Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance

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Myeloid antigen-presenting cells (APC) are known to cross-present exogenous antigen on major histocompatibility class I molecules to CD8+ T cells and thereby induce protective immunity against infecting microorganisms. Here we report that liver sinusoidal endothelial cells (LSEC) are organ-resident, non-myeloid APC capable of cross-presenting soluble exogenous antigen to CD8+ T cells. Though LSEC employ similar molecular mechanisms for cross-presentation as dendritic cells, the outcome of cross-presentation by LSEC is CD8+ T cell tolerance rather than immunity. As uptake of circulating antigens into LSEC occurs efficiently *in vivo*, it is likely that cross-presentation by LSEC contributes to CD8+ T cell tolerance observed in situations where soluble antigen is present in the circulation.

The outcome of immune responses, that is, immunity or tolerance, depends on factors such as the nature of the antigen, route of antigen administration, type of antigen presenting cells (APC) and local micro-environment. Lymphatic tissues are the prototype of an immune-stimulatory microenvironment where professional APC induce T-cell activation[1]. Professional APC are APC that are well characterized, express all costimulatory molecules, prime immune responses and induce immunity. The constitutive expression of costimulatory molecules and the exceptional capacity of dendritic cells and macrophages to cross-present exogenous antigens via major histocompatibility (MHC)-class I molecules to CD8+ T cells are crucial for a successful defense against pathogenic micro-organisms and tumors[2]. Cross-presentation, initially identified by Bevan[3], is a function found exclusively in myeloid cells, thereby restricting CD8+ T-cell immunity to professional APC (ref. 2). After antigen uptake, the molecular mechanism of cross-presentation involves an unidentified transport system for proteasomal processing[4]. Although mostly associated with immune stimulation, cross-presentation of antigen by professional APC in draining lymph nodes does not necessarily lead to activation of CD8+ T cells, but may result in tolerance[5].

Particulate antigens and subcutaneous antigen application induce immunity[6], but soluble antigen injected intravenously induces tolerance[7,8]. Similar to tolerance induction after intravenous injection of antigen, intraportal antigen application results in systemic antigen-specific tolerance, indicating that the liver is particularly capable of actively inducing peripheral tolerance[9,10]. T cells pass the liver on estimation several hundred times per day and the unique hepatic microcirculation allows for interaction between T cells and cells of the hepatic sinusoids, that is, Kupffer cells and liver sinusoidal endothelial cells[11,12] (LSEC). Once arrested in the liver, T cells are exposed to tolerogenic mediators—such as IL-10, TGF-β and prostaglandins—that are part of the physiological hepatic micro-environment[13]. Along the hepatic sinusoids MHC-class II positive cells have been detected by immunohistochemistry[14] indicating that regulation of immune responses occurs constitutively in the liver. In studies of immunity against allo-antigens, Kupffer cells[15] as well as special subsets of dendritic cells[16,17], the liver dendritic cells (LDC), were shown to be involved in hepatic tolerance induction. Regulation of the immune response in the liver against soluble antigens, however, has not been addressed in detail, although food antigens and bacterial products from the gastrointestinal tract are abundantly present in portal venous blood and are cleared together with circulating antigens from the blood by the liver.

The induction of tolerance in the liver towards soluble antigens requires an APC that efficiently scavenges antigen and is able to present it on MHC-class I and II molecules to T cells. Candidate cells fitting all requirements are the liver sinusoidal endothelial cells (LSEC) lining the hepatic sinusoids. These cells not only have intimate contact with T cells passing the liver, but in addition are reported to scavenge circulating antigens via pattern recognition receptors[18]. A series of surface molecules associated with professional APC are constitutively expressed by LSEC, such as MHC-class I and II, CD54 (ICAM-I), CD106 (VCAM-I) and the costimulatory molecules CD80, CD86 and CD40, indicating an immunological role of this resident hepatic cell population[19]. Indeed, LSEC are capable of presenting soluble antigen
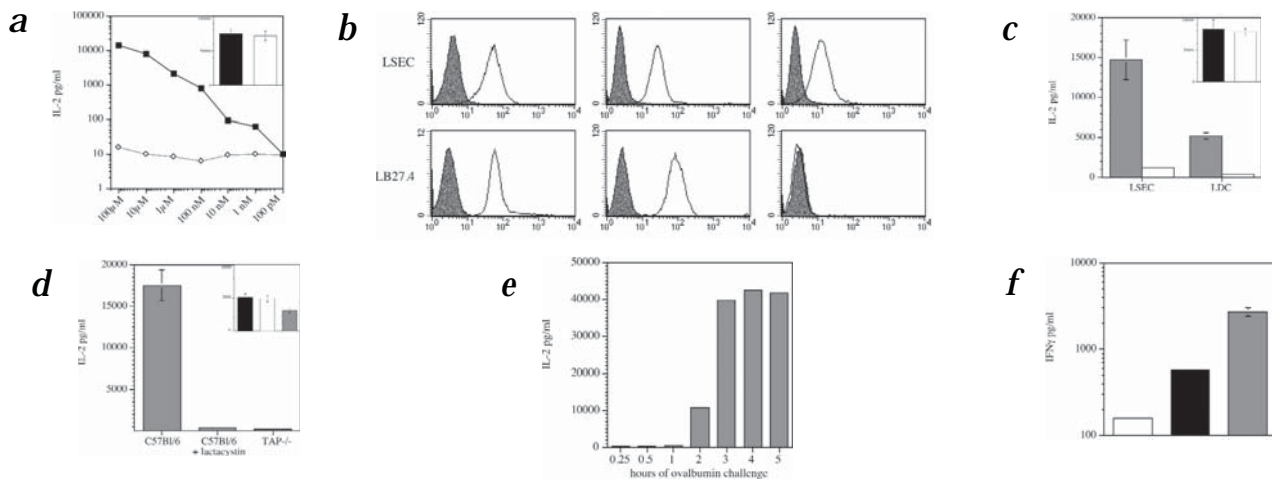
Fig. 1 Cross-presentation in LSEC is efficient and depends on the proteasome and TAP. *a*, IL-2 release by B3Z cells after contact with primary cultures of LSEC or LB27.4 pulsed with different concentrations of ovalbumin. LSEC, ■ ; LB27.4, ◇. Inset: IL-2 release by B3Z cells after peptide loading of LSEC and LB27.4 (LSEC,■; LB27.4, □). *b*, Comparison of antigen-uptake (left), MHC-class I expression (middle) and expression of K[b+-SIINFEKL] (right) between LSEC and LB27.4. Shaded areas represent cells not challenged with antigen. *c*, Ability of ovalbumin-pulsed LSEC or LDC to induce IL-2 release from B3Z cells (■, 1×10^4 antigen presenting cells per well; □, 6×10^2 antigen presenting cells per well). Inset: IL-2 release from B3Z cells after peptide loading of LSEC (■) or LDC (□). *d*, Cross-presentation of exogenous ovalbumin was measured in LSEC isolated from TAP-deficient mice or in LSEC isolated from C57BL/6 mice pre-incubated with lactacystin. Inset: IL-2 release from B3Z cells after peptide-loading of LSEC (LSEC, ■; lactacystin-treated LSEC, □; LSEC from TAP-deficient^{-/-}, ■). *e*, Time kinetics of LSEC cross-presentation to B3Z cells. All experiments shown are representative of at least three independent experiments. *f*, Cross-presentation of a non-glycosylated antigen (β-gal) to specific CD8^{+} T cells. T cell stimulation is measured by release of IFN-γ (CD8^{+} T cells + β-gal,□; CD8^{+} T cells + LSEC + β-gal, ■; CD8^{+} T cells + LSEC + specific peptide, ■).

to naive CD4^{+} T cells and induce a regulatory phenotype in them[19].

The contribution of dendritic cells to regulation of immune responses has been well defined. Here we report that non-myeloid, organ resident endothelial cells of the liver are capable of cross-presentation of soluble exogenous antigens on MHC-class I molecules to CD8^{+} T cells. The functional outcome, however, of CD8^{+} T cell stimulation by cross-presenting LSEC is tolerance.

Presentation of exogenous protein to CD8^{+} T cells by LSEC

To investigate cross-presentation in LSEC, we established pure primary cultures of murine LSEC. Here we show that LSEC efficiently cross-presented exogenous ovalbumin on K[b] molecules, as judged by IL-2 release from the CD8^{+} T-cell hybridoma line B3Z, recognizing the specific peptide SIINFEKL (Fig. 1*a*). The amount of IL-2 released by T cells depended on the antigen concentration used to pulse LSEC (Fig. 1*a*). Ovalbumin concentrations as low as one nM (45 ng/ml) used for pulsing of LSEC were sufficient to induce IL-2 expression in B3Z cells, demonstrating that antigen uptake and antigen processing occurred efficiently in LSEC.

Antigen uptake alone, however, is necessary but not sufficient to endow a cell with the capacity for cross-presentation. A B-cell line (LB27.4) that endocytosed ovalbumin as efficiently as LSEC and expressed comparable levels of MHC-class I molecules on their surface (Fig. 1*b*) still did not induce IL-2 release from CD8^{+} T cells (Fig. 1*a*). MHC-class II restricted presentation was, however, detected in these cells (data not shown). Unambiguous evidence for cross-presentation of ovalbumin in LSEC was obtained from staining of K[b] molecules loaded with SIINFEKL (K[b-SIINFEKL]) at the cell surface with the monoclonal antibody 25-D1.16. The inability of B cells to process endocytosed ovalbumin for MHC-class I presentation is reflected by the absence of K[b+-SIINFEKL],

whereas a homogenous cell population of ovalbumin-pulsed LSEC was positive for K[b-SIINFEKL] (Fig. 1*b*). Titration experiments further revealed that less than 100 cross-presenting LSEC were sufficient to induce IL-2 release from B3Z cells (data not shown). At this cell number, contaminating LDC are unlikely to be responsible for the observed cross-presentation. We found, however, that LDC are generally capable of cross-presenting exogenous ovalbumin to B3Z albeit with lower efficiency than LSEC (Fig. 1*c*). At concentrations likely to be a contaminating cell population in LSEC (≤ 1%), we did not detect any significant stimulation of B3Z by LDC (Fig. 1*c*).

As in dendritic cells, LSEC process and cross-present exogenous antigens by mechanisms that depend on the proteasome and the peptide transporter associated with antigen processes TAP (ref. 4). After incubation of LSEC with the proteasome inhibitor lactacystin (1 μM), cross-presentation was reduced by more than 90% compared to untreated controls (Fig. 1*d*). Furthermore, lack of cross-presentation was observed when LSEC from mice deficient in the gene encoding TAP (TAP-deficient^{-/-}) mice were used (Fig. 1*d*). Cross-presentation by LSEC was not only efficient but also proved to occur rapidly after antigen challenge. Two hours after ovalbumin pulse and fixation with paraformaldehyde, LSEC cross-presented ovalbumin to CD8^{+} T cells, reaching a maximum of cross-presentation four hours after antigen challenge (Fig. 1*e*).

Cross-presentation by LSEC was not limited to ovalbumin but was observed for other glycoproteins such as lymphocytic choriomeningitis virus (LCMV) glycoprotein (data not shown). Glycosylation, however, was not necessary for protein antigens to become cross-presented by LSEC, because β-galactosidase (β-gal)-specific CD8^{+} T cells (0805B) were stimulated by LSEC pulsed with *Eschericia coli* derived β-gal (Fig. 1*f*) to release interferon gamma (IFN-γ). Together, these findings support the rapid and
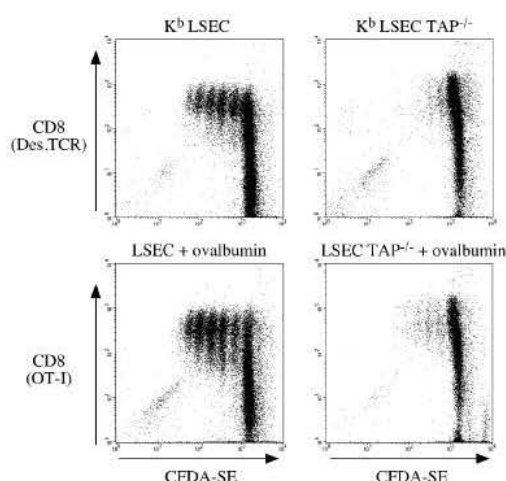
Fig. 2 LSEC induce proliferation of naive CD8^{+} T cells. K[b] LSEC or K[b] LSEC pulsed with ovalbumin were incubated with CFDA-SE labeled naive T cells from either Des.TCR or OT-I mice, respectively. LSEC from TAP-deficient^{-/-} mice were used accordingly.

efficient scavenger function described for LSEC (ref. 18) further stressing the importance of cross-presentation by LSEC.

Cross-priming of naive CD8^{+} T cells through LSEC

Because LSEC constitutively express costimulatory molecules such as CD80, CD86 and CD40 (ref. 19) we investigated whether LSEC had the ability to prime naive CD8^{+} T cells. We used two different transgenic mouse lines expressing K[b]-restricted T-cell receptors that recognize different antigens. Though CD8^{+} T cells of Des.TCR mice recognize an endogenous peptide on K[b] (ref. 20), CD8^{+} T cells of OT-I mice recognize the peptide SIINFEKL on K[b] after uptake and processing of ovalbumin by the APC (ref. 5). The T cells of both TCR transgenic lines were labeled with the fluorescent dye CFDA-SE and incubated with either LSEC from C57BL/6 or TAP-deficient^{-/-} mice. Antigen-presenting LSEC induced proliferation of CD8^{+} T cells *in vitro* and presentation of endogenous and exogenous antigen on K[b] molecules by LSEC required the presence of TAP (Fig. 2).

LSEC cross-present ovalbumin *in vivo*

The liver is known to take up the bulk of circulating antigens[21]. We assume cross-presentation by LSEC is relevant *in vivo* because intravenously injected ovalbumin was found to accumulate in sinusoidal lining cells (Fig. 3a and ref. 22) that were

identified as LSEC by uptake of the endothelial-cell–specific substrate, acetylated LDL (Fig. 3b). We did not observe a comparable uptake of ovalbumin into other organs such as spleen (Fig. 3a), kidney or lung (data not shown). Professional APC such as dendritic cells, however, can present antigen although antigen uptake[23] is below the level of detection. Our inability to detect antigen uptake in our system does not exclude the possibility that cells in other organs (cross-)present ovalbumin to T cells.

In order to determine the contribution of sinusoidal cells and hepatocytes to cross-presentation after contact with antigen *in vivo*, we isolated hepatocytes, Kupffer cells and LSEC from mice that had been injected intravenously with ovalbumin (10 μmol/mouse) two hours previously. In contrast to hepatocytes, LSEC cross-presented ovalbumin to CD8^{+} T cells (Fig. 3c). Because hepatocytes cannot be completely separated from LSEC (1–2% in hepatocyte cultures), we assume that the small amounts of IL-2 detected after incubation of B3Z cells with hepatocyte cultures (Fig. 3c) resulted from contaminating LSEC. We detected antigen uptake and cross-presentation by Kupffer cells at high antigen concentrations (data not shown) which corroborates the capacity of macrophages to cross-present soluble antigens[24].

To prove the relevance of the cross-presentation by LSEC observed *in vitro*, we established a new model system in which LSEC from one animal were adoptively transferred into another animal and orthotopically implanted in the hepatic sinusoid (Fig. 4a). When transferred into mutant B6.C-H2[bm1] mice that harbor point mutations in the K[b]-binding groove preventing presentation of SIINFEKL on K[b], ovalbumin-pulsed LSEC from wild-type C57BL/6 mice were the only APC population capable of cross-presenting SIINFEKL. Three days after adoptive transfer of T cells from (SIINFEKL-specific) T-cell receptor transgenic mice (OT-I) into B6.C-H2[bm1] mice transplanted with LSEC from C57BL/6 mice, we observed proliferating CD8^{+} T cells in the liver (Fig. 4b). We further detected proliferating CD8^{+} T cells in the peripheral blood, whereas only small numbers of proliferating CD8^{+} T cells were found in spleen or lymph nodes (data not shown). We conclude that LSEC can cross-present antigen to CD8^{+} T cells *in vivo* and induce proliferation of naive CD8^{+} T cells outside of lymphatic tissue.

Antigen-specific induction of tolerance in CD8^{+} T cells by LSEC

Bone-marrow–derived APC are required for cross-presentation leading to induction of a protective immune response against viral infection[2]. As LSEC in adult mice are not derived from bone marrow (P. Knolle and B. Arnold, unpublished observation), we
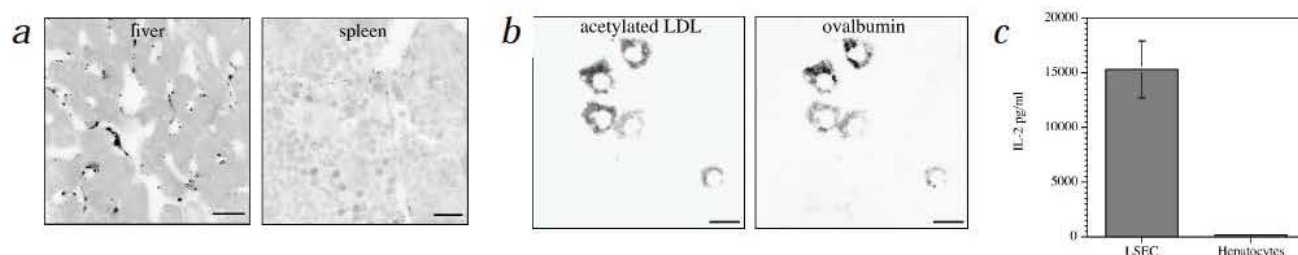

Fig. 3 LSEC cross-present antigen after antigen uptake *in vivo*. Antigen uptake at the cellular level in different organs by confocal microscopy following intravenous injection of ova-TxRed (**a** and **b**). **a**, Liver and spleen sections after perfusion fixation with paraformaldehyde (scale bar, 20 μm). **b**, Uptake of Bodipy-FL–labeled, acetylated LDL by ova-TxRed–positive liver cells (scale bar, 10 μm). **c**, Comparison of cross-presentation to B3Z cells *in vitro* by either LSEC or hepatocytes isolated from mice previously challenged with ovalbumin intravenously.
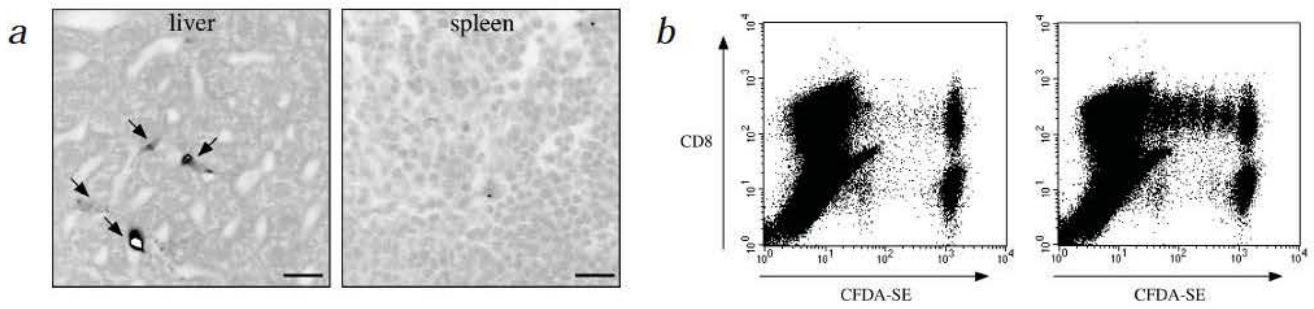
Fig. 4 LSEC cross-present ovalbumin to CD8⁺ T cells *in vivo.a,* Detection of LSEC in hepatic sinusoids following adoptive transfer. Fluorescent images of organ sections were recorded by confocal microscopy (scale bar, 20 μm). *b,* Three days after adoptive transfer of ovalbumin pulsed LSEC and CFDA-SE

naive OT-I T cells into B6.C-H2^bm1 mice, lymphocytes were isolated from liver and analyzed for proliferation. Left panel: control animal injected with CFDA-SE labeled OT-I T cells. Right panel: animal injected with ovalbumin-pulsed LSEC followed by injection with CFDA-SE labeled OT-I T cells.

investigated whether the functional outcome of CD8⁺ T-cell priming by LSEC might be different from that by conventional APC. Following incubation with antigen-presenting LSEC *in vitro,* CD8⁺ T cells gradually lost the ability to express IFNγ and IL-2 upon clonotypic restimulation (Fig. 5*a*). Though CD8⁺ T cells, after three days of coculture with LSEC, still secreted IFNγ and IL-2 after restimulation, more extended coculture of T cells with LSEC (4–5 days) led to loss of cytokine expression in CD8⁺ T cells (Fig. 5*a*). In contrast, when CD8⁺ T cells were incubated with antigen-presenting splenocytes, CD8⁺ T cells retained the ability to express IFN-γ and IL-2 at all time points investigated (Fig. 5*a*). Downregulation of cytokine production in CD8⁺ T cells strictly depended on presentation of antigen by LSEC. Coculture of CD8⁺ T cells with LSEC not presenting the antigen did not result in loss of cytokine expression after clonotypic restimulation (data not shown).

K^b-specific CD8⁺ T cells primed by antigen-presenting LSEC did not show K^b-specific cytotoxicity whereas CD8⁺ T cells primed by splenocytes displayed specific cytotoxicity of more than 50% at an effector:target ratio of 50:1 (Fig. 5*b*). We did not detect secretion of suppressive cytokines (such as IL-4, IL-10 or TGF-β) from CD8⁺ T cells primed by LSEC that could explain the loss of specific T-cell cytotoxicity (data not shown). Therefore, we assume that contact with antigen-presenting LSEC rendered CD8⁺ T cells tolerant. The phenotype of CD8⁺ T cells primed by antigen-presenting LSEC was not distinct from CD8⁺ T cells primed by splenocytes. Upregulation of surface activation markers on CD8⁺ T cells (such as CD25, CD69

andCD44) was already detected 24 hours after stimulation and was not observed in the absence of antigen or on CD4⁺ T cells (data not shown). However, supplementation of LSEC/T cell cocultures with interleukin 2 (IL-2) (10 ng/ml) but not interleukin 12 (IL-12) (10 ng/ml), interferon gamma (IFN-γ) (10 ng/ml) and tumor necrosis factor alpha (TNF-α) (10 ng/ml) prevented induction of tolerance in T cells (Fig. 5*c*). This finding indicates that absence of sustained IL-2 expression in T cells was involved in LSEC-induced tolerance. Mediators involved in tolerance likely to be present in the hepatic microenvironment such as TGF-β and PGE₂ downregulated T-cell cytotoxicity even further than LSEC alone (Fig. 5*c*).

T-cell tolerance is induced by cross-presenting LSEC *in vivo*

To investigate the ability of cross-presenting LSEC to induce T-cell tolerance *in vivo*, we adoptively transferred LSEC pulsed with ovalbumin into OT-I mice and challenged the animals with a syngeneic tumor cell line transfected with ovalbumin (RMA-ova). Most untreated OT-I mice (5/6) rejected RMA-ova whereas OT-I mice after adoptive transfer of ovalbumin-pulsed LSEC accepted RMA-ova (6/6). Thus, LSEC cross-presenting soluble antigen induced antigen-specific immune tolerance. To demonstrate participation of LSEC in tolerance induction to circulating soluble antigens, we injected C57BL/6 mice intravenously with ovalbumin (60 μmol/mouse), isolated LSEC 24 hours later and adoptively transferred these LSEC into C57BL/6 mice. After one week the procedure was repeated and the mice were challenged with RMA-ova as described above. Only mice harboring ovalbu-
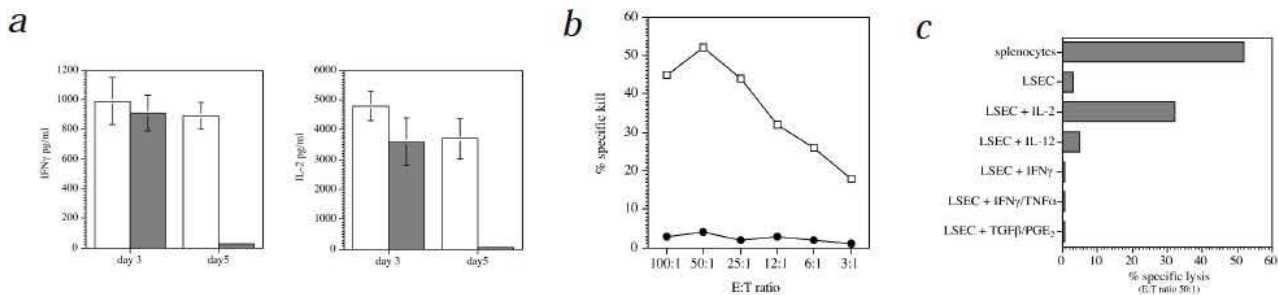

Fig. 5 Antigen-presentation by LSEC leads to tolerance induction in CD8⁺ T cells.*a,* Release of cytokines IFN-γ and IL-2 from Des.TCR CD8⁺ T cells after incubation with either K^b LSEC (■) or irradiated K^b splenocytes (□) and clonotypic restim-ulation.*b,* Specific cytotoxicity of Des.TCR CD8⁺ T cells after coculture with either K^b LSEC (●) or K^b splenocytes (◇). *c,* Influence of exogenous cytokines during priming of Des.TCR CD8⁺ T cells through K^b LSEC for specific T-cell cytotoxicity.

min-pulsed LSEC accepted the tumor (3/6), whereas all untreated mice (6/6) showed tumor rejection. This indicates that T-cell tolerance can be induced by cross-presenting LSEC in animals with a normal T-cell receptor repertoire and that tolerance to intravenously applied antigen is mediated by LSEC.

Discussion

Cross-presentation of exogenous antigens on MHC-class I molecules is crucial to the induction of immunity against pathogens or tumors and has been shown to occur in myeloid APC such as dendritic cells[2,25]. The ability of macrophages and B cells to cross-present is in controversy. Some have reported that macrophages cross-present particulate antigen preferentially[24,26], while cross-presentation by B cells appears to be restricted to antigens internalized via specific membrane bound immunoglobulins (mIg) (ref. 27) . Here we report that LSEC, non-myeloid, organ-resident cells, cross-present soluble exogenous antigens to CD8^{+} T cells. Cross-presentation by LSEC is a fast and efficient process that uses the conventional proteasome- and TAP-dependent pathway for loading of MHC-class I molecules. While myeloid APC such as macrophages and dendritic cells require relatively high concentrations of soluble antigen ($\geq 20\ \mu M$) to cross-present antigen *in vitro*[24,28], low antigen concentrations are sufficient for cross-presentation through LSEC (≤ 1 nM). As APC from different organs may have distinct functions[29], we investigated the ability of LDC to cross-present antigen. Although a contribution of LDC to cross-presentation by LSEC was excluded (see Fig.1), we observed that LDC *in vitro* indeed could cross-present exogenous antigen. Because antigen-presentation by DC may either result in immunity or in immune tolerance depending on the cell type, differentiation status and the microenvironment[30], we investigated the functional outcome of antigen-presentation by LSEC to CD8^{+} T cells. In contrast to conventional APC, LSEC induced tolerance rather than immunity in CD8^{+} T cells suggesting that LSEC in addition to LDC (ref. 17) contributes to peripheral hepatic immune tolerance .

Detection of activation markers and proliferation[31] shows that CD8^{+} T cells have been activated by LSEC. The lack of IL-2 production after restimulation and the observation that tolerance does not occur in the presence of exogenous IL-2 indicate that tolerance induction by LSEC is an active process preventing sustained autocrine stimulation via IL-2. Although the molecular mechanism of tolerance induction through LSEC is currently unknown, the hepatic microenvironment, which is a rich supply of immunoregulatory mediators such as IL-10 or TGF-β (ref. 13), is likely to contribute to local induction of immune tolerance.

Strategically-positioned LSEC in the liver sinusoid scavenge circulating antigens and interact with passenger T cells. T cells can be estimated to pass several hundred times per day through the hepatic meshwork of narrow sinusoids, where interaction with LSEC occurs without the need for prior activation of LSEC (LSEC constitutively express CD54 and CD106, refs. 12,32). Using a new method of orthotopic LSEC transplantation, we demonstrated that LSEC cross-presented soluble antigens *in vivo* to naive CD8^{+} T cells leading to T-cell proliferation in the liver (Fig. 4). Because we did not detect significant CD8^{+} T cell proliferation in lymphatic tissues, it is unlikely that adoptively transferred LSEC were integrated into lymphatic tissue. Previous reports indicate that presentation of antigen in lymphatic tissue can result either in immunity or in tolerance, depending on antigen dose and environmental stimuli[33,34]. Our results that LSEC mediate antigen-specific tolerance, attribute a new role to the liver and LSEC: control of immune responses to circulating soluble antigens.

Induction of immune tolerance following intravenous application of soluble antigen has been observed in different experimental systems[7,35,36]. Furthermore, orally ingested antigens that access the liver via the portal vein are reported to induce systemic tolerance[8,37]. All observations confirm that initial proliferation of T cells is detected before induction of specific T-cell anergy and clonal elimination by apoptosis. Although it has been suggested that soluble antigen is undetected by myeloid APC *in vivo* in the absence of adjuvant[34,38], our results indicate that LSEC may be responsible for tolerance induction to soluble antigen. We have previously reported that MHC-class II restricted antigen presentation by LSEC to naive CD4^{+} T cells leads to differentiation into IL-4/IL-10 expressing T cells with regulatory function[19]. Here we demonstrate that LSEC induce CD8^{+} T-cell proliferation (Fig. 4) and that adoptive transfer of LSEC, isolated from mice intravenously challenged with antigen, results in antigen-specific tolerance. T-cell proliferation, however, was followed by a reduction in T-cell number (A. Limmer, unpublished observation). Given the constitutive surface expression of death receptors such as CD95L, TRAIL and membrane-bound TNF-α on LSEC (A. Limmer, unpublished observation), we assume that T-cell apoptosis occurred as a result of interaction with LSEC. Downregulation of T-cell activity has been shown to occur preferentially in the liver in response to circulating antigen (during systemic viral infection or after peptide administration) either by induction of tolerant T cells[39] or by T-cell apoptosis[40].

Hepatic tolerance induction may also be operative in confinement of T-cell immunity once antigen is systemically distributed via the blood and generalized T-cell activation threatens to harm the organism. Though tolerance induction through antigen-presenting LSEC may have a role in avoidance of unwanted autoimmune reactions or against food antigens, this mechanism may also be involved in tolerance to allogeneic organ transplants[41]. Tolerance induction through LSEC may be abused by infecting non-cytopathic microorganisms that release large amounts of soluble antigens but do not cause tissue destruction, such as hepatitis-B viruses or HIV (ref. 42). Rather than limiting potentially harmful immune responses this would lead to viral immune escape.

The data presented here support a novel concept that LSEC promote the induction of hepatic immune tolerance. In this model, LSEC represent the sessile, organ-resident APC inducing local tolerance in the liver towards soluble antigens such as food antigens or self proteins. LDC take up antigen in the liver as well, but migrate to local lymph nodes mediating immune tolerance in the lymphoid compartment[17].

Methods

Mice, cell lines and reagents. BALB/c, CBA, C57BL/6 and mutant B6.C-H2[bm1] mice were purchased from Jackson laboratories (Bar Harbor, Maine). Des.TCR (ref. 20), OT-I (ref. 5) and TAP-deficient, (TAP-deficient^{−/−}, ref. 43) mice have been described previously. Mice were kept under SPF conditions at the animal facility of the ZMBH (Heidelberg, Germany).

The ovalbumin-specific T-cell hybridoma B3Z was provided by N. Shastri and the B-cell line LB27.4 was provided by G. Hämmerling. The peptide SIINFEKL (ovalbumin 257-64) was synthesized by the bio-engineering department at ZMBH. Ovalbumin (Grade VII) and β-gal (Grade VII, produced in *E. coli*) were purchased from Sigma (München, Germany). Ovalbumin labeled with TexasRed was obtained from Molecular Probes (Leiden, The Netherlands). β-gal–specific, CD8^{+} T-cell

clone (0805B) and specific peptide (TPHPARIK) were provided by H. Rammensee. LCMV glycoprotein-specific T cells (GP33Hyb) were generated as described[44]. Lactacystin was purchased from ICN (Eschwege, Germany). P7/7, Y3P (monoclonal antibodies against I-A^b), K10.56.1 (monoclonal antibody against K^b) and clonotype-specific antibody Désiré were provided by G. Hämmerling. Antibody 25-D1.16 against K$^{b-SIINFEKL}$ was provided by L. Eisenbach and A.Porgador. All other monoclonal antibodies were purchased from Pharmingen (Hamburg, Germany).

Isolation of primary cell populations from murine liver. LSEC were isolated from murine liver as described[19]. LDC were isolated and enriched as reported[45]. LDC-enriched cell populations were used for cross-presentation assays analogous to LSEC. Cells were incubated with ovalbumin (20 μM) for 2 h at 37 °C, were extensively washed, and subsequently transferred to 96 well plates and incubated with 1×10^5 B3Z cells for 18 h. Parenchymal cells were isolated according to standard protocol.

Isolation of T cells. T cells of OT-I or Des.TCR transgenic mice were isolated from spleen and depleted of macrophages, DC's and B cells by plastic adhesion and Y3P (mouse antibody against I-A^b)/(mouse antibody against IgG)–coated magnetic beads (Paesel & Lorei, Hanau, Germany). Resulting cells were ≥ 95% pure as determined by FACS-staining for CD45R (RA3-6B2) and MHC-class II (P7/7).

Cross-presentation in vitro. LSEC were seeded at a final concentration of 1 $\times 10^5$ cells/well in 24 collagen-I–coated well plates. 3 d later LSEC were pulsed for 2 h with ovalbumin or specific peptide SIINFEKL (ova 257-64) and incubated with 5×10^5 ovalbumin-specific CD8$^+$ T-hybridoma cells (B3Z) for 18 h. Hepatocytes and LB27.4 cells were treated accordingly. IL-2 concentration in supernatants was measured by ELISA. To investigate (cross)priming, LSEC from C57BL/6 and from TAP-deficient$^{-/-}$ mice were incubated for 3–5 d with naive T cells from OT-I or Des.TCR transgenic mice.

LSEC Transplantation. Ova-TxRed-(2 μM)–pulsed LSEC (2×10^6) were intravenously injected and perfusion fixed organs were investigated for implantation of LSEC by confocal microscopy. 1–7 d after adoptive transfer orthotopic implantation of LSEC was observed only into hepatic sinusoids but no cell implantation was observed in spleen, kidney or lung.

Cross-presentation in vivo. K^b-LSEC–pulsed in vitro with ovalbumin (20 μM) for 3 h were adoptively transferred into B6.CH-2^{bm1} mice that are unable to present SIINFEKL on K^b to CD8$^+$ T cells. Naive OT-I T cells (1×10^7) stained with the fluorescent dye CFDA-SE (10 μM) were injected (IP) into LSEC-transplanted B6.CH-2^{bm1} mice. After 72 h, lymphocytes were re-isolated from spleen, lymph nodes, liver and peripheral blood. Cells from spleen and lymph nodes were isolated by mechanical cell dispersion, peripheral blood lymphocytes were isolated by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation of whole blood. Lymphocytes from the liver were isolated by collagenase perfusion, mechanical dispersion followed by filtration (70 μm) and Percoll gradient (40%/80%) centrifugation (800 g) for 20 min. Cell populations isolated from different organs were stained for CD8 and CFDA-SE. Fluorescence intensity was determined by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany).

Confocal microscopy. Organs were fixed by perfusion with 3% paraformaldehyde, 50 μm thick liver sections were cut with a vibratome (VT1000S, Leica, Darmstadt, Germany) and mounted on cover slides. Fluorescent images of organ sections were recorded with a confocal laser-scanning microscope (Leica) and were subsequently imported into Adobe Photoshop for labeling. To analyse living cells, LSEC were plated onto collagen-I–coated borosilicate chamber slides (Nunc, Wiesbaden, Germany). Following excitation at 488 nm or 568 nm, green and red fluorescence images, respectively, were separately recorded to avoid artefacts.

Analysis of T-cell function in vitro. To determine the potential of LSEC to influence T-cell function, Des.TCR T cells were isolated as described above and incubated with K^{b+} LSEC or irradiated K^{b+} splenocytes. At days 3, 4 and 5, supernatants of T cells that were restimulated with a clonotype-specific monoclonal antibody (Désiré) (10 μg/ml) for 48 h, and

were analysed for IL-2, IL-4, IL-10, IFN-γ and TGF-β concentrations by ELISA. At day 5, K^b-specific lysis by T cells was determined with a [^{51}Cr]-release assay according to standard procedure.

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