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Cross-presentation of soluble Ag on MHC class I molecules to naive CD8 T cells by liver sinusoidal endothelial cells (LSECs) leads to induction of T cell tolerance that requires interaction between coinhibitory B7-H1 on LSECs and programmed cell death-1 on CD8 T cells. In this study, we investigate whether cross-presentation of high as well as low Ag concentrations allowed for LSEC-induced tolerance. Ag concentration directly correlated with the cross-presentation capacity of murine LSECs and thus strength of TCR stimulation. Although LSEC cross-presentation at low-Ag concentrations resulted in tolerance, they induced differentiation into effector T cells (CTL) at high-Ag concentrations. CTL differentiation under these conditions was not caused by increased expression of costimulatory CD80/86 on cross-presenting LSECs but was determined by early IL-2 release from naive CD8 T cells. B7-H1 signals from LSECs and TCR avidity reciprocally controlled early T cell release of IL-2 and CTL differentiation. B7-H1 expression directly correlated with cross-presentation at low- but not high-Ag concentrations, indicating an imbalance between TCR and coinhibitory signals regulating T cell release of IL-2. Exogenous IL-2 overrode coinhibitory B7-H1–mediated signals by LSECs and induced full CTL differentiation. Our results imply that LSEC-mediated T cell tolerance can be broken in situations where T cells bearing high-avidity TCR encounter LSECs cross-presenting high numbers of cognate MHC class I peptide molecules, such as during viral infection of the liver. Furthermore, we attribute a novel costimulatory function to IL-2 acting in a T cell autonomous fashion to promote local induction of immunity in the liver even in the absence of CD80/86 costimulation. The Journal of Immunology, 2010, 184: 4107–4114.

Antigen presentation by local APC populations in the liver, such as dendritic cells (DCs), Kupffer cells, and liver sinusoidal endothelial cells (LSECs), is known to result in induction of T cell tolerance rather than in induction of T cell immunity (1, 2). Among these cell populations, LSECs are most prominent with respect to receptor-mediated uptake of soluble Ags from the blood-stream and cross-presentation on MHC class I (MHC-I) molecules to CD8 T cells (3–5). Induction of tolerance in CD8 T cells by LSECs relies on a mutual licensing step, where Ag-specific interaction leads to upregulation of B7-H1 on LSECs and programmed cell death (PD)-1 on CD8 T cells leading to delivery of predominant coinhibitory signals in T cells, in combination with low expression levels of costimulatory molecules, such as CD80/86 (6). Stimulation of naive CD8 T cells by APCs requires significant numbers of peptide-loaded MHC-I molecules to trigger sufficient TCR signaling to sustain stimulation for subsequent differentiation into effector T cells or induction of tolerance (7). In particular, the strength of TCR signaling triggered by peptide-loaded MHC-I molecules has been shown to be critical for proper activation of T cells (8, 9). We therefore addressed the question whether exposure of LSECs to different Ag concentrations and subsequent different levels of peptide-loaded MHC-I molecules as consequence of cross-presentation uniformly resulted in tolerogenic stimulation of naive CD8 T cells. Our experiments demonstrate that high levels of peptide-loaded MHC-I molecules and therefore strong TCR stimulation overcomes tolerance induction in naive CD8 T cells by a novel T cell-intrinsic costimulatory mechanism.

Materials and Methods

Mice

C57BL/6, B7-H1−/−, H2-Kb–SIINFEKL−resticted CD8 + OT-1 TCR, and H2-Dd–SGSNITTPFEL–restricted St42 and St35 TCR-transgenic mice (10) were bred under specific pathogen-free conditions according to the Federation of European Laboratory Animal Science Association guidelines in the central animal facility of the University Hospital Bonn. Six- to 20-wk-old mice were used in accordance with local animal experimentation guidelines.

Abs, reagents, and flow cytometry

Ab-coated magnetic microbeads for cell purification were from (Miltenyi Biotec, Bergisch Gladbach, Germany), OVA from (Serva, Heidelberg, Germany), and was further purified via PD-10 column to exclude peptide-contamination. Abs for flow cytometry, ELISAs, Western blotting, and neutralization of IL-2 were purchased from ebiosciences (San Diego, CA), Santa Cruz Biotechnologies (Santa Cruz, CA), Sigma-Aldrich (Munich, Germany), or BD Biosciences (Heidelberg, Germany). For flow cytometric analysis, cells were stained with saturating concentrations of fluorochrome-labeled Abs for 30 min in FACS buffer (1% FCS, 2 mM EDTA) on ice. Abs were blocking Fc receptor (clone 2.4G2). For proliferation experiments, naive CD8 + T cells were labeled with 1 μM CFSE (Invitrogen, Mannheim, Germany) and incubated with OVA-pulsed APCs for 72 h before CFSE-dilution was measured by flow cytometry. Dead cells were excluded by Hoechst-33258 staining (Sigma-Aldrich). Measurements were
conducted with an LSR II or Canto II (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Cell isolation and T cell stimulation
LSECs were isolated as described previously (6) and purified by immunomagnetic separation using αCD146-beads (Miltenyi Biotec) according to the manufacturer’s guidelines. Isolated LSECs were 98% pure as determined by AcLDL uptake or CD146 staining, seeded at 10^5/well into 24-well plates (Costar, Cambridge, MA) in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS and glucose and quiescent cells were used 2 d after isolation for experiments. DCs were obtained from spleens digested with 0.05% collagenase solution by immunomagnetic separation with αCD11c-beads. CD8 T cells were isolated from spleen, mesenterial, and inguinal lymph nodes by αCD8 microbeads. For activation of naive T cells, LSECs and DCs were pulsed with peptide or Ag for 1 h before addition of 10^6 freshly purified naive CD8 T cells, in an APC/T cell ratio of 1:2. Cytokine release by T cells into the cell culture supernatant was quantified using commercial ELISA assays.

Restimulation of T cells and in vitro cytotoxicity assay
T cells were recovered from cocultures with LSECs or DCs on day 4, and viable T cells were purified by gradient-centrifugation (Nycomed-Pharma, Bochum, Germany). The 10^5 T cells were restimulated by plate-bound αCD3 Ab (145.2C11, 10 μg/ml) in 96 flat-bottom well plates. Release of IFN-γ or IL-2 into supernatant was measured by commercial ELISAs after 16 h. To assess specific cytotoxicity, target RMA cells were loaded with 10 μM SIINFEKL peptide, control RMA cells were kept in PBS for 30 min at 37°C and subsequently washed three times. SIINFEKL-loaded target and control cells (RMA cells) were differentially labeled with 1 µM and 0.1 µM CFSE, respectively, and a 1:1 of target/control cell mixture was incubated with CD8 T cells at different E:T ratios. After 5 h Ag-specific cytotoxicity was measured by flow cytometry and calculated as reduction in CFSE^low target cells compared with CFSE^low control cells: % specific kill = 100 × (CFSE^high/CFSE^low)target/(CFSE^high/CFSE^low)control.

Statistical analysis
Student t test was used to determine statistical significance of results. Results are shown as mean ± SD for representative experiments or ± SEM (SEM), p values < 0.05 were considered significant, *p < 0.05, **p < 0.01, ***p < 0.001.

Results
Cross-presenting LSECs induce CD8 T cell tolerance at low but not high Ag concentrations
Stimulation of naive CD8 T cells by LSEC cross-presenting soluble Ag led to initial T cell stimulation characterized by expression of T cell activation markers and T cell proliferation (Fig. 1A, 1B), as shown previously (6). However, at later time points LSEC-stimulated CD8 T cells became tolerant to reactivation with plate-bound αCD3 Abs and failed to release cytokines, such as IFN-γ/IL-2, or to develop Ag-specific cytotoxicity (Fig. 1C), as previously reported (6). We sought to investigate whether induction of tolerance in naive CD8 T cells through cross-presenting LSEC occurred over a wide range of Ag concentrations. First, we established whether increasing the concentration of Ag (OVA) available for cross-presentation also increased the number of peptide loaded MHC-I molecules by using the H-2K^b-SIINFEKL^-restricted T cell hybridoma B3Z. There was a linear correlation between Ag concentration, Ag uptake by LSECs (data not shown), and subsequent cross-presentation of OVA-derived SIINFEKL-peptide on H-2K^b to B3Z T cells leading to IL-2 release (Fig. 2A). As B3Z cells require only H-2K^b-SIINFEKL^-molecules to trigger IL-2 release, the linear correlation between Ag concentration and IL-2 release allowed us to study whether small as well as large numbers of H-2K^b-SIINFEKL^-molecules presented by LSECs equally led to CD8 T cell tolerance. We found that cross-presenting LSECs induced tolerance in naïve OVA-specific OT-1 T cells at low concentrations of OVA (10 and 100 μg/ml) (Fig. 2B). However, at high-Ag concentration (1000 μg/ml) cross-presentation by LSECs failed to induce tolerance, as OT-1 T cells responded to restimulation with expression of IL-2 and IFN-γ (Fig. 2B, 2C). Similar results were obtained when LSECs were pulsed with increasing concentrations of SIINFEKL-peptide (Fig. 2D). Interestingly, T cells stimulated by LSECs at high-Ag concentration displayed higher expression levels of the activation marker CD25 at day 4 of coculture (Fig. 2E). However, CD62L

FIGURE 1. Phenotype of LSEC-tolerized and DC-activated CD8 T cells. A, Flow cytometric evaluation of surface marker expression on OT-1 T cells at day 1 of coculture with DC or LSEC cross-presenting OVA (100 μg/ml). B, CFSE-dilution profiles of OT-1 T cells on day 3 after priming by cross-presenting DCs or LSECs. C, LSEC- or DC-primed T cells were restimulated with αCD3 at day 4 and cytokine expression was determined by ELISA or specific cytotoxicity against SIINFEKL-pulsed target cells was determined as described in Materials and Methods. One of four independent experiments.
was still high on those T cells (Fig. 2E), indicating differences in immunogenic T cell priming by LSECs and DCs. Taken together, these results suggested that increased TCR signals through more H-2Kb-SIINFEKL–molecules on LSECs were operative in loss of tolerance induction. To more broadly evaluate the relevance of TCR signals for T cell differentiation, we investigated the influence of Ag titration on the ability of DCs to initiate immunogenic CD8 T cell differentiation. Ag concentration directly correlated with the ability of DCs to cross-prime naive CD8 T cells as shown by the numbers of CD69/CD44 positive cells at 24 h (Fig. 3A) and by T cell proliferation at day 3 (Fig. 3B), as expected. Most importantly, cross-priming by DCs at 0.1 or 1 mg/ml OVA both induced T cell differentiation into effector cells that expressed IFN-γ on restimulation (Fig. 3C). Thus, Ag concentration limits the ability of DCs to stimulate T cell differentiation in a quantitative fashion that is restricting the numbers of T cells that can be stimulated, whereas the quality of T cell activation, namely, differentiation into effector T cells is not affected by Ag concentration.

Early expression of IL-2 by CD8 T cells overcomes tolerogenic signals from LSECs

Tolerance induction by LSECs is governed by balanced expression of more coinhibitory B7-H1 molecules over less costimulatory CD80/86 molecules (6). To identify the molecular mechanism involved in the loss of tolerance induction by LSECs, we tested the
a lower TCR avidity also developed into effector cells when primed by cross-presenting LSECs at high-Ag concentrations. St42 and St35 TCR-transgenic T cells, bearing different levels of the same TCR and less TCR molecules than wild-type C57BL/6 or OT-1 mice (Fig. 5A), were all stimulated by peptide-pulsed LSECs and DCs (Fig. 5B). However, LSECs loaded with high-peptide concentrations did not induce CTL differentiation in St35 T cells bearing low TCR levels as compared with St42 T cells bearing much higher TCR levels, which released IFN-γ on restimulation (Fig. 5C). This finding indicates that LSEC-induced CTL differentiation did not occur in T cells with low-avidity TCR. Interestingly, addition of exogenous IL-2 during T cell priming by LSECs again allowed for development of effector function, such as IFN-γ production, even in low-avidity St35 T cells (Fig. 5D). Taken together, these results suggested that increasing signaling strength through the TCR allowed for CTL differentiation through Ag-presenting LSECs by early induction of IL-2 expression in T cells.

Coinhibitory B7-H1–dependent signals from LSECs regulate early IL-2 release by CD8 T cells and control full CTL differentiation

Tolerance induction by LSECs is known to involve the mutual upregulation of B7-H1 on LSECs and PD-1 on TCR-specific interaction (6). We found a direct correlation between Ag-concentration and Ag uptake leading to increased cross-presentation (Fig. 2A) and increased B7-H1 expression on LSEC on Ag-specific T cell interaction (Fig. 6A). At low-Ag concentrations a linear correlation with increasing B7-H1 expression was observed, whereas at high-Ag concentration B7-H1 expression levels ceased to further increase (Fig. 6A). This suggested that curtailed coinhibitory signals in the presence of continuously increasing TCR signals were responsible for early IL-2 release and subsequent T cell differentiation.

We have recently reported that in the absence of coinhibitory B7-H1 signals on LSECs, tolerance induction in T cells could not be achieved (6). Therefore, we studied whether T cells primed by B7-H1−/− LSECs initially released more IL-2 compared with T cells primed by B7-H1+ LSECs. Indeed, there was strong and early IL-2 production by T cells stimulated by cross-presenting B7-H1+/− LSECs (Fig. 6B), which led to differentiation into IFN-γ-expressing T cells (Fig. 6C). Interestingly, in B7-H1−/− LSECs even cross-presentation of low-Ag concentrations, that failed to influence tolerogenic priming by B7-H1+ LSECs, also led to CTL differentiation (Fig. 6C). IL-2 expression was essential for CTL differentiation even in the absence of coinhibitory signals by B7-H1, because neutralizing anti-IL-2 Abs prevented CTL differentiation (Fig. 6D).

Although cross-priming of naive CD8 T cells by LSECs at high-Ag concentration led to CTL differentiation allowing IFN-γ expression on TCR-mediated restimulation (Fig. 2B), these cells failed to show cytotoxic activity (Fig. 7A), in contrast to T cells stimulated by B7-H1+/− LSECs (Fig. 7A). Importantly, Ag titration experiments revealed that induction of T cell cytotoxicity by B7-H1+/− LSECs occurred even at low-Ag concentrations (Fig. 7A). These results indicated that dynamic regulation of TCR signals through coinhibitory B7-H1–mediated signals control IL-2 expression in LSEC-stimulated CD8 T cells and subsequent acquisition of CTL function. We wondered whether B7-H1–mediated signals controlled CTL differentiation by inducing distinct coinhibitory signals in T cells or by restricting early IL-2 expression in T cells. To address this question, we incubated cocultures of cross-presenting LSECs (at low-Ag concentration) and naive CD8 T cells with increasing concentrations of exogenous IL-2 and then determined cytotoxicity 4 d later. Clearly, gain of Ag-specific
cytotoxicity directly correlated with the concentration of exogenous IL-2 present during LSEC-mediated T cell priming even in the presence of B7-H1 (Fig. 7B). These results indicated that a linear and yet dynamic correlation exists between IL-2 induced through strong TCR signals and full CTL differentiation, and identifies IL-2 as a T cell-autonomous costimulatory factor promoting CTL differentiation by cross-presenting LSECs in the absence of strong costimulatory signals, such as CD80 and CD86 or IL-12.

Discussion

The liver is known to induce tolerance rather than immunity in situations, such as organ transplantation or during physiologic situations, when autoantigens or innocuous exogenous Ags are presented by various tolerogenic hepatic cell populations to T cells (1, 2, 12). Whereas conditions are well-defined that are responsible for the functional state of DCs, that is, tolerogenic or immunogenic maturation (13–15), we lack information on the conditions defining immunogenic versus tolerogenic priming by liver-resident APCs, such as LSECs. Signaling through the TCR does not dictate the differentiation of CD8 T cells by itself. The necessity for complimentary signals in addition to the TCR is clearly illustrated by the complex interplay between Ag-presenting DCs, CD4 T cells, and cytokines during the priming period of CD8 T cells (16). Still, duration and strength of TCR signals are equally important for induction of T cell immunity (9), that is, differentiation of naive CD8 T cells as well as expansion and functionality of effector and memory cell populations, and for induction of T cell tolerance (17, 18). This led us to investigate whether the numbers of peptide-loaded MHC-I molecules and thus the strength of TCR signaling influenced tolerogenic T cell priming by cross-presenting LSECs.

LSECs represent a unique liver-resident cell population that is even more potent in cross-presentation than CD8α⁺ splenic DCs when compared at a per-cell basis (19). Cross-presentation of soluble Ag by LSECs in vivo is functional and sufficient to cause Ag-specific retention of circulating naive CD8 T cells to the liver (5). We found that Ag uptake and cross-presentation by LSECs correlated directly with the concentration of soluble Ag, which did not reach saturation demonstrating that Ag processing and peptide-loading onto MHC-I molecules was not restricted even at high-Ag concentrations. We expected to find that induction of T cell tolerance would be compromised at lower concentration of soluble Ags, for example, 10 μg/ml, as induction of T cell
tolerance by cross-presenting LSECs is an active process requiring coinhibitory signaling through B7-H1/PD-1 molecules (6). However, exactly the opposite was found. Even at low-Ag concentration cross-presentation by LSECs was sufficient to drive tolerogenic T cell differentiation. At these low-Ag concentrations, initial T cell stimulation was accompanied by upregulation of CD69, CD44, and PD-1 similar to Ag presentation by DCs (6). In contrast, at high-Ag concentrations cross-presenting LSECs failed to induce T cell tolerance. Instead T cell differentiation into IFN-γ-expressing effector cells occurred. High-dose Ag has been reported to result in CD4 T cell anergy (20), whereas (cross)presentation of high-dose self-Ag by immature DCs or viral Ags during persistent viral infection leads to clonal elimination of CD8 T cells or T cell anergy (18, 21). However, the main factors determining development of CD8 T cell tolerance or immunity are the functional state of the DCs, supporting immunity after functional maturation by delivery of costimulatory signals, as well as the persistence of Ag rather than the Ag dose itself (22).

Induction of CTL differentiation by cross-presenting LSECs at high-Ag concentration was not caused by increased costimulatory signals through CD80/86, IL-12, IFN-α/β, or increased expression of CD40, 4-1BB, ICOS-L, or CD70 (not shown). Tolerogenic signals by cross-presenting LSECs were overcome by IL-2 that was expressed by CD8 T cells themselves early during Ag-specific interaction with LSECs. How was such early IL-2 expression then achieved at high-Ag concentrations? Our data indicate that high-Ag concentration and cross-presentation by LSECs was sufficient to drive tolerogenic signaling complex and thereby restricted downstream signaling events (not shown) suggesting that rather the expression levels of coinhibitory B7-H1 on cross-presenting LSECs were decisive whether CD8 T cell tolerance was induced. It is unclear why LSECs fail to further increase B7-H1 expression under these conditions. We hypothesize that the mechanism causing the restriction in dynamic regulation of B7-H1 expression in response to very strong TCR stimulation by peptide-loaded MHC-I molecules may relate to reverse signaling through B7-H1, which was already described to influence DC function (25).

The early induction of IL-2 by T cells, either as a consequence of strong TCR signals not controlled by sufficiently strong coinhibitory B7-H1 signals or in the total absence of B7-H1 expression on LSECs, triggered CTL differentiation, and thus provided a new paradigm how T cell activation can be achieved in the absence of conventional costimulatory signals through CD80/86 and IL-12. PD-1 controlled TCR signals by recruiting the phosphatase SHP-2 to the TCR signaling complex and thereby restricted downstream signaling events (26). We assume that IL-2 prevented LSEC-mediated tolerogenic programming of naive CD8 T cells, because only the presence of IL-2 during the first 36 h of Ag-specific interaction between tolerogenic LSECs and naive CD8 T cells inhibited tolerance induction (A. Limmer, unpublished observation). Programming of naive CD8 T cells into fully functional CTL has been reported to occur within 24 h of interaction with immunogenic DCs (27), which again emphasizes the role of early IL-2 in the overcoming of LSEC-induced tolerance. Our findings further imply that IL-2 serves as a T cell autonomous costimulatory signal to drive T cell differentiation and

FIGURE 5. Influence of TCR avidity on T cell-differentiation by LSEC. A, TCR surface levels determined with TCRβ-specific Ab for CD8+ T cells from C57BL/6, OT-1, St42, and St35 mice. B, Increase in total CD44 expression on CD8+ T cells primed by peptide-pulsed LSECs or DCs (SIINFEKL 10 nM for OT-1 and SGSNTPPEI 10 nM for St42 and St35). C, Restimulation of equal numbers of CD8+ T cells from B, IFN-γ release was determined by ELISA. D, St35 T cells primed by peptide-pulsed LSECs or DCs ± exogenous IL-2 (10 ng/ml) were restimulated for 24 h at day 4 and IFN-γ release was determined. One of three independent experiments.
may constitute a novel mechanism whereby T cell immunity can be generated by organ-resident APCs lacking conventional cosstimulation. Our findings also suggest that this mechanism may play a role during viral infection of the liver where large Ag quantities are present in combination with T cells with high-avidity TCRs against foreign viral Ags.

**FIGURE 6.** B7-H1 on cross-presenting LSECs restricts IL-2 expression by T cells. A, The dynamics of OVA uptake by LSECs pulsed for 15 min and B7-H1 expression after 24 h on LSECs cross-presenting different concentrations of OVA to naive OT-1 T cells was determined. B, IL-2 release from naive OT-1 T cells during the first 24 h of contact with cross-presenting wild-type or B7-H1−/− LSECs. C, IFN-γ-release by OT-1 T cells on day 4 after priming by wild-type or B7-H1−/− LSECs at different Ag concentrations. D, Neutralizing αIL-2 was added during cocultures of cross-presenting B7-H1−/− LSECs with naive OT-1 T cells. Specific cytotoxicity of OT-1 T cells against SIINFEKL-pulsed target cells was determined at day 4 (E:T ratio 50:1). One of three independent experiments.

**FIGURE 7.** IL-2 is the key molecule inducing CTL-differentiation through cross-presenting LSECs. A, OT-1 T cells were primed by wild-type or B7-H1−/− LSEC cross-presenting OVA at high (1000 μg/ml) or low (100 μg/ml) concentration for 4 d before specific cytotoxicity was determined. B, OT-1 T cells were primed by cross-presenting (100 μg/ml) LSECs in the presence of exogenous IL-2; specific cytotoxicity was determined at day 4 of coculture. One of three independent experiments.
Importantly, exogenous IL-2 overrode the tolerogenic signals provided by LSECs. This implies that IL-2 released from other immune cells with different Ag specificity could change the outcome of intrahepatic T cell priming. Thus, memory CD4 T cells known to produce significant concentrations of IL-2 (28) may provide costimulatory IL-2 to induce local CTL immunity, if they were recruited to the liver. Although LSECs are unlikely to Ag specifically recruit CD4 T cells to the liver due to low MHC class II expression and inefficient MHC class II-restricted Ag presentation (29–31), local activation of memory CD4 T cells in the liver by Ag-presenting Kupffer cells or hepatic DCs may occur and modulate the outcome of LSEC-induced T cell priming. Indeed, in the presence of activated memory CD4 T cells providing exogenous IL-2, LSECs also induced differentiation of naive T cells into effector CTLs (not shown). Thus CTL differentiation by LSECs is clearly different from DC-induced T cell immunity (32, 33), which primarily leads to functional maturation of DCs that further drives CD8 T cell immunity (34). Taken together, our experiments demonstrate a unique mechanism of inducing CD8 T cell immunity by liver-resident Ag-presenting LSECs.

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Disclosures
The authors have no financial conflicts of interest.

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