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IL-6 *trans*-Signaling-Dependent Rapid Development of Cytotoxic CD8$^+$ T Cell Function

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SUMMARY

Immune control of infections with viruses or intracellular bacteria relies on cytotoxic CD8$^+$ T cells that use granzyme B (GzmB) for elimination of infected cells. During inflammation, mature antigen-presenting dendritic cells instruct naive T cells within lymphoid organs to develop into effector T cells. Here, we report a mechanistically distinct and more rapid process of effector T cell development occurring within 18 hr. Such rapid acquisition of effector T cell function occurred through cross-presenting liver sinusoidal endothelial cells (LSECs) in the absence of innate immune stimulation and known costimulatory signaling. Rather, interleukin-6 (IL-6) *trans*-signaling was required and sufficient for rapid induction of GzmB expression in CD8$^+$ T cells. Such LSEC-stimulated GzmB-expressing CD8$^+$ T cells further responded to inflammatory cytokines, eliciting increased and protracted effector functions. Our findings identify a role for IL-6 *trans*-signaling in rapid generation of effector function in CD8$^+$ T cells that may be beneficial for vaccination strategies.

INTRODUCTION

The induction of T cell immunity requires innate immune activation that generates inflammation and leads to maturation of professional antigen-presenting cells (APCs), such as dendritic cells (DCs). Such mature APCs cross-prime naive CD8$^+$ T cells and elicit differentiation into cytotoxic T lymphocytes (CTLs) (Kurts et al., 2010). Ideally, naive T cells receive membrane-associated and soluble costimulatory signals through CD28 together with receptors for interleukin-12 (IL-12) and type I interferon (IFN) in addition to T cell receptor (TCR) stimulation (Curtsinger and Mescher, 2010). Such combination of signals is necessary to achieve optimal stimulation to induce sustained T cell proliferation and acquisition of T cell effector function. This differentiation process takes several days and is governed by a complex network of transcriptional regulators that control cell proliferation, effector function, and survival (Kaech and Cui, 2012). Effector function of CTLs is accomplished by secretion of anti-infectious cytokines such as tumor necrosis factor (TNF) and IFNγ together with expression of death-inducing molecules such as granzyme B (GzmB) or perforin that are crucial for elimination of infected cells (Trapani and Smyth, 2002; Zhang and Bevan, 2011). Key to CTL differentiation and effector cell function are the T-box transcription factors T-bet and eomesodermin (Eomes) (Intlekofer et al., 2005; Pearce et al., 2003). In the absence of T-box transcription factors, T cells fail to correctly differentiate into functional CTLs.

IL-6 is a cytokine with pleiotropic functions that contributes to anti-infectious immunity. IL-6 signals through the IL-6 receptor (IL-6R) and β subunit glycoprotein 130 (gp130). IL-6R exists as a membrane-anchored protein (classic signaling) as well as in a soluble form (*trans*-signaling) that can be detected at sites of inflammation. Because of the restricted expression of IL-6R, many of the biological activities of IL-6 are attributed not to the cytokine alone but to the action of a soluble complex of IL-6 and IL6R, which initiates IL-6 *trans*-signaling by binding to ubiquitously expressed gp130 (Jones et al., 2011). IL-6 has been shown to induce expression of acute-phase proteins, regulate development of DCs, contribute to T helper 17 cell differentiation, and foster B cell development and antibody responses (Jones, 2005; Jones et al., 2011). A contribution of IL-6 signaling to development of CD8$^+$ T cell immunity beyond the induction of inflammation has been suggested (MacLeod et al., 2011) but has not been characterized in detail.

In vivo, priming of naive T cells by mature APCs occurs in lymphoid tissues such as lymph nodes and spleen, which facilitates the encounter of antigen-loaded APCs and naive T cells in highly specialized compartments, i.e., the T cell zones (Junt et al., 2008). Alternatively, naive CD8$^+$ T cells are also stimulated outside of lymphoid tissues in peripheral organs such as the liver (Thomson and Knolle, 2010). Here, a highly abundant population of liver-resident cells, i.e., liver sinusoidal endothelial cells

(LSECs), function as scavenger cells to clear antigens from the circulation and as APCs to cross-present those antigens to circulating CD8$^+$ T cells (von Oppen et al., 2009). Naive T cell stimulation in the liver occurs without the requirement for innate immune stimulation and in the absence of conventional costimulatory signals. Such T cell stimulation by antigen-presenting LSECs results over a period of several days in the generation of memory T cells, thus complementing conventional memory T cell generation induced during inflammation (Böttcher et al., 2013). These memory T cells generated by antigen-presenting LSECs over a period of several days did not show any direct cytotoxic effector function and, similar to conventional memory T cells, required combinatorial stimulation through costimulatory receptors for reactivation (Böttcher et al., 2013).

Here, we report on the discovery of a mechanism that facilitates rapid induction of GzmB expression and effector function within 18 hr in naive CD8$^+$ T cells. This rapid gain of effector function was triggered by antigen-presenting LSECs, but not by mature DCs. Such rapid LSEC-mediated expression of GzmB in T cells was independent of conventional costimulatory signals but required IL-6 *trans*-signaling. Mechanistically, IL-6 *trans*-signaling together with TCR signaling was sufficient for direct and rapid GzmB expression in T cells, thus identifying a unique role for IL-6 *trans*-signaling in the development of effector T cell function.

RESULTS

Naive CD8$^+$ T Cells Rapidly Express Granzyme B after Antigen Presentation by LSECs, but Not DCs

LSECs are potent antigen (cross)-presenting cells resident in the liver. Whereas cross-priming by DCs requires several days for cytotoxic effector T cell differentiation, cross-presentation by LSECs to naive CD8$^+$ T cells leads to an accelerated generation of memory T cells (Böttcher et al., 2013). Here, we investigate the early phase (i.e., the first 18 hr) of the interaction between cross-presenting LSEC and naive CD8$^+$ T cells compared to DCs. Coculture of naive H-2K^b-restricted ovalbumin (OVA)-specific TCR-transgenic CD8$^+$ T cells (OT-I) with LSECs cross-presenting OVA resulted in rapid T cell activation. After 18 hr of T cell contact with cross-presenting LSECs (LSEC-stimulated T cells), increased expression levels of CD25, CD44, and CD69 were observed compared to T cells cultured with LSECs without antigen (Figures 1A and 1B). Such increased expression of activation markers was indistinguishable from that following activation by cross-presenting mature DCs for 18 hr (DC-stimulated T cells) (Figures 1A and 1B). Similarly, activation-induced reduction of CD62L expression was similar between LSEC- and DC-stimulated T cells (Figures 1A and 1B). Although LSECs and DCs were equally efficient in cross-presentation and initial stimulation of naive OT-I T cells leading to expression of cytokines such as IL-2, TNF, and IFNγ during the first 12 hr (Figure 1C), we found one fundamental difference when analyzing proteins important for T cell cytotoxic effector function. Stimulation by cross-presenting LSECs within 18 hr led to strong upregulation of the serine protease GzmB (Figure 1D), which is key for cytolytic function of effector T cells (Heusel et al., 1994; Trapani and Smyth, 2002). In contrast, cross-priming by mature DCs did not induce such rapid GzmB expression within 18 hr (Figure 1D). This finding was corroborated by the prominent upregulation of *gzmb* mRNA expression in LSEC-stimulated, but not DC-stimulated, T cells within 18 hr (Figure 1E). Acquisition of GzmB expression and development of effector function upon classical T cell priming by DCs was only observed after T cells had proliferated (Figure 1F) (Curtsinger et al., 2005b; van Stipdonk et al., 2001). The rapid GzmB expression in LSEC-stimulated T cells, however, occurred before proliferation started, since no carboxyfluorescein succinimidyl ester (CFSE) dilution was observed in GzmB-expressing T cells (Figure 1F), which suggests a distinct developmental process. Time kinetic analysis of *gzmb* gene expression between T cells stimulated by LSECs or DCs confirmed the rapid GzmB induction by LSECs, but not DCs (Figure 1G). These experiments further demonstrated that DC-stimulated T cells had sustained GzmB expression after 48 hr, whereas *gzmb* levels in LSEC-stimulated T cells declined to baseline at this point (Figure 1G). Taken together, these results indicate a fundamental difference between LSECs and mature DCs in the dynamics of GzmB induction, namely a rapid but transient expression induced by LSECs and a later but sustained expression induced by DCs. These two time points (i.e., 18 hr and 48 hr) are referred to here as early and late GzmB induction, respectively.

We next investigated whether antigen presentation in vivo also led to rapid GzmB expression in T cells. We adoptively transferred fluorescence-activated cell-sorted CD45.1$^+$ naive CD44^{low}CD62L$^+$ OT-I T cells into CD45.2$^+$ recipients and challenged these mice with soluble endotoxin-free OVA. After 18 hr, GzmB-positive OT-I T cells were observed in liver, but not lymphoid, tissues (Figure 1H). Consistent with their antigen-specific activation, GzmB-positive T cells also expressed activation markers such as CD44 and CD69 (not shown). Not all transferred T cells isolated from the liver showed increased GzmB expression, which may relate to the fact that in vivo, only some naive T cells engage in closer interaction with cross-presenting LSECs in hepatic sinusoids (von Oppen et al., 2009). Since we transferred only naive CFSE-labeled CD44^{low} T cells, we can exclude that rapid GzmB induction in vivo resulted from reactivation of CD44$^+$ memory T cells. GzmB-positive T cells isolated from liver had not entered the cell cycle, as demonstrated by the absence of CFSE dilution (Figure 1I), similar to LSEC-stimulated GzmB-positive T cells in vitro. These results suggested that LSECs cross-presenting OVA in vivo could be responsible for the rapid GzmB induction in naive OT-I T cells. To address this question, we used a chimeric mouse model where H-2K^b expression is restricted to nonmyeloid cells (bm1 → C57BL/6), which together with the injection of endotoxin-free OVA restricts cross-presentation to liver-resident LSECs in vivo (Böttcher et al., 2013; von Oppen et al., 2009). Transfer of fluorescence-activated cell-sorted naive OT-I T cells into (bm1 → C57BL/6) chimeric mice followed by injection of endotoxin-free OVA resulted in GzmB induction in T cells isolated 18 hr later from liver that was indistinguishable from T cells stimulated in wild-type mice with ubiquitous H-2K^b expression (Figure 1J). No GzmB expression was observed 18 hr after T cell transfer into OVA-challenged (C57BL/6 → bm1) chimeric mice, where only bone marrow-derived APCs,

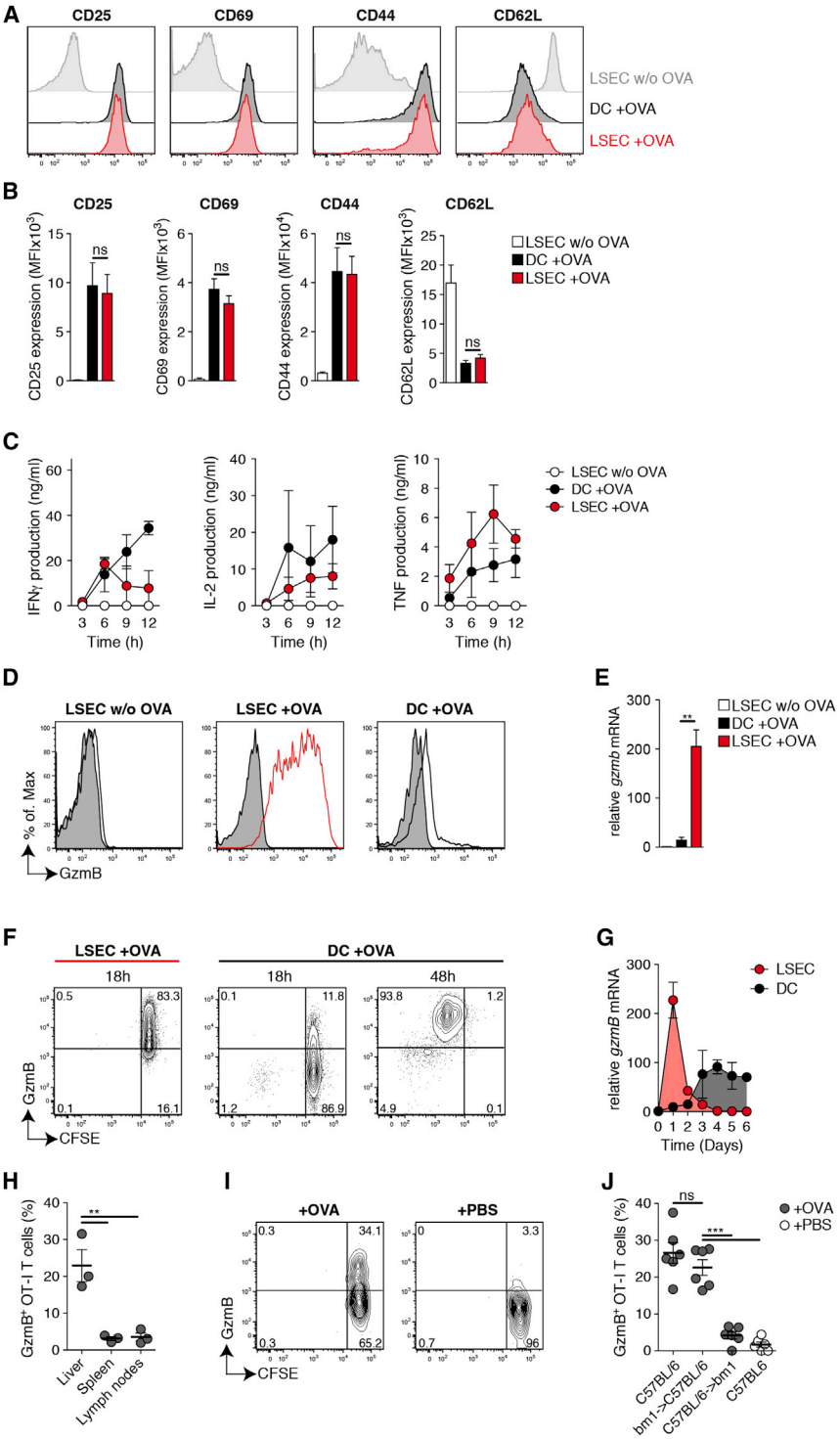

Figure 1. CD8⁺ T Cell Stimulation by Cross-Presenting LSECs, but Not DCs, Leads to Rapid GzmB Induction

(A–E) Analysis of naive OT-I T cell activation 18 hr after in vitro coculture with OVA-cross-presenting LSECs or mature DCs. T cells cultured on LSECs without OVA served as control. (A) Flow cytometric analysis and (B) quantification of surface marker expression. (C) OT-I T cells were cocultured with OVA-loaded splenic DCs or OVA-loaded LSECs for 3–12 hr in vitro. Production of T cell effector cytokines IFNγ, IL-2, and TNF was analyzed by ELISA. Data pooled from two or three independent experiments are shown, and error bars depict mean ± SD. (D) Intracellular GzmB protein. (E) Quantification of *gzmB* mRNA levels.

(F) Analysis of GzmB expression and proliferation in CFSE-labeled T cells.

(G) Time kinetic of *gzmB* mRNA expression in LSEC- or DC-stimulated T cells.

(H and I) Adoptive transfer of fluorescence-activated cell-sorted naive CD45.1⁺CD44^{low} OT-I T cells labeled with CFSE into C57BL/6 wild-type mice receiving 1 mg endotoxin-free OVA. (H) Frequency of GzmB⁺ CD45.1⁺ T cells and (I) analysis of GzmB expression versus CFSE dilution in CD45.1⁺ T cells from the liver 18 hr after OVA application.

(J) GzmB expression in transferred CD45.1⁺ OT-I T cells 18 hr after antigen-specific priming in C57BL/6 wild-type mice, [bm1- > C57BL/6] chimeric mice, or [C57BL/6- > bm1] chimeric mice in vivo. For (B), (E), (G), and (J), data pooled from two to four separate experiments are shown and error bars depict mean ± SEM. In (A), (D), (F), and (I), flow cytometric graphs representative of at least three independent experiments are shown.

LSEC-Stimulated GmzB-Positive T Cells Gain Effector Functions

Analysis of GzmB expression suggested that naive T cells stimulated by cross-presenting LSEC rapidly gained cytotoxic effector function. Consistent with this assumption, TCR restimulation of LSEC-stimulated T cells in vitro resulted in localization of LAMP1 molecules to the cell surface (Figure 2A), indicating that LSEC-stimulated T cells have the potential to secrete GzmB from intracellular stores (Blott and Griffiths, 2002). LSEC-stimulated GzmB-expressing T cells efficiently killed peptide-loaded target cells, whereas unloaded control cells were not affected (Figure 2B). The rapid development of antigen-specific cytotoxicity within 18 hr required cross-presentation, because OT-I T cells cocultured with LSECs in the absence of OVA did not kill target cells (Figure 2B). In contrast, DC-stimulated T cells were not cytotoxic at 18 hr after T cell priming (Figure 2B) but acquired cytotoxic function later (Figure 2C). This suggests but not LSECs, cross-present circulating OVA (Figure 1J). Taken together, these results support the notion that LSEC cross-presentation leads to a unique differentiation process in naive CD8⁺ T cells characterized by rapid GzmB induction within 18 hr.

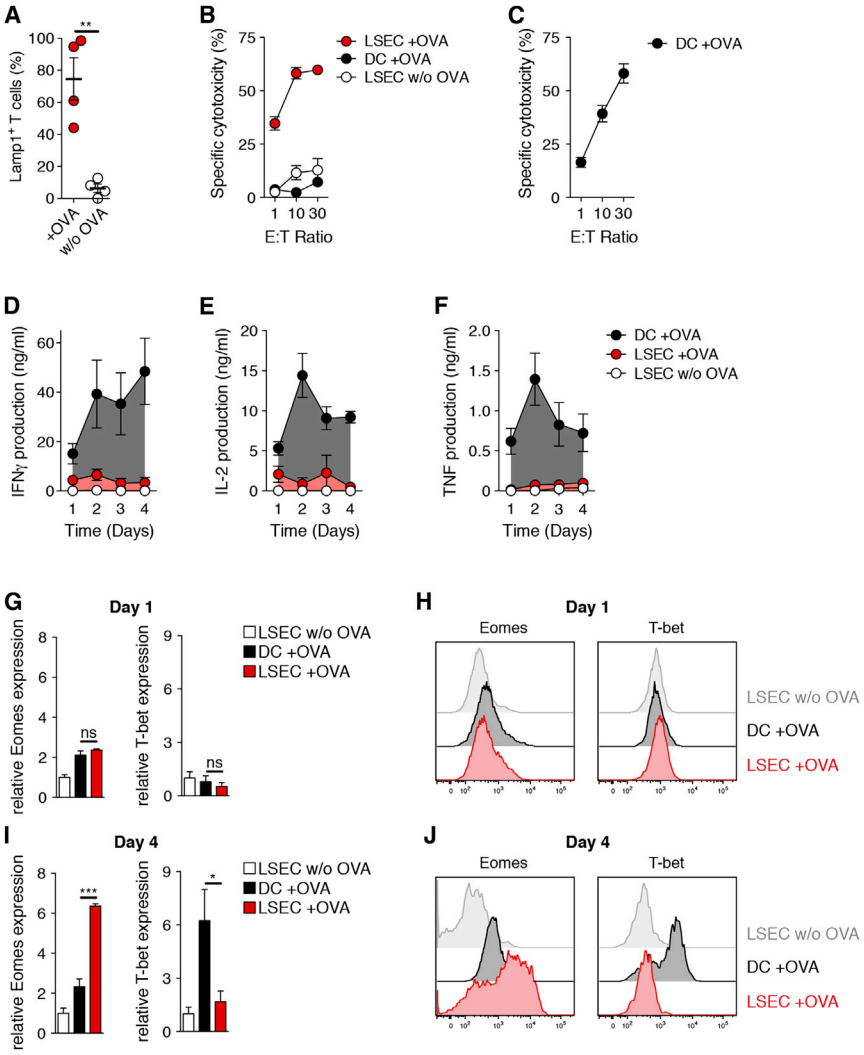

Figure 2. Rapid Acquisition of Effector Functions in LSEC-Stimulated T Cells

(A) Flow cytometric quantification of anti-CD3 induced externalization of LAMP1 surface expression on OT-I T cells cultured with cross-presenting LSECs for 18 hr.

(B and C) Antigen-specific T cell cytotoxicity after 18 hr (B) or 72 hr (C) of coculture with cross-presenting LSECs or DCs.

(D–J) Time kinetic analysis of cytokine release by ELISA (D–F) and flow cytometric analysis of expression of T-box transcription factors Eomes and T-bet (G–J) in OT-I T cells cocultured with cross-presenting splenic DCs or LSECs. In (B), (C), (H), and (J), data from one out of three separate experiments are shown as mean ± SD. In (A), (D)–(G), and (I), data pooled from three to four experiments are shown and error bars represent mean ± SEM.

that development of effector T cell functions occurs via different means through cross-presenting LSECs compared to mature DCs.

Cytotoxic CD8$^+$ T cells are known to also secrete effector cytokines such as IFNγ, IL-2, and TNF (Zhang and Bevan, 2011). LSEC-stimulated GzmB-positive T cells, despite their potent cytotoxic function, did not produce significant amounts of IFNγ, IL-2, or TNF compared to DC-stimulated T cells (Figures 2D–2F), demonstrating that cytokine production does not correlate with rapid acquisition of cytotoxicity. The T-box transcription factors T-bet and Eomes are involved in cytotoxic T cell differentiation by mature DCs (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; Pearce et al., 2003). During the first 18 hr of T cell stimulation by LSECs or mature DCs, no difference in expression of Eomes or T-bet was observed (Figures 2G and 2H). After 4 days, however, we found a reciprocal regulation of these two transcription factors, i.e., increased Eomes expression in LSEC-stimulated T cells and increased T-bet expression in DC-stimulated T cells (Figures 2I and 2J). While these results confirm previous reports on the regulation of T-bet and Eomes expression during canonical cytotoxic T cell differentiation (Böttcher et al., 2013; Intlekofer et al., 2005; Rao et al., 2010), they suggest that rapid GzmB expression and cytotoxic function in LSEC-stimulated T cells may not depend on T-bet or Eomes.

IL-6 *trans*-Signaling Elicits Early GzmB Expression in T Cells

We wondered which signals were responsible for LSEC-induced rapid expression of GzmB in T cells. We first investigated the contribution of TCR signaling strength. LSECs and DCs were equally efficient in cross-presentation over a wide OVA concentration range (Figure 3A), i.e., delivery of signals through the TCR. However, augmenting antigen concentrations did not result in rapid GzmB induction in DC-stimulated T cells (Figure 3B). Notwithstanding the direct correlation of TCR activation and GzmB induction in LSEC-stimulated T cells, the results therefore indicate that the extent of TCR signaling alone does not determine rapid GzmB upregulation in T cells. It rather suggested that LSECs provided a distinct signal to T cells for rapid GzmB induction within 18 hr that is not provided by mature DCs.

We therefore analyzed the contribution of cosignaling molecules to rapid GzmB induction in LSEC-stimulated T cells. Even in the absence of CD28 signals, in T cells stimulated by cross-presenting CD80/86^{dko} LSECs, we observed a robust induction of rapid GzmB expression (Figure 3C). Similarly, neutralizing antibodies blocking the interaction between receptor-ligand pairs relevant for T cell costimulation, such as 4-1BB–4-1BBL, CD40-CD40L, CD70-CD27 or OX40–OX40L, or ICAM-1 did not influence LSEC-induced rapid GzmB expression (Figure 3D). Interestingly, blockade of the coinhibitory receptor PD-1, which controls TCR signaling (Francisco et al., 2010), also did not increase GzmB expression levels (Figure 3D),

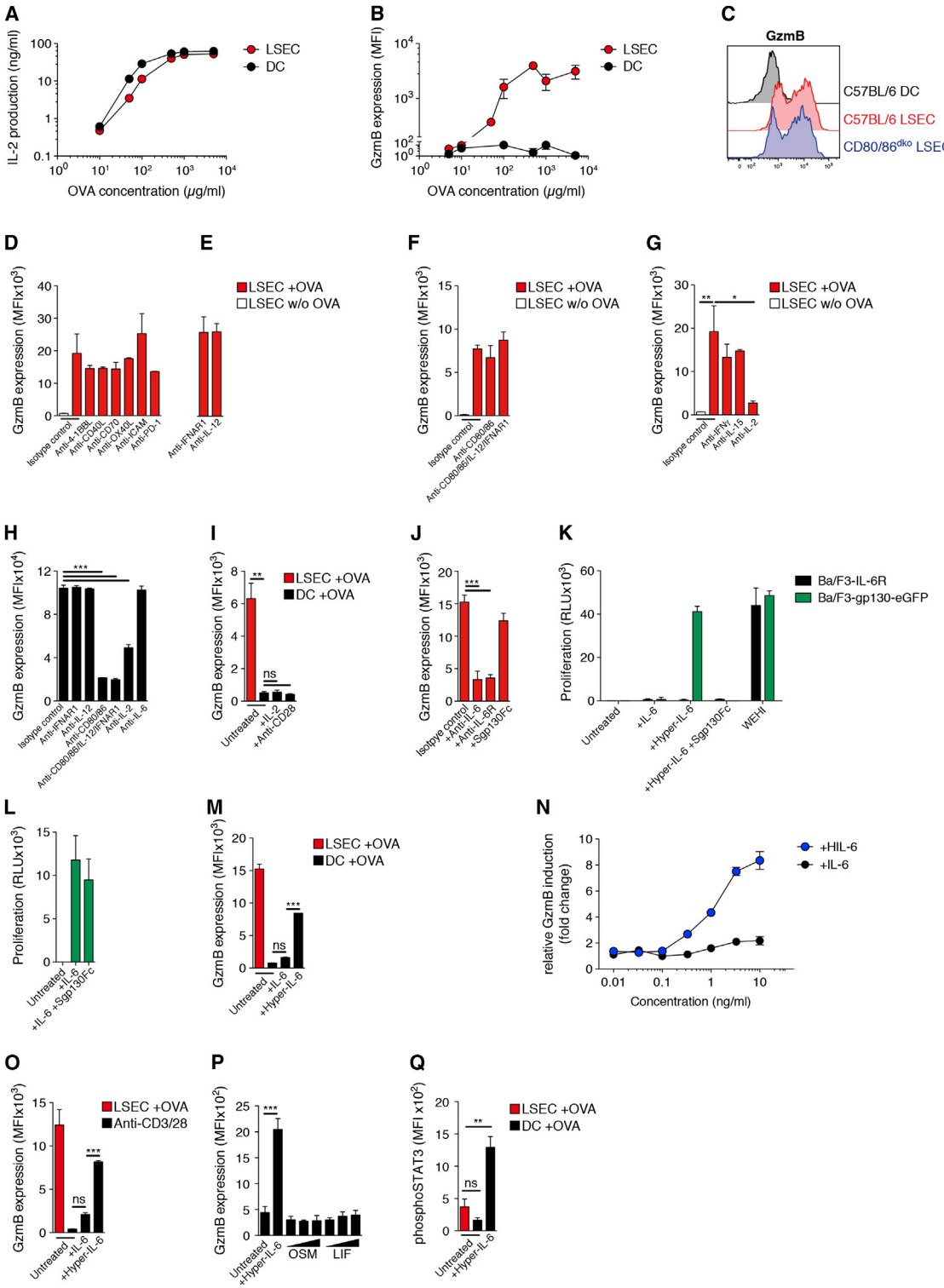

Figure 3. Rapid GzmB Induction in T Cells Is Mediated by IL-6 *trans*-signaling

(A) Cross-presentation determined by IL-2 release from T cells measured by ELISA.

(B) Antigen dose kinetic of GzmB expression in LSEC- or DC-stimulated T cells.

(C) GzmB expression in T cells stimulated by wild-type or CD80/86^{dko} cross-presenting LSECs.

(D–H) Flow cytometric determination of GzmB expression in T cells stimulated by LSECs in the presence of neutralizing antibodies for 18 hr (D–G) or by mature DCs for 72 hr (H).

(legend continued on next page)

strengthening again the notion that GzmB expression in LSEC-stimulated T cells was not regulated by the strength of TCR signaling.

Next, we analyzed the contribution of soluble mediators that serve as cosignaling molecules for T cell activation (Curtsinger and Mescher, 2010). Blockade of IL-12 did not affect GzmB expression (Figure 3E), consistent with lack of IL-12 expression by LSECs (Kern et al., 2010). Similarly, neutralization of type I IFN using anti-IFN alpha receptor antibodies did not modify GzmB expression levels in LSEC-stimulated T cells (Figure 3E). To exclude redundant functions of these cosignaling molecules, we simultaneously blocked CD28, IL-12, and type I IFN signals. This concurrent blockade did not influence rapid LSEC-mediated GzmB induction in T cells (Figure 3F). Also, blockade of other soluble mediators known to enhance T cell effector functions (Freeman et al., 2012), i.e., IFNγ or IL-15, had no effect, but blocking IL-2 strongly reduced rapid GzmB induction in LSEC-stimulated T cells (Figure 3G). Whereas CD28 signaling was dispensable for rapid GzmB induction in LSEC-stimulated T cells (see Figure 3C), it was crucial for late GzmB induction in DC-stimulated T cells (Figure 3H). Neutralization of IL-2 also impaired late GzmB induction in DC-stimulated T cells (Figure 3H). Supplementation of cocultures of mature antigen-presenting DCs and T cells with exogenous IL-2 did not enable rapid GzmB induction in T cells within 18 hr (Figure 3I), which suggests that IL-2 is not sufficient for early GzmB induction but rather acts as a cofactor. Since we observed that LSECs and DCs released large concentrations of IL-6 during cross-presentation to naive T cells (not shown), we tested whether IL-6 signaling was involved in rapid GzmB induction. IL-6 classic signaling requires the presence of IL-6R and the ubiquitously expressed signaling transducing unit gp130. Cells that do not express membrane-bound IL-6R can be activated through IL-6 *trans*-signaling, a process whereby the complex of IL-6/sIL-6R triggers gp130 activation (Jones et al., 2011). Neutralization of IL-6 or blockade of the IL-6R both abrogated rapid GzmB-induction in LSEC-stimulated T cells (Figure 3J), raising the question whether classical or IL-6 *trans*-signaling was involved. The addition of soluble gp130 (sgp130Fc) to cocultures of cross-presenting LSECs and naive T cells did not abrogate rapid GzmB induction (Figure 3J), although *trans*-signaling by soluble IL-6/IL-6R complexes is completely blocked by sgp130Fc (Jones et al., 2011). We therefore determined whether IL-6R expression on cells in *trans* could also lead to IL-6 signaling on an IL-6R-deficient cell population, such as $CD8^+$ T cells (Jones et al., 2011). To address this issue, we used Ba/F3 cells stably transduced with either gp130 (Ba/F3-gp130) or IL-6R (Ba/F3-IL-6R). Whereas Ba/F3-gp130 cells only proliferated in response to hyper-IL-6, but not IL-6 alone (Figure 3K), the addition of IL-6 to Ba/F3-gp130 cells cocultured together with Ba/F3-IL-6R cells was sufficient to induce proliferation in Ba/F3-gp130 cells (Figure 3L). Furthermore, such proliferation even occurred in the presence of sgp130Fc (Figure 3L). These data indicate that not only soluble IL-6/IL-6R complex but also surface-bound IL-6/IL-6R was sufficient to induce IL-6 *trans*-signaling in IL-6R-deficient cells. If IL-6 *trans*-signaling was relevant for rapid GzmB-induction, then IL-6 coupled to its receptor (hyper-IL-6) should lead to GzmB induction in T cells stimulated by antigen-presenting DCs. Indeed, addition of hyper-IL-6, but not IL-6 alone, triggered GzmB expression within 18 hr in DC-stimulated T cells (Figures 3M and 3N). The addition of hyper-IL-6 to anti-CD3/CD28-coated microbeads as artificial APCs also induced GzmB expression within 18 hr (Figure 3O), which demonstrates that IL-6 *trans*-signaling acting on T cells was required and sufficient to drive rapid development of effector functions. Interestingly, other ligands for gp130 such as oncostatin M (OSM) or leukemia inhibitory factor (LIF) failed to induce GzmB expression (Figure 3P). Furthermore, hyper-IL-6 treatment induced STAT3 phosphorylation in T cells in combination with anti-CD3/CD28-coated microbeads within 18 hr, whereas LSEC-stimulated T cells only showed a nonsignificant increase in STAT3 phosphorylation (Figure 3Q). These results indicate that hyper-IL-6 initiates an as-yet-undefined signaling cascade relevant for rapid effector T cell differentiation that needs to be further identified in the future.

Rapid Acquisition of Effector Cell Function Attributes Superior Activation Potential to T Cells

Next, we asked whether the rapid but transient induction of effector T cell function by LSECs (see Figure 1) changed the responsiveness toward further stimulation. Whereas GzmB expression declined in LSEC-stimulated T cells over time (see Figure 1), reactivation of these T cells 18 hr after their initial stimulation through LSECs by mature DCs or anti-CD3/CD28 microbeads led to a sustained and further increase in GzmB expression over several days (Figures 4A and 4B). During such treatment, naive T cells required more than 48 hr to reach similar levels of GzmB expression compared to LSEC-stimulated T cells (Figures 4A and 4B). Also, IFNγ expression was increased upon such reactivation of LSEC-stimulated T cells (Figures 4C and 4D). These findings were confirmed by challenge of T cells stimulated by LSECs for 18 hr or naive T cells with phorbol myristate acetate (PMA)/ionomycin followed by determination of cytokine

(I) Influence of exogenous IL-2 (10 ng/ml) or stimulatory anti-CD28 antibody (10 μg/ml) on GzmB expression in DC-stimulated T cells after 18 hr.
(J) Impact of neutralizing antibodies blocking IL-6 or IL-6R-signaling on rapid GzmB induction in LSEC-stimulated T cells.
(K) Proliferation of Ba/F3-IL-6R or Ba/F3-gp130-eGFP cells after 2 days of in vitro culture in the presence of human IL-6, hyper-IL-6 (both 10 ng/ml), or hyper-IL-6 and 1 μg/ml Sgp130Fc. Murine IL-3 served as a positive control.
(M–O) GzmB expression in T cells 18 hr after stimulation by (K and L) cross-presenting mature DCs or (M) anti-CD3/28 microbeads supplemented with exogenous IL-6 or hyper-IL-6. For (K) and (M), IL-6 and hyper-IL-6 were used at 5 ng/ml and Sgp130Fc was used at 10 μg/ml. Data from one of three independent experiments are shown as mean ± SD.
(P) Analysis of GzmB expression in OT-I T cells 18 hr after stimulation by CD3/28 microbeads supplemented with the gp130-ligands oncostatin M (OSM) and leukemia inhibitory factor (LIF), each at 1–100 ng/ml. Stimulation with CD3/CD28 beads plus 5 ng/ml hyper-IL-6 served as positive control. Data are representative of two independent experiments is shown as mean ± SD.
(Q) Intracellular expression of phospho-STAT3 18 hr after stimulation of naive OT-I T cells with OVA-loaded LSECs, OVA-loaded DCs, or OVA-loaded DCs supplemented with 5 ng/ml hyper-IL-6. Data are representative of two independent experiments and shown as mean ± SD.

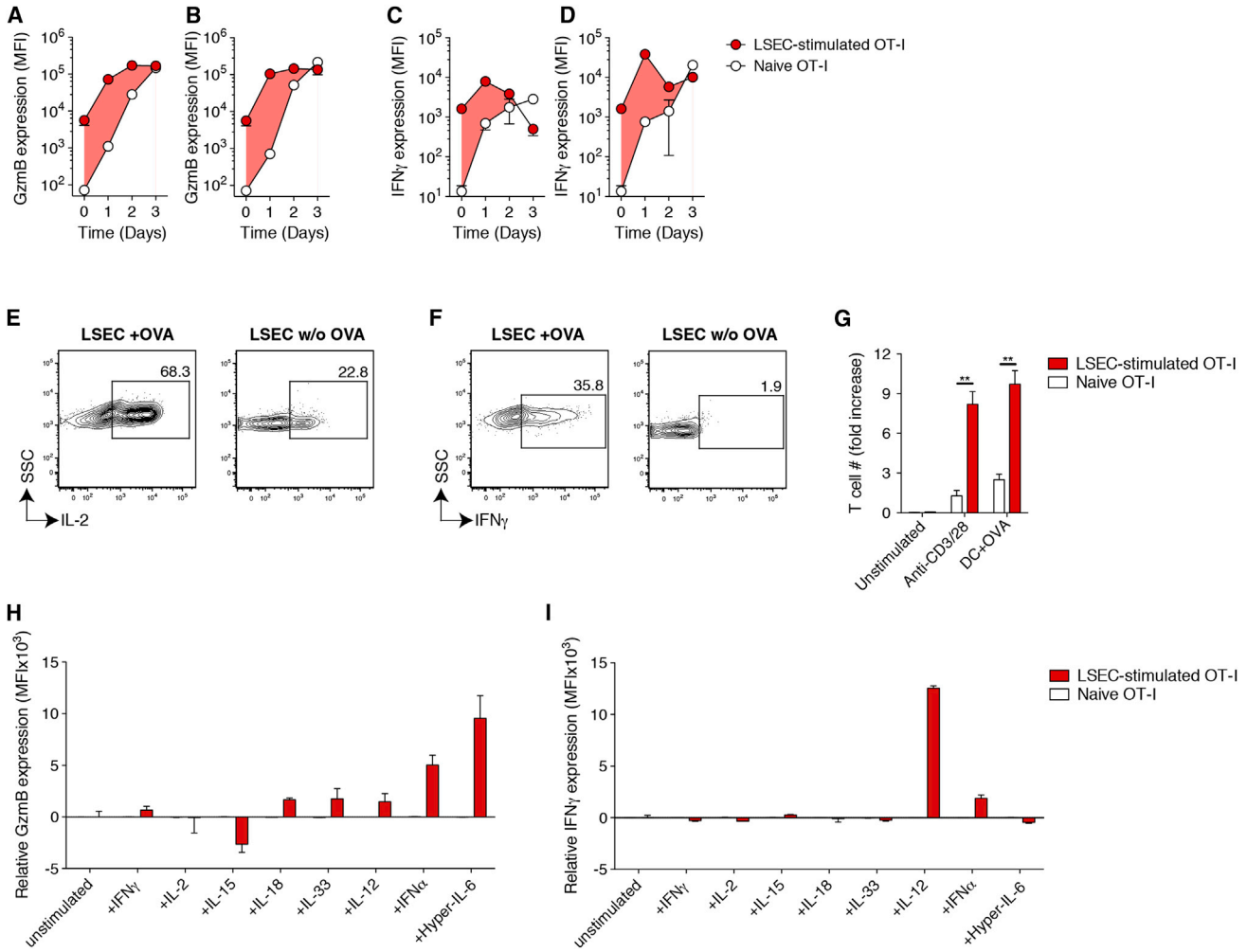

Figure 4. LSEC-Stimulated GzmB-Expressing T Cells Are Responsive to Further Stimulation

(A–D) Naive T cells or LSEC-stimulated GzmB-expressing T cells (after 18 hr) were restimulated with cross-presenting mature DCs (A and C) or anti-CD3/28 microbeads (B and D) for 3 days and analyzed for GzmB (A and B) or IFNγ expression (C and D) after restimulation with PMA/ionomycin.

(E and F) IL-2 (E) and IFNγ (F) production 4 hr after PMA/ionomycin restimulation of LSEC-stimulated GzmB-positive T cells.

(G) Expansion (fold increase) of naive or LSEC-stimulated GzmB-expressing T cells after 3 days of stimulation with anti-CD3/28 microbeads or cross-presenting mature DCs.

(H and I) Increase in GzmB (H) or IFNγ production (I) in naive or LSEC-stimulated GzmB-expressing T cells after 8 hr incubation with inflammatory cytokines. The increase in MFI compared to unstimulated T cells is shown. Data from one of three independent experiments are shown; error bars depict mean ± SD.

production within 4 hr. Under these conditions, LSEC-stimulated T cells showed strong expression of IL-2 and IFNγ, whereas naive T cells did not produce any cytokines (Figures 4E and 4F), demonstrating that GzmB-expressing T cells are more responsive to reactivation than naive T cells. Such increased IL-2 expression together with expression of its receptor CD25 (see Figure 1) suggested a capacity for rapid proliferation. Indeed, LSEC-stimulated T cells showed vigorous expansion within 72 hr of restimulation with antigen-presenting DCs or anti-CD3/CD28 microbeads that were more prominent than proliferation of naive T cells (Figure 4G).

T cell effector function is triggered by antigen-specific stimulation but can also be evoked by cytokines released during inflammation in an antigen-independent fashion (Berg et al., 2003; Freeman et al., 2012; Raué et al., 2004). We therefore determined whether LSEC-stimulated T cells were susceptible for such "innate activation" by inflammatory cytokines. Indeed, we observed a further upregulation of GzmB expression if LSEC-stimulated T cells were incubated with type I IFN or hyper-IL-6 in the absence of antigen (Figure 4H) and a moderate upregulation of GzmB expression upon incubation with IFNγ, IL-12, IL-18, or IL-33 but no effect upon incubation with IL-2 or IL-15 (Figure 4H). Importantly, naive OT-I T cells did not show any GzmB expression under these conditions (Figure 4H). Likewise, both IL-12 and type I IFN evoked production of the T cell effector cytokine IFNγ in LSEC-stimulated T cells, but not in naive T cells (Figure 4I). These results indicate that rapid induction of effector T cell functions by cross-presenting LSECs in the liver may

provide an advantage to mount protective immunity against systemically circulating pathogens.

DISCUSSION

Mature DCs are well known to provide costimulatory signals that initiate development of naive $CD8^+$ T cells into functional cytotoxic effector T cells (Zhang and Bevan, 2011). In particular, certain stimulatory cytokines like interferons and IL-12 are crucial for the acquisition of T cell effector function like GzmB expression and IFNγ production (Curtsinger and Mescher, 2010). This developmental process takes at least 48 hr and correlates with T cell proliferation (Curtsinger et al., 2005a; van Stipdonk et al., 2001). Here, we report the existence of an as-yet-unrecognized developmental process for generation of effector T cells, which occurs much faster and independent of those conventional stimulatory signals through signals delivered by nonimmune antigen-presenting cells. Naive $CD8^+$ T cells stimulated by cross-presenting LSECs acquired within 18 hr transient GzmB expression and killed target cells in an antigen-specific fashion. Although we could not provide formal evidence for expression of perforin, the combination of GzmB induction and the ability to kill target cells strongly suggests that LSEC-primed T cells have a cytotoxic effector function. Such rapid induction of GzmB expression occurred together with a rapidly induced production of cytokines, preceded T cell proliferation, and was independent of signaling through CD28, IL-12R, or IFN alpha receptor. Since T-box transcription factors were not differentially regulated between LSEC-stimulated and DC-stimulated T cells at this early time point, a critical contribution of these factors to rapid GzmB induction appears unlikely, although we have not provided formal evidence for this here. Inhibition of other less prominent costimulatory signals such as 4-1BB or OX40 also did not contribute to this rapid GzmB induction. However, we found that IL-2 was necessary, but not sufficient, for rapid GzmB induction, indicating that IL-2 may serve as an important cofactor. Taken together, these results suggest the existence of another LSEC-derived mediator triggering rapid GzmB induction.

We discovered that IL-6 *trans*-signaling was required for LSEC-induced rapid GzmB induction in T cells and was also sufficient to establish rapid GzmB expression in T cells when coadministered together with cross-presenting DCs. LSECs produced IL-6 during cross-presentation and stimulation of naive $CD8^+$ T cells. IL-6 signaling is only initiated upon association with the IL-6R and binding to the ubiquitously expressed gp130. Since $CD8^+$ T cells do not express IL-6R, *trans*-signaling through soluble hyper-IL-6 (i.e., IL-6 complexed to the IL-6R) is the only means to establish proinflammatory IL-6 signaling in these cells (Rose-John, 2012). Since 50 to 100 IL-6/IL-6R complexes suffice to initiate *trans*-signaling (Jones et al., 2011), it is difficult to directly demonstrate expression of these complexes on the surface of $CD8^+$ T cells stimulated by cross-presenting LSECs. However, hyper-IL-6, but not other gp130 ligands such as OSM or LIF in combination with anti-CD3/28-coated microbeads, sufficed to induce rapid GzmB expression in naive $CD8^+$ T cells, demonstrating that IL-6 *trans*-signaling directly acts on T cells. The complementation of conventional vaccine protocols with hyper-IL-6 may therefore provide an additional benefit by rapid induction of effector functions in combination with the conventional developmental process for the acquisition of cytotoxic T cell effector function by mature DCs.

After several days, LSEC-stimulated T cells resemble central memory T cells with respect to their localization in lymphoid tissue and their requirements for activation and generation of new effector T cells during a recall response (Böttcher et al., 2013). The rapid GzmB induction during the first 18 hr of contact with LSECs may therefore represent an intermediate developmental stage or may serve a particular function during immune responses initiated locally in the liver. It seems unlikely, however, that transient acquisition of cytotoxicity may serve any direct function given the low frequencies of particular antigen-specific $CD8^+$ T cells. Nevertheless, similar to cytokine-induced activation of certain memory T cell populations (Berg et al., 2003; Kupz et al., 2012; Soudja et al., 2012), we found that contact with inflammatory mediators such as interferons or antigen-specific restimulation of GzmB-positive LSEC-stimulated T cells prolonged GzmB expression and led to strong T cell proliferation. Since LSEC-stimulated T cells rapidly relocate from the liver to lymphoid tissues via CCR7 and CD62L (Böttcher et al., 2013), it is possible that these cells predominantly exert their effector functions in lymphoid tissues. As LSEC-stimulated T cells upregulate CXCR3 (Böttcher et al., 2013) and CXCR3-expressing T cells are critical for defense against microbial infections in the subcapsular region of lymph nodes (Kastenmüller et al., 2012), LSEC-stimulated T cells may contribute to antimicrobial defense in the lymph node rather than in the liver.

Taken together, rapid generation of GzmB-positive T cells in the liver may constitute an as-yet-unrecognized arm of antigen-specific immunity against pathogens that are systemically distributed and whose antigens are cross-presented by LSECs, like viruses infecting the liver or lung. The rapid yet transient induction of effector functions in LSEC-stimulated T cells is consistent with our previous observation that these cells at later time points are nonresponsive to restimulation via the TCR (Diehl et al., 2008) but show memory-like functions to support anti-infectious immunity upon combinatorial restimulation through the TCR and costimulatory molecules (Böttcher et al., 2013). The knowledge of the importance of IL-6 *trans*-signaling for this rapid generation of T cells with effector functions may help to implement novel vaccination strategies to increase the efficiency of protective immunity.

EXPERIMENTAL PROCEDURES

Mice

B6.CH-2^{bm1}, CD80/86^{dko}, C57BL/6, CD90.1^+ C57BL/6, TCR transgenic OT-I, and CD45.1^+ OT-I mice were bred under specific-pathogen-free (SPF) conditions in the central animal facility of the University Hospital Bonn. Chimeric animals were generated as described previously (von Oppen et al., 2009). Mice were kept under SPF conditions, and in vivo experiments were approved by the local animal care commission.

Analysis of T Cell Priming In Vivo

Splenic OT-I T cells were enriched by autoMACS using the untouched CD8 T cell isolation kit (Miltenyi), and $CD8^+$CD45.1^+CD44^{low} OT-I T cells were sorted by fluorescence-activated cell sorting (FACS). A total of 1×10^6 were injected intravenously into C57BL/6 wild-type mice or chimeric animals, which

received 1 mg endotoxin-free OVA (Hyglos). Then, 18 hr later, lymphocytes from spleen, liver, or lymph nodes were purified as described previously (Böttcher et al., 2013), and the expression of GzmB was analyzed within $CD3^+CD8^+CD45.1^+$ T cells. In some experiments, fluorescence-activated cell-sorted naive T cells were labeled with CFSE prior to adoptive transfer.

Ba/F3 Cell Experiments

Ba/F3 cells were stably transduced with human IL-6R cDNA and Ba/F3-gp130 cells with enhanced GFP cDNA as described (Ketteler et al., 2002). Expression and purification of IL-6, hyper-IL-6, and sgp130Fc has been described previously (Fischer et al., 1997; Mackiewicz et al., 1992). For the coculture experiment, 5×10^4 cells were cultivated at the indicated ratios for 2 days. Cell viability was determined with Cell Titer Blue Cell viability assay reagent (Promega) following the manufacturer's protocol.

Cell Isolation and Coculture Experiments

Dendritic cells or naive OT-I T cells used in in vitro coculture experiments were isolated from spleen and purified by autoMACS (Miltenyi Biotec). LSECs were isolated by gradient centrifugation followed by immunomagnetic sorting (CD146) (Diehl et al., 2008). Cocultures of naive OT-I cells with LSECs or mature DCs were conducted as described previously (Böttcher et al., 2013). LSECs or mature DCs were loaded with 100 μg/ml OVA, and OT-I T cells were added to antigen-presenting cells in a ratio of 1:1. In some experiments, OT-I T cells were labeled with 1 μM CFSE (Invitrogen) for 15 min at 37°C prior to the coculture assay. Determination of cross-presentation was done by incubation of OVA-loaded APCs with $H\text{-}2K^b$-restricted OVA-specific B3Z cells and analysis of IL-2 release by ELISA after 20 hr. In some experiments, neutralizing antibodies for 41BBL (clone TKS-1), CD40L (MR1), CD70 (FR70), OX40L (RM134L), ICAM (YN1/1.7.4), PD-1 (J43), CD80 (16-10A1), CD86 (GL1), IL-6 (MP5-20F3), IL-6R (polyclonal, AF1830, R&D Systems), IFNγ (XMG1.2), IL-15 (A10.3), IL-2 (JES6-1A12), IL-12 (clone C17.8), or type I IFN signaling (anti-mouse IFN alpha receptor 1, clone MAR1-5A3) were added to cocultures at 10 μg/ml. In some experiments, stimulation of naive T cells by mature DCs or anti-CD3/28 microbeads was done after the addition of recombinant cytokines IL-2, IL-6, hyper-IL-6 (HIL-6), or stimulating anti-CD28 antibody (10 μg/ml, eBioscience).

Quantification of *gzmB* Gene Expression by Real-Time PCR

Total mRNA was isolated using the RNeasy Micro Kit (QIAGEN; including DNase digestion) and transcribed reversely into cDNA using the AffinityScript multiple-temperature reverse transcriptase (Stratagene). Real-time PCR was performed using the absolute QPCR SYBR green PCR mix (Abgene) in combination with specific primers (QuantiTect primer assay; QIAGEN) for murine GzmB and GAPDH. GAPDH was used as reference gene. All real-time PCR reactions were performed in a C1000 Thermal Cycler (Bio-Rad).

Flow Cytometry and Fluorescence-Activated Cell Sorting

Flow cytometric analyses and assessment of mean fluorescence intensity (MFI) were conducted with a LSR Fortessa or Canto II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). LIVE/DEAD fixable violet or near-IR dead cell stain kit (Invitrogen) was used to exclude dead cells in all experiments, and anti-CD16/32 antibody (2.4G2) was used to block unspecific antibody binding via Fc receptors. Antibodies were purchased from Biolegend or eBioscience. The following antibodies were used: CD3 (17A2), CD8α (clone 53-6.7), CD25 (3C7), CD27 (LG.7F9), CD44 (IM7), CD45.1 (A20), CD62L (MEL-14), CD69 (H1.2 F3), CD90.2 (HIS51), $H\text{-}2K^b$ (5F1), and Lamp1 (1DB4). For intracellular staining of cytokines, cells were fixed in 4% PFA and intracellular staining by anti-IFNγ antibodies (XMG1.2) or anti-IL-2 antibodies (JES6-5H4) was performed in permeabilization buffer (eBioscience) for 30 min. Staining of T-bet (eBio4B10), Eomes (Dan11mag), or granzyme B (anti-human, cross-reactive with mouse, clone GB11) was performed using the Foxp3/transcription factor staining buffer set from eBioscience. Quantification of total T cell numbers was done with fluorochrome-labeled microbeads (CountBright absolute counting beads; Life Technologies, Invitrogen). FACS of naive T cells was performed with a DIVA cell sorter (BD). Expression of phospho-STAT3 was determined by flow cytometry after intracellular staining with anti-STAT3pY705 (clone4/P-STAT3) using the phosflow kit from Becton Dickinson. Staining with the corresponding isotype antibody served as control.

Analysis of T Cell Effector Functions

Analysis of GzmB expression was performed directly after isolation of T cells ex vivo or from in vitro cocultures without further stimulation. T cell coculture with αnti-CD3/CD28 microbeads (Invitrogen) was done in the presence of recombinant mouse IL-2 and IL-12 (5 ng/ml). In order to determine the potential of T cells to produce effector cytokines (see Figures 4E and F), T cells were stimulated with PMA (5 ng/ml; Sigma Aldrich) and ionomycin (200 ng/ml, Sigma Aldrich). To analyze T cell proliferation, naive $CD8^+$ T cells were labeled with 1 μM CFSE (Invitrogen) before coculture experiments or adoptive transfer, and CFSE-dilution was measured by flow cytometry. Analysis of cytokine-mediated T cell activation was done by incubating purified T cells for 8 hr at 37°C with cytokines IL-2, IL-15, IL-18, IL-33, IFN-γ, TNF (all 10 ng/ml), IL-12 (5 ng/ml), hyper-IL-6 (5 ng/ml), or IFNα (type 4, 1,000 U/ml) on $CD90.1^+$ splenocytes as feeder cells. Brefeldin A and monensin were added during the last 2 hr of stimulation. Expression of GzmB and IFNγ was subsequently analyzed within $CD90.2^+$ OT-I T cells by flow cytometry. Determination of antigen-specific specific cytotoxicity was determined in vitro (Diehl et al., 2008). Lipopolysaccharide-free OVA (Hyglos) was used for in vivo experiments at a concentration of 1 mg/mouse.

Statistical Analysis

Data were compared using a one-way ANOVA or the unpaired two-tailed Student's t test. Data are shown as mean ± SEM or mean ± SD with $^*p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

AUTHOR CONTRIBUTIONS

J.P.B., O.S., A.Z., S.H., M.B., and W.K. designed and performed experiments with LSEC-primed T cells; C.G. and S.R.J. designed and performed experiments with hyper-IL-6; C.K. and J.L.S. provided key reagents; and J.P.B., S.R.J., and P.A.K. wrote the paper.

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