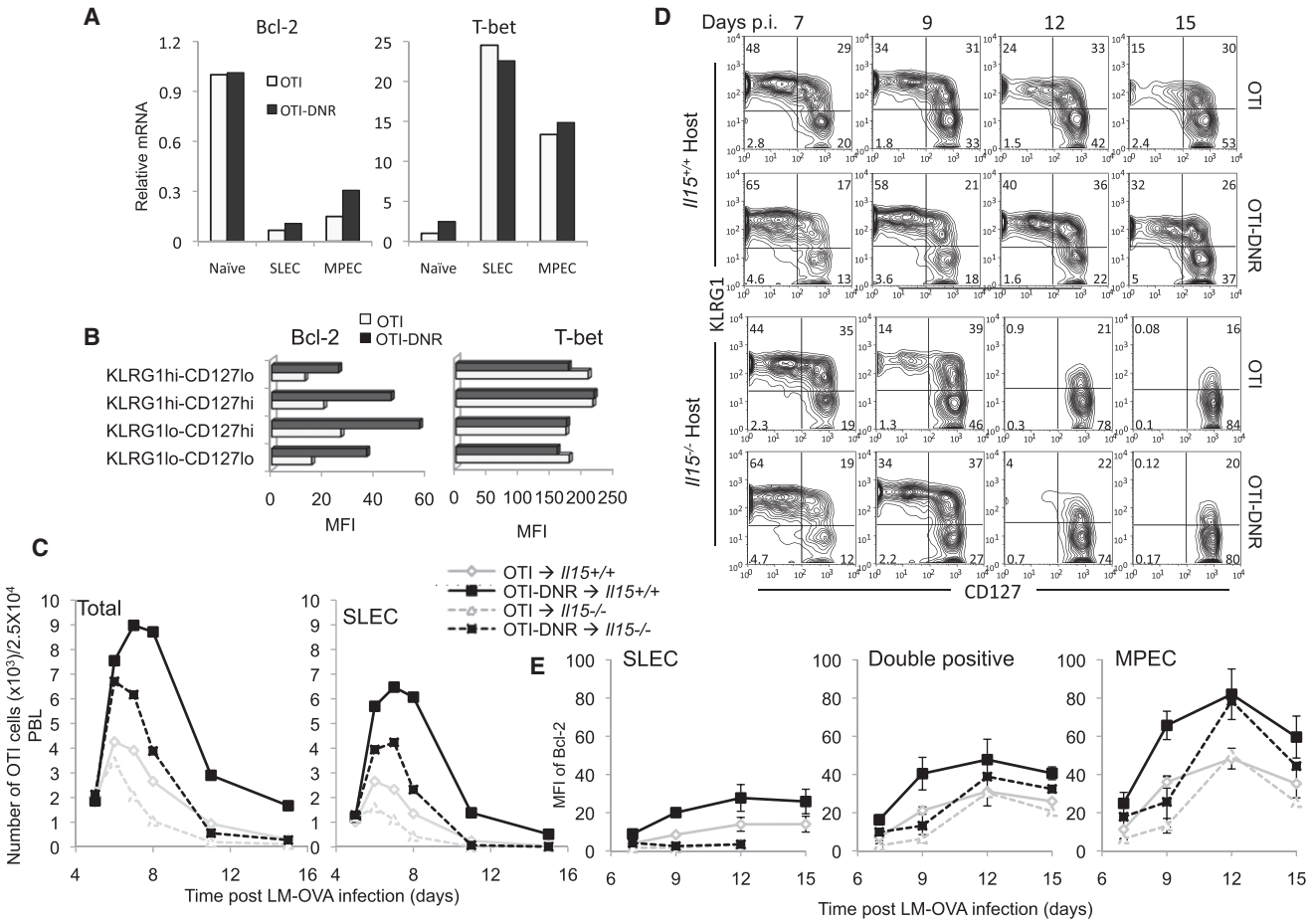

Figure 7. TGF-β and IL-15 Control the Number of SLECs by Exerting Opposing Effects on Bcl-2 Levels In Vivo

(A) Quantitative RT-PCR comparing mRNA expression of Bcl-2 and T-bet in sorted naive (prior to adoptive transfer) and effector (day 7 p.i.) OTI and OTI-DNR SLECs and MPECs. Samples were first normalized to HPRT. Fold change from naive OTI cells is shown. Data are representative of 1 out of 3 independent experiments with similar results.

(B) Intracellular staining of Bcl-2 and T-bet protein in day 7 effector OTI and OTI-DNR subpopulations from a cotransfer experiment. Data are representative of 1 out of 3 independent experiments with similar results.

(C) 2.5 × 10^4 each OTI and OTI-DNR cells were cotransferred into either *Il-15*$^{+/+}$ or *Il-15*$^{-/-}$ hosts followed by LM-OVA infection. Fraction of total (left) and SLEC subpopulation (right) of OTI and OTI-DNR cells were determined among total PBLs and converted to numbers of each cell type per 25,000 PBL.

(D) Representation of the difference seen in the percentage of each OTI and OTI-DNR subpopulations from the spleens of infected *Il-15*$^{+/+}$ and *Il-15*$^{-/-}$ hosts at the indicated time points.

(E) Comparison of intracellular Bcl-2 protein levels in each subpopulation of OTI and OTI-DNR cells isolated from the spleen of *Il-15*$^{+/+}$ or *Il-15*$^{-/-}$ hosts.

major inducer of Bcl-2 in effector CD8$^+$ T cells, we wanted to better understand the intertwined relationship between the survival of SLECs, IL-15, TGF-β, and Bcl-2 expression.

To better understand the in vivo dependence of TGF-β on IL-15 for promoting apoptosis of SLECs, we began by performing longitudinal cotransfer experiments comparing the ratio of OTI and OTI-DNR effector cell subpopulations among total PBLs in *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts (Figures 7C–7D; Figures S7B–S7D). Up to day 5 postinfection, no difference was observed between the ratios of all OTI and OTI-DNR effector cell subpopulations in both *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts, suggesting that proliferation and survival of early effector CD8$^+$ T cells in response to *Listeria* infection is independent of both TGF-β and IL-15. This is consistent with a previous report where, with a similar OTI-LM-OVA infection system, the same numbers of CD127^{hi} and CD127^{lo} OTI cells were found in both *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts at the peak of the immune response (Yajima et al., 2006).

Starting at around day 6 postinfection, the apoptotic effect of TGF-β was observed in both *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts, suggesting that TGF-β can promote apoptosis of clonally expanding T cells independent of IL-15 signaling. In fact, the magnitude of the difference between OTI and OTI-DNR SLECs remained the same in both *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts up until day 11 postinfection. However, the absence of IL-15 had a dramatic effect on the total number of SLECs starting at the peak of clonal expansion and into the contraction phase (around day 7 p.i.) (Figure 7D; Figures S8A, S8C, and S8D). In our OTI-LM-OVA system, similar to previous reports, the clonal expansion of effector cells was not altered by the absence of IL-15; however, IL-15 was essential for the survival of effector cells, in particular SLECs, during the

contraction phase (D'Cruz et al., 2009; Yajima et al., 2006). Together, these data suggest that in response to *Listeria* infection, TGF-β promotes the apoptosis of SLECs during CD8+ T cell clonal expansion independently of IL-15 signaling; however, the eventual survival of the remaining SLECs during the contraction phase is dependent on the combination of prosurvival and proapoptotic effects of IL-15 and TGF-β, respectively.

TGF-β Dampens Bcl-2 Expression Induced by IL-15 in SLECs

To address the mechanism by which TGF-β selectively promotes the apoptosis of SLECs during the contraction phase, we further explored the combinatorial effect of IL-15 and TGF-β on Bcl-2 levels. We performed adoptive cotransfer experiments in *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts and measured intracellular Bcl-2 expression in each subpopulation of OTI and OTI-DNR effector cells, focusing on the peak of clonal expansion and contraction, between days 7 and15 p.i. (Figure 7E; Figure S8B). Once again, the independence of SLECs on IL-15 up until day 7 p.i. and the dependence of SLECs on IL-15 during the contraction phase is depicted in the similarity between cell numbers in *Il15*$^{+/+}$ and *Il15*$^{-/-}$ mice at day 7 p.i. and the dramatic decline of both OTI and OTI-DNR SLECs in *Il15*$^{-/-}$ hosts by day 12 p.i., respectively (Figure 7C; Figure S8A, S8C, and S8D). The rapid contraction of both OTI and OTI-DNR SLECs by day 12 p.i. in *Il15*$^{-/-}$ hosts correlated with an absence of Bcl-2 expression in this subpopulation in both cell types (Figure 7E; Figure S8B). In the presence of IL-15, the Bcl-2 continued to slowly rise in SLECs, demonstrating the dependence of this subpopulation on IL-15 for the induction of Bcl-2. Importantly, OTI-DNR cells continued to have twice as much Bcl-2 at all time points in the *Il15*$^{+/+}$ hosts, which also correlated with better survival of OTI-DNR SLECs during the contraction phase (Figures 7D and 7E). Interestingly, IL-15 also contributed to the total Bcl-2 amounts in CD127^{hi} subpopulations (double positives and MPECs) between days 7 and 12 of the contraction phase (Figure 7E; Figure S8B). These results suggest that IL-15 plays a critical role in promoting the survival of all effector CD8+ T cells during the early phases of contraction. IL-15 and IL-7 signaling appear to have an additive effect on the induction of Bcl-2 in the MPEC subpopulation, whereas IL-15 signaling alone is responsible for inducing Bcl-2 in the SLEC subpopulation. Taken together, we propose a model where TGF-β can inhibit Bcl-2 expression in all effector cell populations; however, it has its most dramatic effect on SLECs. Because of their sole dependence on IL-15 for survival signals, SLECs express very low amounts of Bcl2. By further reducing the already low amounts of Bcl-2, TGF-β drops the Bcl-2 expression in SLECs below the threshold of survival, and therefore selectively promotes apoptosis of this subpopulation (Figure S9).

DISCUSSION

In this study, we addressed the role of TGF-β signaling in effector CD8+ T cells under infectious conditions with *Listeria*. We did not find any differences in OTI function and cell differentiation in CD11c-DNR versus wild-type hosts; however, we cannot formally and completely exclude minor contributions from the CD11c-DNR transgene on our findings. Nevertheless, we describe that the process of clonal expansion of CD8+ T cells is a combination of cell proliferation and cell death, and we identified a role for TGF-β as a key inducer of apoptosis of the short-lived effector CD8+ T cells during an active immune response. Our data suggest that both IL-15 and TGF-β are responsible for controlling the number of short-lived effector CD8+ T cells, where TGF-β promotes their apoptosis during both clonal expansion and contraction and IL-15 promotes their survival during contraction. These findings substantially enhance our basic knowledge of how the number of effector CD8+ T cells is regulated in response to an acute infection and emphasizes the tightly regulated mechanisms involved in the contraction of effector cells.

The apoptotic effects of TGF-β become apparent around day 6 after *Listeria* infection. Several mechanisms can explain why the early effector cells are not influenced by TGF-β. Under appropriate inflammatory conditions and upon antigen encounter, TCR signaling supports the very early survival and proliferation of CD8+ T cells. Shortly after, induction of IL-2 promotes the upregulation of CD25 on early effector cells, supporting their further proliferation and differentiation as well as memory recall response (D'Souza and Lefrancois, 2004; Williams et al., 2006). When clonally expanding T cells were cultured in the presence of IL-2 or a combination of IL-2 and TGF-β, no difference was seen in total numbers of cell recovery or CFSE dilution. By contrast, when cells were cultured with IL-15 in combination with TGF-β, cell recovery dropped to 30% of the recovery seen when cells were cultured with IL-15 alone, which was mostly due to increased cell death. These data suggest that IL-2 may be able to overcome the inhibitory and apoptotic effects of TGF-β and may provide an explanation for the mechanism by which early effector cells in vivo are not influenced by TGF-β. However, once the infection is resolved and IL-2 amounts subside, effector CD8+ T cells lose CD25 expression and their ability to respond to IL-2 is decreased. In the periphery, downregulation of CD25 is followed by a peak in CD122 expression around day 4 p.i. Because, in response to LM-OVA, the absence of IL-15 does not seem to influence the survival and the overall numbers of effector cells during clonal expansion, the heightened CD122 expression around day 4 p.i. may serve to make the effector cells more responsive to residual amounts of IL-2, thus further protecting them from apoptotic effects of TGF-β. Interestingly, effector T cells that migrate to nonlymphoid tissues remain more dependent on IL-2 than those circulating in lymphoid organs (D'Souza et al., 2002); IL-2 may be the arsenal protecting these effector cells from the apoptotic effects of TGF-β. Between days 5 and 7, the effector cells become sensitive to apoptotic affects of TGF-β, in particular the SLECs that do not express IL-7Rα. Several events likely contribute to this sensitivity. First, systemic TGF-β amounts go up, tripling in amount by day 7 postinfection, while at the same time the majority of the effector population becomes KLRG1^{hi}-CD127^{lo} and dependent on IL-15 for survival (Joshi et al., 2007; Yajima et al., 2006). Meanwhile, CD122 and IL-15Rα amounts that had peaked by day 4 now drop to 40%–50% by day 7, making the SLECs less able to compete for limiting amounts of IL-2 and IL-15. The combination of these events probably shifts the balance between the survival signals that SLECs can receive and the proapoptotic signals that they

receive via TGF-β, resulting in the death of the majority of SLECs around the peak of proliferation.

The fact that OTI-DNR cells do contract with similar kinetics as wild-type OTI cells suggests that another TGF-β-independent mechanism, such as cytokine withdrawal, is responsible for the eventual contraction of all effector CD8+ T cells. Comparing the survival of contracting effector CD8+ T cells in *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts, or in the presence of exogenously administered recombinant IL-15, has highly suggested that IL-15 may be the factor that determines the ultimate fate of the SLECs, and our data further support these findings (D'Cruz et al., 2009; Waldmann, 2006; Yajima et al., 2006).

The activity of the proapoptotic molecule Bim is controlled by the relative expression of antiapoptotic molecules such as Bcl-2, Bcl-XL, and MCL-1 and consequently, Bcl-2 and MCL-1 have been shown to be necessary for the survival of memory T cells (Opferman et al., 2003; Wojciechowski et al., 2007). Bcl-2 is downregulated at the peak of infection in activated T cells, and transgenic expression of Bcl-2 can rescue T cells from contraction (Hildeman et al., 2002; Pellegrini et al., 2003). It is thought that cγc cytokines promote the survival of effector T cells by inducing the expression of Bcl-2 (Nakajima et al., 1997; Vella et al., 1998). We observed much higher Bcl-2 expression in MPECs compared to SLECs during early contraction, which seemed to be the result of signaling induced by both IL-7 and IL-15. However, toward the latter part of the contraction phase, Bcl-2 expression in SLECs remained nonexistent in *Il15*$^{-/-}$ hosts and low in *Il15*$^{+/+}$ hosts and Bcl-2 expression stayed high in MPECs and became equivalent in both *Il15*$^{+/+}$ and *Il15*$^{-/-}$ hosts, suggesting that IL-7 eventually becomes the major source of Bcl-2 induction for MPECs. Similar to forced expression of Bcl-2, administration of IL-2, IL-7, or IL-15 can prevent the apoptosis of both CD127^{hi} and CD127^{lo} effector T cells during contraction (Rubinstein et al., 2008; Yajima et al., 2006). This indirectly suggests that toward the middle of the contraction phase, IL-15 becomes limiting even in the *Il15*$^{+/+}$ hosts, thereby further limiting the only Bcl-2-inducing signal that SLECs receive. Meanwhile, the 2-fold impact of TGF-β on Bcl-2 expression becomes much more critical for the SLEC population compared to the MPEC population, causing the already low expression of Bcl-2 to fall below the survival threshold, which ultimately results in the selective apoptosis of SLECs. This model reconciles our current data and provides an explanation for the selective impact TGF-β has on promoting the apoptosis of SLECs during contraction, while allowing the survival and differentiation of MPECs.

TGF-β is synthesized as an inactive latent form that is unable to bind to its receptor. This suggests that high levels of TGF-β in plasma, possibly produced by effector T cells, T regulatory cells, or antigen-presenting cells, do not necessarily translate to a systemic increase in TGF-β responsiveness. Notably, the cells that can activate TGF-β may also be different than those that produce this potent cytokine, and thus this activation step provides a means for TGF-β to integrate signals from multiple cell types (Li and Flavell, 2008a, 2008b). Dendritic cells (DCs) may play a role in activating T cell-produced TGF-β through their surface-expressed αvβ8 integrin (Li and Flavell, 2008a; Travis et al., 2007). In addition, DCs have been suggested to be the prominent source of *trans*-presented IL-15 during acute infection, as indicated by the fact that they upregulate the IL-15-IL-15Rα complex in response to inflammatory stimuli (Dubois et al., 2002; Waldmann, 2006). It is therefore plausible to hypothesize that another mechanism whereby TGF-β selectively induces apoptosis of SLECs relates to the means by which these cells come in contact with *trans*-presented IL-15, which brings them into the vicinity of αvβ8 integrins expressed on the surface of DCs. As a result, the latent form of TGF-β that is abundantly available in plasma during the peak of infection, or made by either the DC or the effector T cell itself, may become locally activated by the αvβ8 integrins. This model would suggest that as the SLECs come in contact with DCs to receive a survival signal through *trans*-presented IL-15, they also become simultaneously exposed to active form of TGF-β in the same local environment. In contrast, because the MPECs can rely on both *trans*-presented IL-15 and soluble IL-7 for survival, they can receive survival signals through IL-7 signaling without exposing themselves to the active forms of TGF-β that potentially exists in the local environment near the *trans*-presented IL-15 on DCs.

The current findings in this study have several potentially important clinical implications. Autoimmunity and inflammatory diseases can be caused by decreased immune suppression, whereas cancers and certain infectious diseases are often associated with increased immune suppression. Induction of TGF-β-producing regulatory T cells has been associated with better prognosis in several autoimmune diseases. For example, a promising immunotherapy for type 1 diabetes (T1D) involves the administration of a CD3-specific monoclonal antibody (Herold et al., 2002), which functions by eliminating autoreactive T cells and promoting the expansion of TGF-β-producing regulatory T cells (Chatenoud and Bluestone, 2007). Our data suggest that a mechanism by which this immunotherapy may function is through a TGF-β-dependent induction of apoptosis of self-reactive effector T cells. Such depleting immunotherapy also generates a lymphopenic environment where IL-15 presumably becomes highly abundant. We therefore would predict that neutralization of IL-15 under such lymphopenic conditions may help to further destabilize self-reactive effector T cells whose survival may be dependent on IL-15. Targeting IL-15 as a means of immunotherapy against many autoimmune diseases has been suggested and is currently under trial (Waldmann, 2006). Our findings in this study support and provide further mechanistic insights into the success of a combinatorial therapy of simultaneously inducing TGF-β and blocking IL-15 to achieve the best immunotherapeutic results against T1D.

Elimination of most tumors by the immune system involves functional CD8+ cytotoxic T lymphocytes. Studies in our laboratory and by other investigators provide compelling evidence that inhibition of TGF-β signaling may address several key mechanisms of resistance and improve the efficacy of tumor immune therapy (Gorelik and Flavell, 2001; Wrzesinski et al., 2007). In the current study we describe a mechanism whereby tumors may prevent their immune-mediated destruction by producing TGF-β and directly promoting the apoptosis of tumor antigen-specific effector CD8+ T cells. In addition, many studies have already demonstrated the efficacy of therapeutic uses of IL-15 for eradicating tumors (Klebanoff et al., 2004; Morris et al., 2006). Our findings strongly support these notions and provide a cellular mechanism by which the combinatorial effects of

blocking TGF-β signaling in addition to administering IL-15 may have the most beneficial therapeutic outcomes during vaccination or tumor immunotherapy. Such combinatorial therapy would most likely lead to enhanced numbers of effector $CD8^+$ SLECs as well as $CD4^+$ Th1 cells (Li et al., 2006a).

EXPERIMENTAL PROCEDURES

Mice

CD4-TGF-βRII-DNR mice (Gorelik and Flavell, 2000) were bred on to OTI Vα2/Vβ5 TCR transgenic specific for $OVA_{257-264}$ that recognizes SIINFEKL epitope in the context of MHC class I $H\text{-}2K^b$. These mice were then crossed to *Rag1*$^{-/-}$ (JAX) and CD45.1 (NCI) congenic markers. CD11c-DNR transgenic mice (Laouar et al., 2005) were maintained on a C57BL/6 background. *Il-15*$^{-/-}$ mice were purchased from Taconic and bred onto the CD11c-DNR mice. All animal experimentation was conducted in accordance with institutional guidelines.

Adoptive Cotransfer and LM-OVA Infection

Every cotransfer experiment consisted of 1.5–2.5 × 10^4 sorted naive OTI and OTI-DNR cells each on a different congenic marker mixed at a 1:1 ratio. One to two days after adoptive transfer, mice were infected with 1 × 10^5 recombinant *Listeria monocytogenes*, expressing OVA (LM-OVA), which was a generous gift from H. Shen (U. Penn).

Isolation of Peripheral Blood Lymphocytes and Calculating Cell Populations among PBLs

100–150 μl of blood from tail vein of infected mice was added to 30 μl of 1× heparin (500 units/ml). 500 μl of 1× ACK lysis buffer (Lonza) was added directly to the cells and incubated at room temperature for 2–3 min. Cells were centrifuged at 4000 rpm for 5 min. The top layer was aspirated and another 500 μl of 1× ACK lysis buffer was added followed by centrifugation. Cells were resuspended in 500 μl of FACS buffer (PBS + 0.5% FBS) and divided into 2–4 wells of a 96-well V-bottom plate and stained with surface antibodies. The same gate was set on a FACS Calibur machine to collect 25,000 live lymphocytes for every time point. The same gate was also set during analysis of the data, and thus the starting number of PBLs was set to 25,000 for every calculation. Total number of host $CD8^+$ T cells was measured as an internal control, which remained roughly around 4,000/25,000 in early and late time points, but increased to 5,000–6,000 at the peak of T cell proliferation. Based on the frequencies obtained from FACS analysis, we then calculated the number of each subpopulation out of 25,000 PBLs.

Isolation of Lymphocytes from Nonlymphoid Organs

Peripheral blood was isolated from tail vein of infected mice as described above. Mice were then euthanized and perfused with 30 mL of cold PBS to remove all the blood from the liver and lung. Liver and lung were removed, and cell homogenates were digested for 1 hr at 37°C with digestion buffer (RPMI + 5% FBS + 100 U/ml DNase I [Sigma] + 0.2 mg/ml Collagenase D [Roche]). Liver homogenates were centrifuged at 300 rpm for 3 min to remove hepatocytes, and lung homogenates were run through a 70 μm filter mesh. Nonhepatic supernatant and lung lymphocytes were centrifuged at 1500 rpm for 10 min. The cell pellet was resuspended in 1 ml of RPMI + 5% FBS and mixed with 4 ml of 27.5% OptiPrep (Axis-Shield; Oslo, Norway). To make a gradient, 1 ml of RPMI was carefully layered on top of the cells and centrifuged at 2700 rpm for 20 min. Lymphocytes were carefully removed from the interface of the gradient and further analyzed.

Antibodies and Reagents

All FACS antibodies used were from BD PharMingen, except for Biotin or PE-CD127 (eBioscience), APC KLRG1 (eBioscience), APC Granzyme B (CALTAG), PE T-bet (eBioscience), Biotin IL-15Rα (R&D Systems), and PE TGF-βRII and isotype control (R&D Systems). Recombinant cytokines mouse IL-15 (R&D Systems), mouse IL-7 (PeroTech Inc.), mouse IL-2 (BD PharMingen), and human TGF-β1 (R&D Systems) were used.

Measuring Plasma TGF-β1

100–200 μl of tail-vein blood was collected and immediately mixed with 30 μl of 25 mM EDTA and immediately put on ice. Samples were centrifuged at 3,000 rpm for 5 min. Plasma was removed, transferred to a new tube, and spun again at 12,000 rpm for 5 min to remove residual RBCs and platelets. Plasma samples were kept at −70°C for future use. TGF-β1 E_{max} ImmunoAssay System (Promega) was used to measure TGF-β1. Plasma samples were acid treated to activate total TGF-β per manufacturer's instructions. Non-acid-treated samples were diluted 2-fold in the provided 1× Sample Buffer.

BrdU Labeling and AnnexinV + PI Staining

Mice were injected intraperitoneally with a single dose of 1 mg BrdU (5-bromo-deoxyuridine; Sigma) on day 6 postinfection. Spleens were harvested 14 hr later and stained first for surface markers. Cells were fixed and permeabilized overnight in 100 μl of 1% PFA + 0.01% Tween 20 at 4°C. Cells were then treated with DNase I (Sigma) at 37°C for 60 min. FITC anti-BrdU Ab (BD) was used to detect cells that had incorporated BrdU. Annexin V-FITC and -APC Apoptosis Detection Kit I (BD PharMingen) was used per manufacturer's instructions.

Intracellular Cytokine Staining

Splenocytes from day 7 infected mice were incubated with 20 μM of SIINFEKL peptide in the presence of BD GolgiStop for 5 hr at 37°C in CO_2 incubator. BD Cytofix/Cytoperm was used to stain for intracellular cytokines, Granzyme B, Bcl-2, and T-bet.

In Vivo CTL Assay

OTI and OTI-DNR day 7 effector cells were purified by cell sorting and mixed at various ratios with CFSE-labeled target cells as previously described (Ingulli, 2007). The mixture of effector and target cells were injected i.v. into naive hosts. 14–16 hr later, spleens from the recipient mice were harvested and the ratio of $CFSE^{hi}$ and $CFSE^{lo}$ cells was measured by FACS analysis as previously described (Ingulli, 2007).

In Vitro T Cell Cultures and Cell Recovery Assay

Naive OTI and OTI-DNR $CD8^+$ T cells were purified by cell sorting. 100 × 10^6 splenocytes were incubated with 200 nM of SIINFEKL peptide at 37°C for 1 hr followed by 2 washes. 3–5 × 10^6 naive cells were mixed with 30–50 × 10^6 peptide-loaded splenocytes and the mixture was labeled with 5 μM of CFSE. Cells were cultured for 2 days in Complete media (Clicks, 10% FBS, L-Glutamine, BME, pen/strep) in the presence of peptide-loaded APCs. Cells were then washed and mixed again with naive live splenocytes in the presence of various cytokines as indicated. Two days later, one half of the media was removed and replenished with fresh media and cytokines. After a total of 6 days (2 days with peptide and 4 days with cytokines), cells were harvested and stained with PE CD45.1, Percp CD8, and APC-AnnexinV. Cells were kept on ice at all times and resuspended in the same final volume before FACS analysis. To determine the total number of recovered cells, each sample was collected on FACS Calibur for exactly 1 min.

Statistical Analysis

Student's t test (two-tailed) was used to calculate the statistical significance of data comparison. p value of ≤ 0.05 was considered to be significant.

SUPPLEMENTAL DATA

Supplemental Data include nine figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00283-0.

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