Distinct dynamics and kinetics determine efficient antigen-presentation by LSEC and support IL-2 dependent CD8 T cell activation

Dissertation

zur Erlangung des Doktorgrades (Dr. rer. nat.)
der Mathematisch-Naturwissenschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Anna Schurich
aus Bonn

Bonn, Februar 2009

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

Die vorliegende Arbeit wurde am Institut für Molekulare Medizin und Experimentelle Immunologie am Universitätsklinikum Bonn angefertigt.

1. Gutachter: Prof. Dr. Percy A. Knolle

2. Gutachter: Prof. Dr. Waldemar Kolanus

Tag der mündlichen Prüfung: 22. April 2009

Erscheinungsjahr: 2009

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

Für Karen und Peter

Zusammenfassung

Sinusoidale Leber Endothelzellen (LSEC) exprimieren alle zur professionellen Antigenpräsentation nötigen Moleküle wie MHC I und II, geringe Mengen der kostimulatorischen Moleküle CD80/86 und das koinhibitorische Molekül B7-H1. Die Interaktion von LSEC mit naiven T-Zellen führt zur Induktion von Toleranz.

Inhalt dieser Arbeit ist die Beschreibung der Mechanismen der effizienten Antigenaufnahme und Kreuzpräsentation, sowie der daher möglichen Aktivierung von T-Zellen durch LSEC.

Es zeigte sich, dass die Kreuzpräsentation in LSEC und DC eine sehr unterschiedliche Dynamik und Kinetik hat. Systemische, lösliche Antigene wurden bevorzugt in der Leber von LSEC aufgenommen und daraufhin effizient kreuzpräsentiert. Die Kreuzpräsentation durch LSEC war *ex vivo* und *in vitro* deutlich höher als die durch dendritische Zellen (DC). Allerdings verblieben von LSEC aufgenommene Antigene nicht in LSEC, sondern wurden mit einer Halbwertszeit von nur 6 Std aus LSEC eliminiert. Die schnelle Ausschleusung von Antigenen wurde gleichzeitig von einer deutlichen Reduktion der Kreuzpräsentation begleitet. In DC kam es im gleichen Zeitraum zu keiner signifikanten Reduktion der aufgenommenen Antigene und der Kreuzpräsentation.

LSEC verfügten nicht über ein spezialisiertes endosomales Kompartiment für die Kreuzpräsentation löslicher Antigen. Es konnte aber gezeigt werden, dass LSEC mehrere Rezeptoren zur Aufnahme von Antigenen für die Kreuzpräsentation nutzen. Die dadurch hervorgerufene effiziente Antigenaufnahme könnte das Fehlen eines spezialisierten Kompartiments kompensieren. Immunkomplexierte Antigene wurden von LSEC nach Aufnahme über den FcRezeptor nur schlecht kreuzpräsentiert. Dies ist ein möglicher Mechanismus, um eine Toleranzinduktion gegenüber pathogenen Antigenen in Anwesenheit einer humoralen Immunität zu umgehen.

Weiterhin konnte gezeigt werden, dass LSEC naive T-Zellen nicht nur tolerisieren sondern auch aktivieren können. Durch die effiziente Kreuzpräsentation in LSEC wurde ein ausreichend starkes T-Zellrezeptorsignal vermittelt, um trotz geringer Kostimulation eine IL-2 Produktion in T-Zellen auszulösen. Dieses IL-2 konnte die Toleranzinduktion durch LSEC brechen. Die durch LSEC aktivierten T-Zellen zeigten nach Restimulierung eine hohe Zytokinproduktion, aber keine Zytotoxizität solange LSEC koinhibitorische Signale über B7-H1 vermitteln konnten. Wurden T-Zellen von B7-H1-defizienten LSEC aktiviert, so produzierten sie stark erhöhte Mengen an IL-2 und zeigten Zytotoxizität. Die Aktivierung der naiven T-Zellen konnte durch Blocken von IL-2 inhibiert werden. Diese Ergebnisse weisen darauf hin, dass PD-1 die IL-2 Produktion von T-Zellen negativ beeinflusst. In der Interaktion von LSEC mit naiven T-Zellen scheint maßgeblich das Vorhandensein oder Fehlen von IL-2 über die Induktion von Toleranz oder Immunität zu entscheiden.

Contents

1 Introduction

1.1 Brief overview of the immune system

All mammals have a complex immune system to protect themselves from pathogenic agents such as viruses, bacteria and parasites and from mutated cells i.e. tumour cells. The first line of defence is the evolutionary old innate immune system, which is present in all individuals at all times. However as pathogens can developed much faster than mammals, the immune system needs to be able to adapt to and form a memory of encountered disease agents, this difficult task is achieved by the acquired or adaptive immune system.

The two systems are not strictly divided but they closely interact. The innate immune system (components of which are present in all classes of plant and animal life) consists of humoral and cellular defences. Humoral defences consist of the complement system (most factors of which are produced by hepatocytes) which can be activated by immune complexes linking innate and adaptive immunity, chemokines for recruitment of immune cells and cytokines to initiate and shut down immune responses. The cellular defences consist of white blood cells which can regulate immune responses by producing cytokines, cells that phagocytose and destroy pathogens e.g. neutrophils and macrophages and cells that can present antigen e.g. DC and macrophages to elicit an adaptive immune response. Innate immune cells must discriminate between self and non-self and further between dangerous and innocuous non-self. To meet this challenge they carry receptors like Toll like receptors (TLR) and pattern recognition receptors (PRR) recognising conserved pathogen associated molecular patterns (PAMPs) (Palm and Medzhitov, 2009), (Janeway, 1989).

The adaptive immune system also constitutes a humoral and cellular part, B cells and T cells, respectively. The cells of the adaptive immune system show high specificity and are at the same time very versatile due to their expression of receptors recognizing non-conserved molecules. The possibility of the T and B cell receptor to undergo genetic recombination allows a small number of genes to form a nearly infinite number of receptors which will then be able to recognize a molecule never encountered before (Medzhitov and Janeway, 1997a, b). B cells can also undergo somatic hypermutation which makes antibodies more and more specific and increases their affinity over time. Efficiency and magnitude of adaptive immune responses increases with increased encounters, showing a primary and “secondary” (all following) response. Importantly, the adaptive immune response is able to form a memory, conferring life long protection from the respective pathogen to the organism. However, cells of the adaptive immune system cannot reliably discriminate between self and non-self with potentially deleterious consequences to the organism. Therefore adaptive immune responses must

be educated by the innate immune system and tightly controlled (Gallegos and Bevan, 2006; Janeway, 1989; Palm and Medzhitov, 2009).

1.2 Antigen presentation

1.2.1 Conventional presentation on MHC class I and II molecules

The T cell receptor (TCR) cannot recognize foreign antigen by itself. To be recognized by T cells antigen has to be processed and peptides loaded onto major histocompatibility molecules (MHC). In the case of presentation on MHC class I molecules this can be done by every nucleated cell in the body. However, naïve T cells which encounter their cognate antigen in absence of co-stimulatory molecules will not be activated (hernandez 2001, Jenkins Schwartz 1987).

Optimal activation of T cells can only be achieved by antigen presenting cells (APC). These cells can present peptides on MHC class I molecules to $CD8^+$ T cells and on MHC class II molecules to $CD4^+$ T cells in the context of appropriate co-stimulation.

MHC class II molecules are constitutively expressed only on professional APC such as DC, macrophages, B cells, thymic epithelial cells (Jensen, 2007) and also on liver sinusoidal endothelial cells (LSEC). For presentation on MHC class II molecules, exogenous antigens, soluble or particulate, are taken up into endosomes or phagosomes, respectively, which then fuse with lysosomes. Here the protein is enzymatically degraded and loaded onto MHC class II molecules that are recruited to the lysosomes from the ER. MHC class II peptide complexes travel to the cell surface for recognition by $CD4^+$ T cells (Khor and Makar, 2008; Watts, 1997; Wolf and Ploegh, 1995). Activated $CD4^+$ T cells can provide help in the activation of B cells and $CD8^+$ T cells or develop a regulatory phenotype.

Antigen derived from within the APC, e.g. from self proteins, intracellular pathogens like viruses and some bacteria or from mutated proteins in the case of tumours is presented on MHC class I molecules. Proteins are processed within the cytosol by the proteasome. Peptides are then transferred by the transporter associated with antigen processing (TAP) into the ER where they are loaded onto MHC class I molecules (Rock et al., 2004). MHC class I molecules on the cell surface are recognized by $CD8^+$ T cells, which once activated can kill target cells.

1.2.2 Presentation of exogenous proteins on MHC class I molecules (cross-presentation)

However APC are not always infected themselves, therefore presentation of exogenous antigens on MHC class I molecules, a process termed cross-presentation, is important for mounting antigen-specific CD8 T cell immunity. Cross-presented molecules are actively taken up by the APC, processed and cross-presented helping to combat infection and cancer (Bevan, 1976; Heath et al., 2004; Kurts et al., 1996; Shen and

Rock, 2006). APC have been described to acquire antigen for cross-presentation in many different ways. The antigen can be particulate e.g. cell associated when an APC takes up an apoptotic cell and presents its contents, soluble (taken up by receptor mediated endocytosis) or peptides from virally infected cells can even be transferred to APC from cell to cell via gap junctions (Neijssen et al., 2005).

The mechanisms allowing cross-presentation of particulate and soluble antigens by professional antigen presenting cells such as dendritic cells or macrophages are mechanistically distinct (Ackerman and Cresswell, 2004; Burgdorf and Kurts, 2008). Particulate antigens enter phagosomes where MHC class I as well as MHC class II restricted antigen-presentation is initiated (Guermonprez et al., 2003; Savina and Amigorena, 2007). Soluble antigens in contrast enter early endosomes for exclusive presentation on MHC class I molecules. Recently it could be shown by Burgdorf et al that a direct link between endocytosis mechanisms and the cell biology of antigen presentation exists. Uptake of soluble antigen by receptor mediated endocytosis routed antigen into early endosomal compartments for cross-presentation while uptake via pinocytosis delivered antigen into lysosomes for MHC class II presentation. Furthermore in DC and macrophages not all receptors shuttled soluble antigen into endosomes for cross-presentation. The model antigen ovalbumin (OVA) when taken up by the mannose receptor was shown to be delivered into a stable early endosomal antigen ($EEA1^+$) endosomal compartment which also contained TAP. Peptides could thus be loaded onto MHC class I molecules after proteasomal degradation in the cytosol within the original endosomal compartment. However if OVA was taken up by the scavenger receptor (in macrophages) it was delivered into a lysosomal compartment and not cross-presented (Burgdorf et al., 2006; Burgdorf et al., 2008). It has been described that the mannose receptor and scavenger receptors by themselves are not sufficient to mediate adaptive immune responses (Iwasaki and Medzhitov, 2004). This was verified by the observation that TAP which is essential for reintroduction of peptides into endosomes is only recruited if a danger signal such as LPS is present (Burgdorf et al., 2008). This finding suggests that cross-presentation of OVA in DC only ensues when danger is sensed at the same time. Differential receptor expression and the ability to cross-present soluble antigens provides an explanation to the question why different DC subtypes have distinct functional properties (Shortman and Liu, 2002; Villadangos and Schnorrer, 2007). However other cell populations capable of cross-presentation could employ different mechanisms. Loading of proteins within the ER has been described as a further mechanism for cross-presentation in DC (Ackerman et al., 2006; Ackerman et al., 2005) and plasmacytoid DC were shown to be able to mediate proteasome independent cross-presentation of viral antigens (Di Pucchio et al., 2008).

LSEC are also capable of cross-presenting soluble exogenous antigens on MHC class I molecules to CD8 T cells (Diehl et al., 2008; Limmer et al., 2000; Limmer et al., 2005). Furthermore they express the mannose receptor and are very efficient in the uptake of soluble antigens. The molecular mechanism of cross-presentation in LSEC is so far unknown. However my data show that LSEC utilise different receptors for cross-

presentation than DC and macrophages. Furthermore cross-presentation in LSEC follows distinct kinetics and dynamics rendering them highly efficient.

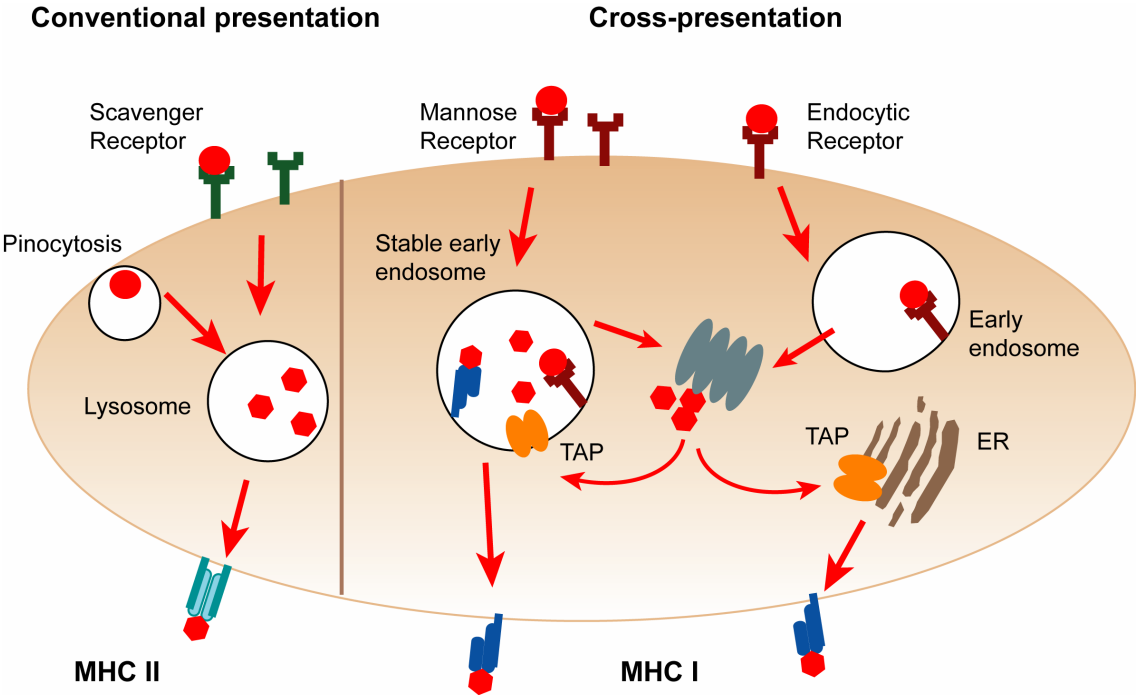

Figure I. Current model for presentation of exogenous antigen on MHC I and MHC II molecules *(adapted from (Burgdorf and Kurts, 2008)). Pinocytosis and scavenger receptor mediated uptake of soluble exogenous antigen leads to presentation on MHC class II molecules in DC and macrophages, respectively (left). OVA taken up by the mannose receptor in DC and macrophages is routed into stable early endosomes, processed by the proteasome and reintroduced into the endosome for loading onto MHC class I (middle). Antigen is taken up by an endocytic receptor shuttled into an early endosome, processed by the proteasome and not reintroduced into the endosome, but loaded onto MHC class I molecules in another compartment, for example in the ER (putative route of cross-presented antigen in LSEC) (right).*

1.3 Induction of T cell immunity

The activation of naïve T cells is controlled by APC of which DC are the most important. In order to fully activate T cells DC must undergo maturation. DC maturation is caused by recognition of pathogen derived products (danger signals) or by CD4+ T cells via CD40/ CD40L interaction (Mescher et al., 2006).

Three signals, required for full T cell activation, have been described. The first, signal 1 is delivered by the TCR specifically recognizing cognate antigen presented on MHC class I molecule (Song et al., 2008). Signal 1 alone is not sufficient for the development of naïve T cells into mature effector T cells (Bevan, 2006; Schwartz, 2005). The second signal is delivered by co-stimulatory molecules expressed on mature APC. Co-stimulation is mainly delivered by molecules of the B7-family (Collins et al., 2005)

such as CD80 (B7.1) and CD86 (B7.2) which are expressed on APC. CD80/86 are upregulated upon DC maturation and stimulate CD28, which is expressed by naïve T cells. CD28 amplifies TCR signalling and is especially important at low TCR occupancy (Acuto and Michel, 2003). The third signal is mediated by proinflammatory cytokines such as IL-12 and type I interferons.

Signalling via CD28 together with TCR induced signalling via the CD3 complex (Lin and Weiss, 2001) activates a complex cascade of events, leading to cell cycle progression and proliferation (Viola and Lanzavecchia, 1996). CD28 signalling also incrases IL-2 receptor expression and production of IL-2, IFNγ and IL-4 (Acuto and Michel, 2003). Furthermore signalling via CD28 induces bcl-2 expression promoting T cell survival (Collins et al., 2005).

The full activation of naïve $CD8^+$ T cells and subsequent development of memory crucially depends on the presence of signal 2 (co-stimulation) and signal 3 (proinflammatory cytokines). Especially IL-12 mediating signal 3 is an important inducer of IFN-γ production and further has a role in the survival of activated $CD8^+$ T cells (Curtsinger et al., 2003). IL-12 is produced by DC in response to danger signals or CD40 ligation. IL-12 or type I interferons as a third signal have been shown to be required for clonal expansion and the development of cytolytic activity in $CD8^+$ T cells (Curtsinger et al., 1999; Curtsinger et al., 2005). Upon APC contact the priming of T cells occurs in 3 phases, the first lasting for about 8h resulting in the upregulation of activation markers. During the second phase, which lasts for approximately 12h, IL-2 and IFNγ production are initiated and during the third phase T cell proliferation is induced (Henrickson et al., 2008).

TCR triggering causes a calcium influx into the T cell activating calmodulin. When calmodulin has bound calcium it can bind and activate calcineurin which can in turn bind to NFAT. NFAT is thereupon dephosphorylated and translocates into the nucleus. In T cells NFATc1 and c2 have been described to be of most importance. In the nucleus NFAT can initiate the transcription of several cytokines, most importantly IL-2 (Macian, 2005). CD28 signalling has been shown to mediate IL-2 mRNA stabilization, thereby supporting its translation. IL-2 induces clonal expansion (Pei et al., 2008) and is crucial for sustained T cell activity (D'Souza and Lefrancois, 2003). Dysfunctions in IL-2 lead to the development of immunodeficiencies as well as autoimmunity in humans and mice, showing that the balance of IL-2 for indcution or prevention of immunity is critical (Lan et al., 2008). IL-2 deficient mice show strong lymphoproliferation which results in fatal autoimmunity (Sadlack et al., 1994). This phenotype has been shown to be due to a severe reduction in $CD4^+CD25^+$ Treg (Malek, 2002; Malek and Bayer, 2004).

The IL-2 receptor is composed of a α, β and common γ chain. The high affinity IL-2 receptor (CD25) consists of all 3 chains together. In a positive feedback loop IL-2 can increase the expression of its own receptor (Goebel et al., 2006). CD25 is not only expressed on regulatory T cells, but is also upregulated on activated T cells, B cells and

NK cells (Lan et al., 2008). IL-2 has been described to be able to prevent or reverse the induction of T cell anergy (Dure and Macian, 2009). As will be shown here, IL-2 can overcome the induction of $CD8^+$ T cell tolerance by LSEC.

1.4 Induction of T cell tolerance

1.4.1 Central tolerance

T cell precursors from the bone marrow develop into naïve T cells in the thymus. Here, a highly diverse set of T cells is generated by the rearrangement of genes that encode the α- and β- chains of the TCR. A repertoire of antigen receptors is generated which can potentially recognize any peptide MHC complex generated, whereas every individual TCR is highly specific for a single combination. However this diversity poses the risk of T cells recognizing self antigens. The primary mechanism to ensure tolerance of T cells towards self antigens is achieved by a process called negative selection. During negative selection T cells are tested for their reactivity to self antigens. To this end they are presented with a wide range of self antigens by the thymic medullary epithelial cells and bone marrow derived macrophages and DC (Gallegos and Bevan, 2006). The transcription of the diverse self antigens presented by the thymic medullary epithelial cells is regulated by the auto-immune regulatory protein (AIRE) (Anderson et al., 2002). Those T cells showing a high affinity towards presented self peptide MHC complexes are deleted, leading to an elimination of more than 95% of the total T cells (Palmer, 2003).

Although in theory negative selection should eliminate all self-reactive T cells, this is not the case, as not all antigens are expressed in the thymus and T cells with low affinity TCR can escape negative selection (Liu et al., 1995). Furthermore T cells passing successfully through central tolerance cannot distinguish between dangerous non self and innocuous non-self antigen (Palm and Medzhitov, 2009). Therefore additional tolerance mechanisms are required.

1.4.2 Peripheral tolerance

Peripheral tolerance is mediated by several mechanisms such as immune ignorance, anergy, peripheral deletion and suppression by regulatory T cells. Cells from the innate immune system which can reliably distinguish between self, non-self and dangerous or innocuous antigens control tolerance induction or activation of T cells in the periphery (Palm and Medzhitov, 2009).

Immunological ignorance either results from spatial sequestration of antigens to immunologically privileged sites like the eye (Alferink et al., 1998; Zinkernagel et al., 1993) or concentrations of the antigen are too low to trigger T cell activation (Kurts et al., 1998). Furthermore self-reactive T cells that have escaped negative selection in the thymus most likely express a TCR of low affinity (Zehn and Bevan, 2006) and thus

binding of the TCR will not result in activation, the cells remain ignorant. However, upon infection and tissue destruction, these self reactive T cells can get into contact with previously sequestered or insufficiently presented antigen, which can result in breaking of ignorance and the development of autoimmune diseases (Oldstone et al., 1991).

Presentation of antigen to naïve T cells in the absence of signalling via co-stimulatory molecules leads to either T cell deletion (Hernandez et al., 2001) or later T cell unresponsiveness called anergy (Jenkins et al., 1987). T cells rendered anergic after antigen encounter can survive but remain in an unresponsive state. Anergy is usually induced by immature DC, which have not upregulated co-stimulatory molecules and do therefore not deliver signal 2 and 3. Endogenous self antigens are constitutively presented on MHC I and II molecules by APC under homeostatic conditions. Furthermore, DC take up soluble exogenous antigens, waste products and antigens from apoptotic cells and can present them on MHC class I molecules leading to the induction of cross-tolerance or deletion of CD8+ T cells (Heath et al., 1998; Kurts et al., 1997; Luckashenak et al., 2008).

Furthermore DC not only passively tolerize T cells by presenting antigen in the absence of co-stimulation, but can actively induce tolerance through signalling via co-inhibititory molecules. B7-H1 and B7-DC expressed by DC can interact with the co-inhibitory molecule PD-1 on T cells (Greenwald et al., 2005) and CD80/86 on DC can induce inhibitory signals via cytotoxic T-lymphocyte antigen 4 (CTLA-4) (Waterhouse et al., 1995). Co-inhibitory molecules are expressed on naïve T cells and further upregulated during priming (Probst et al., 2005).

Tolerance is not only mediated by APCs but also by regulatory T cells and soluble factors (Dhein et al., 1995; Groux et al., 1997). Regulatory T cells (Treg) are characterized by their expression of CD4 and CD25. The development and survival of Treg crucially depends on the presence of IL-2 (Sakaguchi et al., 2008). Like conventional T cells natural $CD4^+CD25^+$ Tregs (nTreg) develop in the thymus. Natural Treg in contrast to induced Treg express the forkhead-box transcription factor Foxp3 (Fontenot et al., 2005). Upon antigen encounter Treg inhibit other T cells in an antigen unspecific manner. Suppression by Treg is dependent on close proximity to the inhibited T cell and has been discussed to be mediated by efficient competition of Treg for IL-2, thus sequestering IL-2 from effector T cells (de la Rosa et al., 2004; Scheffold et al., 2005).

Regulatory T cells can also be induced in the periphery by tolerogenic dendritic cells or by interaction with $CD4^+CD25^+$ Tregs. Induced Treg mediate inhibitory function by the production of immune suppressive cytokines transforming growth factor beta (TGF-β) and Interleukin 10 (IL-10) (Takahashi and Sakaguchi, 2003). Some of these induced regulatory T cells express Foxp3, but show a less stable functional phenotype than natural Treg (Sakaguchi et al., 2008).

Antigen persistence which is seen for self-antigens and in case of a chronic infection will enhance the deletion of activated T cells (Davey et al., 2002; Lopes et al., 2008;

Redmond et al., 2003). Deletion is mediated by a process called activation induced cell death (AICD) which is mediated by the interaction of Fas with Fas ligand expressed by activated T cells (Dhein et al., 1995; Singer et al., 1994).

1.5 The liver

1.5.1 Tolerance mechanisms in the liver

The liver plays an important role in the induction of peripheral tolerance. Foreign and bacterial antigens derived from the gastrointestinal tract are efficiently cleared to avoid the activation of systemic immunity which could lead to generalized organ failure (Knolle and Gerken, 2000). The liver microenvironment contains a tolerizing milieu which is rich in immune suppressive cytokines. The different hepatic APC all contribute to the production of anti inflammatory cytokines. KC, hepatic DC, LSEC and hepatocytes can release IL-10 and TGF-β (Bissell et al., 1995; Goddard et al., 2004). $CD4^+$ T cells primed by hepatic DC and LSEC differentiate towards regulatory T cells and produced IL-10 and IL-4 (Knolle and Gerken, 2000; O'Connell, 2000) and resident NKT cells additionally produce IL-13 (Godfrey et al., 2004). IL-10 inhibits the immune-stimulatory function of APC and T cells in the liver, leading to a decrease in antigen presentation, an inhibition of the production of proinflammatory cytokines like IL-12 and IFN-γ and an increase of Prostaglandin E2 (PGE_2) production.

It could recently be shown that adenosine released by liver cells can inhibit T cell activation (Linnemann et al, in press). In further support of the tolerizing capabilities of the liver is the finding that liver transplants are well accepted and split tolerance towards another organ from the same donor can be observed (Knolle and Limmer, 2003).

Activated T cells are selectively retained in the liver and deleted by apoptosis (Mehal et al., 1999) which could contribute to peripheral tolerance. Two possible mechanisms of T cell deletion within the liver are discussed. The “graveyard” hypothesis (Huang et al., 1994) states that apoptotic cells accumulate in the liver and are then eliminated, while the “killing field” hypothesis suggest apoptosis induction as a result of interaction with liver cells (Crispe et al., 2000).

1.5.2 The Liver microanatomy

The liver has important clearance, metabolic, storage and immune functions. The hepatic blood supply consists of 20% o arterial and 80% venous blood, delivered via the hepatic portal vein (Knolle and Gerken, 2000). Arterial blood is derived from the systemic circulation and is oxygen rich, while venous blood is mostly derived from small and large intestine, spleen, pancreas and stomach. Therefore blood arriving via the portal vein is rich in food derived and bacterial antigens (e.g. endotoxin) from the gut. The liver contains a tight network of small blood vessels, the sinusoids, which are

perfused by a mixed arterial/ venous blood flow, coming from the portal field and leaving the liver via the central veins converging in the vena cava inferior. The portal field contains one biliary duct and one blood vessel derived from the hepatic artery and one from the portal vein. The hepatic lymph vessels are also located in this area. The blood entering the periportal field is especially rich in antigens and hepatic resident macrophages (Kupffer cells) are preferentially located in this area. The entire blood volume moves through the liver 360 times per day which facilitates clearance functions and immune surveillance. The ample amount of foreign antigen present in the liver could easily elicit a local immune response against hepatocytes or, if not cleared, a systemic immune response (Knolle and Gerken, 2000). It is therefore vital that hepatocytes are protected from passenger immune cells to efficiently detoxify and clear waste and bacterial products.

Furthermore hepatocytes are involved in protein synthesis producing plasma proteins, acute phase proteins (Billiar et al., 1992; Knolle and Limmer, 2003), mannose binding proteins, C-type lectins, fibrinogen, complement factors, albumin which is a major osmolar component of the blood and proteins and biochemicals implicated in digestion e.g. bile for emulsifying lipids. Also they play an important role in glucose metabolism (Leclercq et al., 2007), as hepatocytes can build glycogen from glucose (glycogenesis) for subsequent storage. Glycogen can later be broken down into glucose again (gluconeogenesis).

1.5.3 Cell populations of the liver sinusoid

The liver sinusoids make up a mesh of minute blood vessels, with a diameter of only 5-7µm (MacPhee et al., 1995; Wisse et al., 1985) and a very slow flow rate of approximately 25-250µm/ min (MacPhee et al., 1992, 1995). The sinusoids are lined by the sinusoidal endothelial cells (LSEC). LSEC make up a discontinuous endothelium, not forming tight junctions and not containing a basement membrane (Racanelli and Rehermann, 2006). Furthermore LSEC contain little pores called fenestrae with approximately 100nm diameter (Wisse, 1970). LSEC are in direct contact with passenger cells on the luminal side, but are divided from hepatocytes on the apical side by the perisinusoidal space of Dissé (Smedsrod et al., 1994). The space of Dissé contains extracellular matrix (Knolle and Gerken, 2000) produced by the resident hepatic stellate cells (HSC). In absence of inflammation leukocytes cannot move into the space of Dissé and are therefore usually not in direct contact to hepatocytes, as LSEC form a shield in between them (Limmer et al., 1998). However it has been observed that T cells can form little protrusions and touch hepatocytes through the endothelial fenestrae, the function of this interaction is so far unknown (Warren et al., 2006).

The parenchymal hepatocytes constitute the major cell population in the liver. Hepatocytes can contain several nuclei and are mainly responsible for metabolism in the liver. Hepatic stellate cells (HSC) residing in the space of Dissé store vitamin A

contained in lipid droplets within their cytosol. As vitamin A is autofluorescent in ultra violet light HSC are easily identified. Usually HSC are found to be in a quiescent state (Geerts, 2007) expressing long protrusion which are wrapped around the sinusoids and can contract, thus controlling sinusoidal diameter (Oda et al., 2000). HSC have been shown to be able to interact with NKT cells presenting lipid antigens to them (Winau et al., 2007). When activated upon liver injury HSC can transdifferentiate into fibroblasts and produce collagen. They have been implicated in being the main cell population responsible for liver fibrosis.

The liver hosts a large population of resident macrophages the Kupffer cells (KC). KC constitute the largest population of macrophages in the organism (Racanelli and Rehermann, 2006). They efficiently take up particulate antigen by phagocytosis and are responsible for clearance of bacterial products and apoptotic cells (Elvevold et al., 2008b). Furthermore KC can move through the Space of Dissé and clear dead hepatocytes (Racanelli and Rehermann, 2006).

The liver also contains a hepatic DC (HDC) population displaying an immature, tolerogenic phenotype (Banchereau et al., 2000). HDC are mainly located around the central veins. Upon activation they can migrate via the Space of Dissé to the lymphatics in the portal tract and subsequently to extra hepatic lymph nodes (Kudo et al., 1997).

The lymphocyte populations found in the liver are distinct from those found in lymphatic organs. Hepatic lymphocytes show an increased proportion of NK, NKT and γδ T cells. Furthermore the T cell population in the liver is enriched in $CD8^+$ T cells. T cells in the liver, rather than being naïve, mainly exhibit a matured effector or memory phenotype (Crispe et al., 2006).

1.5.4 The liver sinusoidal endothelial cell (LSEC)

The liver contains 3 populations of endothelial cells, which are located macrovascular, periportal and perivenous. They can all be stained by the endothelial cell specific marker ME9 F1 (CD146). However functionally they are distinct. The macrovascular endothelial cells do not express scavenger receptors or C-type lectin receptors and consequently display no scavenger function towards soluble antigen (Knolle and Limmer, 2003). Periportal and perivenous LSEC both express scavenger and C-type lectin receptors, periportal LSEC however have been shown to be more efficient in antigen uptake irrespective of the amount of antigen present (Vidal-Vanaclocha et al., 1993a).

Lining the liver sinusoids LSEC are in direct contact with passenger leukocytes. They have a unique phenotype resembling professional antigen presenting cells, as they express surface molecules usually only found on cells from myeloid origin (Knolle and Limmer, 2003). LSEC constitutively express MHC class II and the co-stimulatory molecule CD40 enabling them to interact with $CD4^+$ T cells, in which they induce a regulatory phenotype (Knolle and Limmer, 2003; Knolle et al., 1999). Furthermore LSEC express MHC class I molecules and the co-stimulatory molecules CD80/86 albeit

the latter at low concentrations. Constitutively the co-inhibitory molecule B7-H1 is expressed shown to be instrumental in induction of tolerance in naïve $CD8^+$ T cells (Diehl et al., 2008). To aid interaction with T cells the adhesion molecules ICAM (CD54) and VCAM are expressed and can be upregulated in response to bacterial endotoxin (Knolle and Gerken, 2000). Importantly LSEC have been shown to be able to cross-present exogenous soluble antigen on MHC class I molecules to $CD8^+$ T cells This finding clearly shows that LSEC function as resident antigen presenting cells (Limmer et al., 2000).

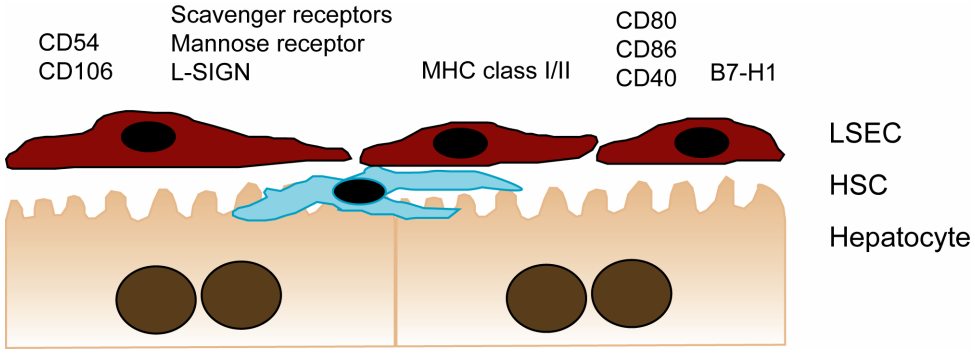

Figure II. LSEC have a unique phenotype, resembling an antigen presenting cell. *LSEC express adhesion molecules like ICAM (CD54) and VCAM (CD106), endocytic receptors and all molecules for professional antigen presentation.*

LSEC play an important role in liver homeostasis as I and others have found that they are the hepatic cell population most important for the clearance of soluble antigen from the circulation (Elvevold et al., 2008b; Malovic et al., 2007). Although LSEC contain fenestrae with a diameter of about 100nm even small particles like colloidal gold of only 15nm size cannot pass freely through them. Colloidal gold after i.v. injection was always only found in the sinusoidal lumen, but never in the space of Disse (Kempka and Kolb-Bachofen, 1988). This finding suggests that clearance of soluble molecules by LSEC is achieved predominantly by receptor mediated endocytosis. In line with this finding, LSEC express several C-type lectin and scavenger receptors.

Receptor	**Ligand**	**Reference**
Mannose receptor (MR)	Mannose, denatured collagen	(Magnusson and Berg, 1989)
L-SIGN, LSECtin (human)	Mannose, glycoproteins (hepatitis C virus)	(Koppel et al., 2005)
mSIGNR1 (mouse)		(Cormier et al., 2004)
oxidised low density lipoprotein receptor 1 (LOX 1)	apoptotic cells, Gram+/- Bac, aged red blood cells, oxidized-low density lipoprotein (OxLDL)	(Shimaoka et al., 2001)

Stabilin I and II	hyaluronan glycosaminoglycans	(Smedsrod et al., 1990)
Macrophage scavenger receptor SR-AI/ II (CD204)	negatively charged ligands, acetylated low densitiy lipoprotein (Ac-LDL), OxLDL, advanced glycation end products (AGEs)	(Smedsrod, 2004)
Scavenger receptor class B SR-B (CD36)	Collagen, OxLDL, fatty acids, *Plasmodium falciparum*	(Tandon et al., 1989) (Nicholson et al., 1995) (Oquendo et al., 1989)

The mannose receptor (MR) has been shown to mediate cross-presentation of soluble ovalbumin (OVA) by DC (Burgdorf et al., 2007). However, on LSEC the very versatile MR has multiple functions. The MR contains 3 distinct binding domains, the CysR domain binds sulphated oligosaccharides (e.g. pituitary hormones), the FNII domain is the collagen binding domain and the CTLD4 domain is a Ca^{2+} dependent sugar binding domain recognising terminal mannose, fucose and N-acetylglucosamine (Boskovic et al., 2006) and plays a role in the recruitment of lysosomal enzymes (Elvevold et al., 2008a). The MR expressed on murine LSEC has been shown to be the main collagen clearance receptor for denatured collagens (Malovic et al., 2007) and functions as a pattern recognition receptor, as for example some viruses are highly mannosylated (Elvevold et al., 2008b).

LSEC and KC also express the FcγRII B1/ 2 (CD32) and FcγR III (CD16) and can bind antibody/ antigen complexes by the Fc domain of immunoglobulin G. Larger immune complexes > 1μm are cleared through phagocytosis by KC and are not taken up by LSEC (Smedsrod, 2004). Small soluble immune complexes can be internalized by LSEC (Lovdal et al., 2000), however, this work shows, that in contrast to molecules taken up by scavenger and C-type lectin receptors, immune complexed antigens are differentially routed and badly cross-presented by LSEC. In contrast, in DC opsonization of antigen was shown to lead to maturation and enhanced antigen uptake and presentation (Regnault et al., 1999).

Molecules taken up by LSEC can be transported through the cell in a process called transcytosis and then be passed on to hepatocytes for metabolism or excretion via the bile. Transcytosis has been demonstrated for transferrin and ceruloplasmin (Tavassoli et al., 1986a; Tavassoli et al., 1986b). However pathogens like hepatitis B and C virus can exploit this mechanism, as they are endocytosed by LSEC and then transferred to hepatocytes in which they replicate (Breiner et al., 2001; Cormier et al., 2004).

LSEC seem not to play a major role in metabolism of endocytosed molecules, as transport to lysosomal compartments was shown to be relatively slow (Hellevik et al., 1998).

Finally LSEC also express the toll like receptor TLR 4 and have been shown to be able to react to very low amounts of endotoxin, resulting in the production of pro inflammatory mediators i.e. IL-6, signalling the hepatocytes (which do not express TLR themselves) to produce acute phase proteins. However, IL-6 production is tightly regulated and LSEC become unresponsive upon repetitive stimulation (Knolle and Limmer, 2003; Knolle et al., 1997).

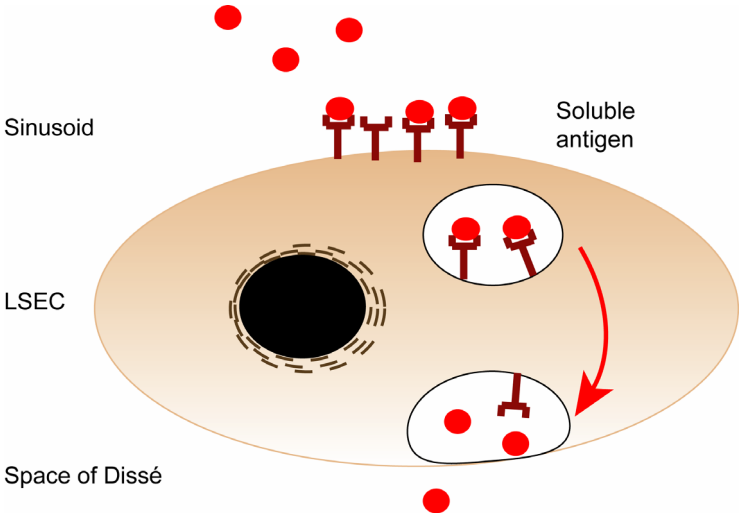

Figure III. Transcytosis in LSEC. *Uptake of soluble antigen from the simusoidal lumen and transfer to the Space of Dissé.*

1.5.5 Tolerance induction by LSEC

An important contribution to the peripheral tolerance established in the liver is made by LSEC. As LSEC line the sinusoids they get into intimate contact with passenger cells. LSEC can suppress neighbouring DC in their capacity to prime naïve T cells (Schildberg et al., 2008) and furthermore LSEC are very efficient antigen presenting cells as will be shown for antigen cross-presentation to $CD8^+$ T cells in this work. LSEC are endowed with extraordinary scavenger activity and take up circulating antigens from the blood to present them to passenger T cells that migrate through the sinusoids. Exogenous soluble antigens can be presented by LSEC in a process termed cross-presentation (described in more detail below) on MHC class I molecules to $CD8^+$ T cells (Limmer et al., 2000). Cross-presentation by LSEC ultimately leads to the induction of T cell tolerance. However naïve $CD8^+$ T cells first undergo an initial activation phase during which they upregulate activation markers like CD44 and CD69 and the high affinity IL-2Rα (CD25). Antigen specific interaction of T cells with LSEC induces a tolerogenic maturation in the latter (Diehl et al., 2008). B7-H1 which is constitutively expressed on LSEC and can deliver an inhibitory signal to T cells via PD-

1 is strongly upregulated upon interaction with T cells. This tolerogenic maturation is unique to LSEC and has not been observed to occur on DC (Diehl et al., 2008). B7-H1 PD-1 interaction has been described to stimulate naïve T cells (Dong et al., 1999) and inhibit activated ones (Freeman et al., 2000). In LSEC B7-H1 is mandatory for the induction of tolerance as B7-H1 deficient LSEC fully activate naïve T cells to become effector cells (Diehl et al., 2008). LSEC upon interaction with T cells do not up regulate the co-stimulatory molecules CD80/86 which are expressed at low levels. Importantly in the contrast to T cells primed by DC, those primed by LSEC produced no detectable amounts of IL-2 upon activation. However, T cells are induced to undergo clonal expansion and the magnitude of proliferation during the first 3 days following priming was indistinguishable to that induced by activating DC (Diehl et al., 2008). When T cells primed by LSEC are restimulated by CD3 triggering on day 4 or 5 post priming they fail to produce IFN-γ and IL-2 and show no cytotoxicity (Limmer et al., 2000). Furthermore T cells show a tolerized phenotype characterized by the expression of CD44 and CD62L but not CD25. However they express the survival factor Bcl-2 and the homeostatic cytokine receptor IL-7Rα suggesting that rather than being deleted LSEC tolerized T cells survive (Diehl et al., 2008). In contrast, the interaction of LSEC with activated T cells has been reported to induce T cell apoptosis (Huang et al., 1994).

2 Objective

The liver is an organ fulfilling important clearance, metabolic and immune functions and has been shown to be instrumental in the induction of peripheral tolerance. LSEC, which line the liver capillaries, are highly efficient scavenger cells for soluble antigen. Furthermore they have a unique phenotype, resembling a professional APC. They constitutively express MHC class I and II molecules, the co-inhibitory molecule B7-H1 and co-stimulatory molecules CD80/86, albeit the latter at low concentrations. LSEC can present exogenous antigens to $CD8^+$ T cells, inducing tolerance. By efficiently clearing foreign antigen from the circulation, LSEC help to sustain peripheral tolerance. However, tolerance induction by LSEC can be exploited by pathogens like Hepatitis B and C virus.

Therefore it is of importance to understand the mechanisms of cross-presentation employed by LSEC to aid the induction of tolerance against self-proteins in case of an autoimmune disease or the induction of immunity in case of a persistent infection.

In this work, the molecular mechanisms allowing cross-presentation in LSEC are investigated and directly compared to the mechanisms employed by dendritic cells, thereby identifying unique mechanisms that allow LSEC to combine scavenger function and high antigen-turnover with cross-presentation. Furthermore the role of the effector cytokine IL-2 in governing the outcome of T cell priming by LSEC was examined.

In this thesis the following questions were addressed:

- Which cells are responsible for the clearance of systemic circulating antigens?
- How efficient is antigen cross-presentation by LSEC in comparison to DC, *ex vivo* and *in vitro*?
- What mechanisms of antigen uptake and routing for cross-presentation do LSEC employ to accommodate antigen turn-over and cross-presentation?
- What is the functional outcome of cross-presentation by LSEC and can the high amount of presented antigen sufficiently stimulate T cells for activation, although co-stimulation is low?
- Which role does IL-2 play in the induction or prevention of tolerance by LSEC?

3 Materials and Methods

3.1 Materials

3.1.1 Mice

C57BL/6	Inbred mouse strain expressing the MHC class I haplotype $H2K^b$
MR-/-	Deficiency of the mannose receptor
OT-1 x Rag	OT-1 x Rag transgenic $CD8^+$ T cells with an $H2K^{b\text{-}SIINFEKL}$ restricted Vα2Vβ5 TCR bred with recombination–activating gene 1-deficient mice
DesTCR	TCR transgenic recognising three endogenous peptides presented in the context of $H2K^b$ (Guimezanes et al., 2001; Schonrich et al., 1991)
B7-H1-/-	Deficiency of the B7-H1 (PD ligand 1) molecule
CD80/86-/-	Deficiency of the CD80 and CD86 (B7.1 and B7.2) molecules
St35	Transgenic CD8 T cells with an $H2K^{b\text{-}SGPSNTPPEI}$ restricted TCR, show low avidity towards the cognate antigen due to low TCR expression. SGP is an adenovirus E1a protein derived peptide.

All mice were on the C57BL/6 background. Experimental animals were bred under specific pathogen free (SPF) conditions according to the FELASA guidelines in the central animal facility "Haus für Experimentelle Therapie" (HET) at the University Hospital Bonn. For all experiments mice between 6-20 weeks of age were used in accordance with local animal experimentation guidelines.

3.1.2 Cell lines

B3Z	$CD8^+$ T cell hybridoma expressing a TCR recognising the $OVA_{257\text{-}264}$ peptide in the context of $H2K^b$, TCR signalling triggers the expression of the bacterial reporter gene *lacZ* (β-galactosidase gene) placed under the control of the NFAT IL-2 enhancer element. Upon activation B3Z produce IL-2 and β-galactosidase
DC2.4	Mouse DC cell line expressing $H2K^b$
RMA	NK cell hybridoma, expressing $H2K^b$
Ag8653	GM-CSF producing hybridoma

3.1.3 Antibodies

3.1.3.1 Flow cytometry

Target	**Host**	**Clone**	**Supplier**
CD146	Rat IgG2a	ME9F1	Own production
CD11c	Hamster IgG1	HL3	BD biosciences Heidelberg
CD8a	Rat IgG2a	53-6.7	BD biosciences, Heidelberg
CD3	Hamster IgG	145-2C11	ebiosciences, San Diego
CD25	Rat IgG2b	3C7	BD biosciences, Heidelberg
CD44	Rat IgG2b	IM7	BD biosciences, Heidelberg
CD62L	Rat IgG2a	MEL-14	BD biosciences, Heidelberg
CD69	Hamster IgG1	H1.2F3	BD biosciences, Heidelberg
CD11b	Rat IgG2b κ	M1/70	BD biosciences, Heidelberg
CD19	Rat IgG2a κ	1D3	BD biosciences, Heidelberg
IFN-γ	Rat IgG1	XMG1.2	ebiosciences, San Diego
H2Kb	Mouse IgG2a	AF6-88.5	BD biosciences, Heidelberg
$H2K^{b:SIINFEKL}$	Mouse	25D1.16	Own prodcution
CD204	Rat IgG2b		Serotec, Düsseldorf
LOX1	Goat	M17	Santa Cruz Biotechnologies, USA
L-SIGN	IgM	ERTR9	BMA, Augst
MR	Rat	MR 5D3	Serotec, Düsseldorf
CD16/32	Rat IgG2b	2.4G2	Own production

TCRβ	Hamster IgG	H57-597	BD biosciences, Heidelberg
Antibodies for flow cytometry were either directly fluorochrome labelled or biotinylated and detected by fluorochrome conjugated Strepavidin (BD biosciences).			

3.1.3.2 Immunofluorescence

Primary Antibodies

Target	**Host**	**Clone**	**Fluorochrome**	**Supplier**
EEA1	Rabbit	D1707	Pure	Santa Cruz Biotechnology, USA
Calnexin	Rabbit		Pure	Abcam, Cambridge
LAMP1, CD107a	Rat IgG2a	1D4B	Pure	ebiosciences, San Diego
MR	Rat IgG2a	MR 5D3	Alexa 647/ A488	Serotec, Düsseldorf
Tap 1	Goat		Pure	Santa Cruz Biotechnology, USA
Rab11	Rabbit	H87	Pure	Santa Cruz Biotechnology, USA
Rab7	Goat		Pure	Santa Cruz Biotechnology, USA

Secondary Antibodies

Target	**Host**	**Clone**	**Fluorochrome**	**Supplier**
Goat	Donkey		Texas Red	Santa Cruz Biotechnology, USA
Rabbit	Sheep		DyLight 649	Serotec, Düsseldorf
Rabbit	Goat		Alexa 568	Invitrogen, Karlsruhe
Rat	Goat		Alexa 568	Invitrogen, Karlsruhe
To block unspecific and FcR mediated binding of antibodies, serum was used for blocking. Mouse, rat and goat sera were purchased from Caltag, Carlsberg, USA.				

3.1.4 Western Blot

Primary Antibodies

Target	Host	Clone	Fluorescence	Supplier
β-Actin	Rabbit		Pure	Sigma, Steinheim
OVA	Rabbit		Serum	Kindly provided by A. Tittel
OVA	Mouse	KB4 F6	Pure	Own production
OVA	Mouse	KB4 3B11	Pure	Own production

Secondary Reagents

Target	Host	Clone	Conjugation	Supplier
Rabbit	Goat		HRP	Santa Cruz Biotechnology, USA
Mouse	Goat		HRP	Santa Cruz Biotechnology, USA

3.1.4.1 Functional antibodies

Target	Host	Clone	Conjugation	Supplier
IL-2	Rat IgG2b	JES6 1A12	pure	ebiosciences, San Diego
CD3	Hamster IgG	145 2C11	pure	Own production
NK1.1	Mouse IgG2a	PK136	pure	Own production

3.1.5 ELISA

Target	Host	Clone	Conjugation	Supplier
IL-2	Rat IgG2b	JES6 1A12	pure	ebiosciences, San Diego
IL-2	Rat IgG2b	JES6 5H4	biotinylated	ebiosciences, San Diego
IFN-γ	Rat IgG1	R46A2	pure	Own production
IFN-γ	Rat IgG1	AN 6A2	biotinylated	Own production

3.1.6 Antibody coated beads

Anti-CD146 (MACS)	Miltenyi, Bergisch Gladbach
Anti-CD11c (MACS)	Miltenyi, Bergisch Gladbach
Anti-CD8 (MACS)	Miltenyi, Bergisch Gladbach

3.1.7 Fluorochrome labelled ligands

OVA Alexa 647/ 488	Invitrogen, Karlsruhe
AcLDL Alexa 488	Invitrogen, Karlsruhe
BSA Alexa 647	Invitrogen, Karlsruhe
Transferrin Alexa 647	Invitrogen, Karlsruhe

3.1.8 Fluorochromes

Hoechst 33258	Sigma, Steinheim
CFSE	Invitrogen, Karlsruhe
Violet dead cell	Invitrogen, Karlsruhe

3.1.9 Enzymes

Collagenase	Sigma, Steinheim
Accutase	PAA, Pasching
Peroxidase	Pierce, Rockford, USA
Trypsin/ EDTA	Gibco BRL, Karlsruhe

3.1.10 Proteins and synthetic peptides

S8L	BWG, Ebersberg
SGP	Kindly provided by H.J. Schild
OVA	Serva, Heidelberg
BSA	Roth, Karlsruhe

3.1.11 Inhibitors

Poly inosinic potassium acid	Sigma, Steinheim
Mannan	Sigma, Steinheim
Brefeldin A	ebiosciences, San Diego
Chloroquine	Sigma, Steinheim
Bafilomycin	Sigma, Steinheim
Epoxomicin	Sigma, Steinheim
Primaquine	Sigma, Steinheim
BNLF2a	Kindly provided by E.Wiertz

3.1.12 Cell culture media

LSEC medium	DMEM high Glucose (4500 mg/l) 10% FCS (v/v) 10^5 U Penicillin, 0.1 g/l Streptomycin 2mM L-Glutamine
T cell medium	RPMI 1640 10% (v/v) FCS 10^5 U Penicillin 0.1g/l Streptomycin 2mM L-Glutamine 50µM β-mercaptoethanol

3.1.13 Cytokines

Interleukin 2	Peprotech, Rocky Hill, USA
Interferon-γ	Peprotech, Rocky Hill, USA

3.1.14 Buffers and Solutions

PBS (phosphate buffered saline)	80g/l NaCl 0.2g/l KCl 1.44g/l $NaHPO_4$*2 H_2O 0.2g/l KH_2PO_4 pH 7.4 (Fa. Biochrom)
GBSS (Gey's balanced salt solution)	137mM NaCl 5mM KCl 1.6mM $CaCl_2$ 0.9mM $MgCl_2$ 0.3mM $MgSO_4$ 0.2mM KH_2PO_4 1.7mM Na_2HPO_4 pH 7.4 2.7mM $NaHCO_3$ 5.5mM D(+)-Glucose 50mM HEPES pH 7.4
MACS/ FACS buffer	PBS 1% (v/v) FCS 2mM EDTA pH 7.2
EDTA (0.5M)	186.1g EDTA approx. 20g NaOH 1000ml H_2O pH 7.8-8.0

ACK Lysis buffer	16.58g NH_4Cl 2g $KHCO_3$ 74.4mg EDTA 2000ml H_2O pH 7.2-7.4
Coating buffer for ELISA	0.1M Na_2HPO_4, pH 9
Blocking buffer for ELISA	1% (w/v) BSA in PBS
Washing buffer for ELISA	0.05% (v/v) Tween-20 in PBS
ABTS buffer for ELISA detection	17.89g citric acid, monohydrate 1000ml H_2O pH 4.35
Perfusion buffer	0.01g L- Aspartic acid 0.02g L-Threonine 0.03g L-Serine 0.04g Glycine 0.05g L-Alanine 0.13g L-Glutamic acid 0.13 g L-Glutamine 3.6g D(+)-Glucose 3.6g D(-)-Fruktose 67.4g Sucrose 0.22g KCl 0.1g NaH_2PO_4* H_2O 0.1g $MgCl_2$* 6 H_2O 2.4g HEPES

	2.0g $NaHCO_3$
	1000ml H_2O
	0.05% (v/v) Collagenase
Protein loading buffer	0.58M Sucrose
	4% (w/v) SDS
	0.04% (v/v) Bromphenol blue
	62.5mM Tris/Hcl, pH 6.8
	60mg/ ml DTT
SDS running buffer	125mM Tris
	192mM Glycin
	0.1% (w/v) SDS
Stripping buffer	100mM 2-mercaptoethanol
	2% (w/v) SDS
	62.5mM Tris/Hcl, pH 6.8
TBS (10x)	200mM Tris
	1.26M NaCl pH 7.6
TBS/ T	TBS
	0.1% (v/v) Tween 20
Transfer buffer	25mM Tris
	192mM Glycin
	0.1% (w/v) SDS
	20% (v/v) Methanol

3.1.15 Chemicals and Reagents

Citric acid monohydrate ($C_6H_8O_7*H_2O$)	Sigma, Steinheim
D(-)-Fructose ($C_6H_{12}O_6$, MW = 180.2)	Sigma, Steinheim
D(+)-Glucose ($C_6H_{12}O_6$, MW = 180.2)	Sigma, Steinheim
Disodium hydrogen phosphate (Na_2HPO_4, MW = 142)	Sigma, Steinheim
EDTA	Roth, Karlsruhe
Ethanol, absolute ($C_2H_4O_2$, MW = 46.07)	Applichem, Darmstadt
Fetal Calf Serum (FCS)	PAA, Pasching, Österreich
Glycine ($C_2H_5NO_2$, MW = 75.07)	Sigma, Steinheim
HEPES ($C_8H_{18}N_2O_4S$, MW = 238.3)	Sigma, Steinheim
Hydrogen peroxide (H_2O_2, MW = 34.0)	Pharmacy of the University of Bonn
L-Glutamine (200 mM) ($C_5H_{10}N_2O_3$)	Cambrex, Verviers, Belgien
Lymphocyte separation medium	Nycomed Pharma, Unterschleissheim
Magnesium chloride ($MgCl_2*6\ H_2O$, MW = 203.3)	Merck, Darmstadt
Magnesium chloride, 50 mM	Invitrogen, Karlsruhe
Magnesium sulfate (MgSO4, MW = 120.4)	Sigma, Steinheim
Monopotassium phosphate (KH_2PO_4, MW = 136.09)	Gerbu, Gaiberg

Monosodium phosphate (NaH_2PO_4, MW = 120.0)	Merck, Darmstadt
Nycodenz	Axis-Shield, Norwegen
Paraformaldehyde (PFA) ($H(-OCH_2)n-OH$)	Fluca, Buchs
PBS	Biochrom, Berlin
Percoll	
Potassium bicarbonate ($KHCO_3$, MW = 100.12)	Sigma, Steinheim
Potassium chloride (KCl, MW = 74.55)	Merck, Darmstadt
RPMI 1640 Medium	Gibco BRL, Karlsruhe
Sodium azide (NaN_3, MW = 65.01)	Sigma, Deisenhofen
Sodium bicarbonate ($NaHCO_3$, MW = 84.01)	Sigma, Steinheim
Sodium chloride (NaCl, MW = 58.44)	Merck, Darmstadt
Sodium hydroxide (NaOH, MW = 40.0)	Merck, Darmstadt
Streptomycin (10 mg/ml)/ Penicillin (10,000 U/ml)	PAA, Pasching, Österreich
Sucrose	Sigma, Steinheim
Tris base	Roth, Karlsruhe
Trypan Blue	Serva, Heidelberg
Tween-20	Merck, Darmstadt
β-mercaptoethanol ($HS(CH_2)_2OH$, MW =78.13)	Sigma, Deisenhofen

3.1.16 ELISA substrates

ABTS	Sigma, Steinheim
TMB	Pierce, Bonn

3.1.17 Equipment

AutoMACS	Miltenyi, Bergisch Gladbach
Centrifuges	Heraeus, Hanau
ELISA reader Spectra MAX 250	MWG Biotech, Hamburg
Elutriator Avanti J25I	Beckman Coulter, Krefeld
FACSorter, DIVA	Becton Dickinson, Heidelberg
Flow cytometer, CantoII	Becton Dickinson, Heidelberg
Flow cytometer, LSRII	Becton Dickinson, Heidelberg
Incubators, Hera cell	Heraeus, Hanau
Microscope IX71	Olympus, Hamburg
Microscope IX81	Olympus, Hamburg
Microscope, Confocal FV 1000	Olympus, Hamburg
Perfusion pump, Masterflex	Cole-Parmer Instrument Company via Novodirect, Kehl/Rhein
pH-meter, pH 523	WTW, Weilheim
Pipette, Multipipette® plus	Eppendorf, Hamburg
Pipetter, cordless	Matrix Technologies CellMate® Thermo Scientific, USA
Pipettes, 0.2-2 µl, 0.5-5 µl, 2-20 µl, 10µl-100µl, 20-200 µl, 100-1000µl	Gilson, Limburg-Offheim
Preparation instruments	Labotec, Göttingen
Shaking Waterbath GFL® 1092	GFL®, Burgwedel
Sieves, steel	University of Bonn, Department „Feinmechanik“

Sonificator	UW2070/Sonoplus (Bandeln electronic, Berlin)
Spectrophotometer Ultrospec 3100 pro	Amersham biosciences, Piscataway
Spectrophotometer, NanoDrop™ ND 1000	NanoDrop Products, Wilmington, USA
Threaded bottles,100 ml, 250 ml, 500 ml, 1000 ml, 1 l, 2 l	Schott, Mainz
Ultrapure water system, NANOpure Diamond, Barnstead	Werner Reinstwassersysteme, Leverkusen
Vibratom VT 1000S	Leica, Wetzlar
Workbench, sterile, Hera safe	Heraeus, Hanau
TE77 Semi-dry transfer unit	Amersham biosciences, Piscataway
Electrophoresis chamber SE600 Ruby	Amersham biosciences, Piscataway

3.1.18 Software

Analysis	Microscopy analysis	Olympus
Excel 2004 for Mac	Data analysis	Microsoft
FACS Diva V6.1.1	FACS analysis	BD
Flowjo V8.7.1	FACS analysis	Tree star, Inc.
Illustrator CS V11.0.0	Graphic design	Adobe
Photoshop	Graphic design	Adobe
Prism 4 for Macintosh	Statisitics and graphic design	GraphPad Software
Scan R	Microscopy analysis	Olympus
Word 2004 for Mac	Data analysis	Microsoft

3.2 Methods

3.2.1 Primary cell isolation

All mice used in the experiments were between 6-20 weeks of age. Mice were sacrificed by CO_2 mediated asphyxiation. Body surfaces were cleaned with 70% ethanol and subsequently the body cavity was opened under semi sterile conditions.

3.2.2 Purification of LSEC

3.2.2.1 Perfusion of the liver

In order to successfully isolate endothelial cells from the liver, the liver tissue needs to be digested, this is best achieved by perfusion of the liver with collagenase.

Material	Perfusion pump, 25G Needle, Perfusion buffer GBBS (Ca^{2+} deprived) containing 0.05% (w/v) collagenase or 4% (w/v) PFA
Method	Cannulation of the portal vein and subsequent opening of the vena cava inferior. The liver is then perfused with perfusion buffer for approx. 10s at a pump speed of 3ml/ min until it turns light. Liver is removed from the abdomen, gallbladder is cut carefully. Liver is kept in GBSS. If the liver is to be fixed to prepare tissue sections for microscopy, it is perfused with 4% w/v PFA. Liver is then cut into cubes and thin 50-100µm slices are cut using a vibratom.

3.2.2.2 Separation of non-parenchymal from parenchymal cells by gradient centrifugation

Material: Scissors, metal filter, GBBS with 0.04% (w/v) collagenase, Nycodenz solution, Percoll solution

Method: Livers are transferred into a petri-dish and softened tissue is torn away using scissor blades, livers are minced. Subsequently, livers are transferred into a 50ml "Falcon", GBSS with 0.04% w/v collagenase tube and shaken in rotary water bath at 240rpm for 17min at 37°C. The cell suspension is passed through a metal filter (mesh size 250µm) and centrifuged (10min, 350xg, 20°C). The supernatant is discarded and 30% w/v Nycodenz is added at 1.23 times the volume to the remaining cell solution (final density of solution 1.089g/ cm3). The suspension is overlaid with approx. 500ul of GBSS. Cells are centrifuged at 1400xg for 20min at room temperature. Cells are recovered from interface and centrifuged (10min, 350xg, 20°C). LSEC are finally isolated via centrifugal elutriation or MACS separation. If liver cells are prepared solely for flow cytometric staining a Percoll gradient is used. Cells are taken up in 40% v/v Percoll and underlaid with 80% v/v Percoll and centrifuged (20min, 800xg, 20°C). Cells are recovered from interface and centrifuged (10min, 350xg, 20°C).

3.2.2.3 Isolation of LSEC by gradient elutriation

The gradient elutriation allows a separation of cells according to their size and density. The separation is achieved by running cells at a certain flow speed through a centrifuge. The speed of flow determines cells of which size are separated, the faster the flow the bigger the cells obtained.

Material: Elutriation centrifuge: J2-MC with Rotor 'JE-6B', LSEC medium

Method: Centrifuge rotor was assembled according to manufacturers's instructions and pump system installed, tubing was sterilised by rinsing with 70% ethanol, then washed with PBS and non-specific binding of cells was blocked by 30min incubation of tubes with LSEC medium. Cells were pumped in at 16ml/ min and a rotor speed of (2500rpm, 24°C). 100ml of each cell fraction was collected increasing the speed successively to 22 and 28ml/ min (recovery of portal and periportal LSEC), 32ml/ min (mixed population containing LSEC and Kupffer cells) and 56ml/ min (Kupffer cells only). Cells were taken up in LSEC medium and seeded into Corning Cellbind® or collagen coated culture plates ($0.8x10^6$, $0.4x10^6$ and $0.15x10^6$ per 24, 48 and 96 well plate, respectively).

3.2.3 Isolation of cell subsets by magnetic activated cell sorting (MACS)

Material AutoMACS, MACS beads, MACS buffer

Method Defined number of cells (manufacturer's instructions) e.g. a mixed population of liver cells, is taken up in ice cold MACS buffer and MACS beads are added. Cells are incubated for 15min at 4°C to allow antigen specific binding. Cells are then centrifuged (300xg, 5min, 4°C), filtered and isolated via AutoMACS cell separation system.

LSEC, 24µl CD146 MACS beads in 200µl buffer per organ

DC, 20µl CD11c MACS beads in 300µl buffer per organ

$CD8^+$ T cells, 20µl CD8α MACS beads in 300µl buffer per organ

3.2.4 Purification of splenic DC

Material Syringe with 25G needle, metal filter, GBBS with 0.04% w/v collagenase, T cell medium

Method Spleens are perfused *in vitro* using a syringe with GBBS 0.04% w/v collagenase and incubated for 30min at 37°C. The spleen is passed through a metal filter (mesh size 250µm) and centrifuged (10min., 350xg, 20°C). Cells are then either stained for FACS analysis or DC are purified by MACS and subsequently cultured in T cell medium.

3.2.5 Purification of T cells

Material metal filter, nylon wool, T cell medium

Method Spleens and lymph nodes are passed through a metal filter (mesh size 250µm), centrifuged (10min, 350xg, 20°C) and taken up in T cell medium. Cells are then incubated for 60min at 37°C on a column (syringe containing nylon wool, blocked by PBS with 2% FCS). DC and macrophages will adhere to the wool, while T and B cells will not, the latter cells are carefully washed off the column with 37°C warm T cell medium. $CD8\alpha^+$ T cells are purified by MACS.

3.2.6 Purification of whole cells from lung, thymus and lymph nodes

Material metal filter, MACS buffer, ACK lysis buffer

Method Respective organs are passed through a metal filter (mesh size 250µm) and centrifuged (10min, 350xg, 20°C). Subsequently cells are filtered and ACK lysis buffer is added for 1min, before ending erythrocyte lysis by addition of FCS. Cells can then be stained for FACS analysis.

3.2.7 Preparation of bmDC

Material: Syringe, GM-CSF containing medium

Method: Bone marrow is recovered from the hind legs, which are cut close to the abdomen. Legs are stored in PBS and muscle tissue is removed. Bone marrow is flushed out with PBS using a syringe and the cell suspension is centrifuged at 300xg for 3 minutes. Supernatant is carefully aspirated and cells resuspended in warm GM-CSF containing medium, then they are seeded into Petri dishes. Cells are split after 3 days. They can be recovered for experiments on day 6-8.

3.2.8 FACSorting of cells for *ex vivo* analysis

Material: FACSorter, MACS buffer, LSEC medium

Method: To obtain highly pure cell populations for comparative analysis *ex vivo*, LSEC and CD8α+ DC are purified by FACSorting. To assess *ex vivo* cross-presentation mice are injected with 1mg OVA i.v., 45min after this initial injection a soluble fluorochrome labeled scavenger ligand such as OVA, AcLDL or BSA is i.v. injected. LSEC and DC are purified according to the conventional purification methods described before (LSEC by elutriation or CD146+ MACS, DC by CD11c+ MACS). DC are then stained with anti-CD8a and anti-CD3 antibodies to exclude T cells. CD8a+CD3-CD11c+ DC and CD8a-CD3-CD11c+ DC are FACSsorted. LSEC with high scavenger activity for fluorochrome ligands *in vivo* are sorted (this excludes macrovascular endothelial cells). Cells are directly sorted into 96 well plates with defined cell numbers. B3Z T cells are then added for assessment of cross-presentation as described.

3.2.9 Staining cells for flow cytometric analysis

Material: CantoII, LSRII, MACS buffer

Method: Cells are stained with saturating concentrations of fluorochrome-labeled antibodies for 15min in MACS buffer on ice after blocking of antibody binding to FcγR using an anti CD16/32 (10µg/ml). Dead cells are excluded from analysis by Hoechst-33258 staining (10µg/ml). Measurements were conducted with an LSR II or Canto II and data were analysed using FlowJo software.

3.2.10 T cell proliferation assay in vitro

Material: CantoII, LSRII, CFSE, T cell medium

Method: For proliferation experiments, naïve primary $CD8^+$ T cells are labelled with 1µM CFSE (10min at 37°C). The labelling reaction is stopped by addition of FCS. T cells are then cultured in T cell medium with OVA-pulsed LSEC or DC for 72h before CFSE-dilution is measured by flow cytometry.

3.2.11 Assessment of cross-presentation

Material: OVA, PD-10 column, ELISA kit, cell culture media, Glutardialdehyde

Method: LSEC or DC are pulsed with OVA *in vitro* for 30min to 4h. To examine the influence of inhibitors on cross-presentation, LSEC and DC are pre-incubated with the respective inhibitor 15min to 1h before OVA addition. If required for the experiment APC are fixed with 0.008% glutardialdehyde (3min, subsequent extensive washing is mandatory). For assessment of cross-presentation *in vivo* or *ex vivo* mice are injected with OVA i.v. or i.p.. The OVA used is purified of contaminating peptides by PD-10 column purification, performed according to manufacturer's guide lines. OVA-specific CD8 T cells (B3Z T cell hybridomas or $CD8^+$ OT-1 T cells) can be used for determination of cross-presentation. T cells are cultured together with APC over night and cross-presentation is determined by measuring the IL-2 release from T cells by ELISA.

3.2.12 Priming and restimulation of T cells *in vitro*

Material: ELISA kit, Lymphocyte separation solution

Method: T cells are cultured in T cell medium with LSEC and DC in a 24 well plate, $1x10^6$ T cells/ well. If OT-I or St35/42 T cells are used, APC are pulsed with antigen prior to co-culture. The culture is examined daily and the medium is partially replaced in case it is used up (turns yellow). DC/ T cell cultures are split on day 2. On day 4 or 5 T cells are recovered from cultures and viable T cells are purified by a lymphocyte separation gradient. T cells are taken up in 5ml T cell medium, transferred into a 15ml tube and are underlaid with 2ml lymphocyte separation solution, gradient is centrifuged at 1400g for 10 min. T cells are recovered from the interface, washed and seeded into anti-CD3 antibody (10µg/ml) coated 96 flat bottom well plates at $1x10^5$ cells/ well. IFN-γ and IL-2 release into supernatant was measured by ELISA after 16h.

3.2.13 Assessment of T cell cytotoxicity *in vitro*

Material: CantoII, LSRII, CFSE, Lymphocyte separation solution

Method: T cells are recovered from cultures on day 4 and viable T cells are purified by a lymphocyte separation gradient, as described in the section "Priming and restimulation of T cells *in vitro*". To assess specific lysis of target cells by OT-I T cells, target RMA cells are loaded with 10µM S8L peptide, control RMA cells are kept in PBS, for 30min at 37°C. Subsequently cells are washed 3 times (centrifuged at 10min, 350xg, 20°C). Target and control cells are then labelled with 1µM and 0.1µM CFSE, respectively. T cells are incubated with a 1:1 mixture of target and control cells at different effector target ratios (e.g. E:T ratios of 50:1, 25:1, 1:1). After 4-5h cells are measured by FACS and specific kill is calculated as the reduction in CFSE high target cells compared to CFSE low control cells:

$$\% \text{ specific kill} = 100x\ [1-(CFSE^{high}/CFSE^{low})_{probe}/(CFSE^{high}/CFSE^{low})_{control}].$$

3.2.14 Immunofluorescence imaging of cultured LSEC

Material: 12mm glass cover slides, 10% v/v collagen R solution, 4% (w/v) PFA, blocking buffer

Method: 12mm glass cover slides are placed in a 24 well plate (1 cover slide per well) and coated for 1h with 10% v/v collagen R in H_2O. LSEC are seeded at a density of $0.8x10^6$ cells per well in LSEC medium. Non-adherent cells are washed off on day 1. On day 2 LSEC are pulsed for 3-15 min with fluorochrome-labeled OVA (5µg/ml), BSA (5µg/ml) transferrin (10µg/ml) or AcLDL (2µg/ml) and subsequently chased for 0min-3h with medium, before fixation with 4% w/v PFA. Cells are permeabilised with 0.1% Triton and blocked with PBS containing milk powder (5% w/v), serum (1-5% v/v) and 0.1% Triton. Cells are then stained with specific antibody, if a combination of primary and secondary antibodies for detection is used, two consecutive staining steps are needed. Nuclei are visualized with DAPI (0.5µg/ml, 5min). During the complete staining process cells are kept at room temperature and for optimal distribution of antibody, cells are rocked on a rocking platform. Immunofluorescence analysis is performed with an IX71, IX81 or FV 1000 confocal microscope. To quantitatively determine colocalization an automated analysis with the ScanR software is performed (Olympus, Germany).

3.2.15 ELISA

Material — ELISA reader, ELISA plate, Coating buffer, blocking solution, substrate

Method — ELISA 96 well plate is coated with 50µl purified monoclonal primary antibody per well in alkaline coating buffer and incubated for 1h at 37°C. Free plastic is blocked with 1% BSA in PBS for 30min. Plate is thoroughly washed and centrifuged upside down to dry completely. Supernatants and a cytokine dilution standard diluted 1/3 are added to wells and incubated for 1h at 37°C. Plate is washed and the biotinylated polyclonal secondary antibody diluted in PBS is added (incubation, 1h at 37°C). Plate is washed again and peroxidase in PBS is added (incubation, 30h at 4°C). Finally the plate is washed and centrifuged upside down and a substrate is added. Measurement of colour reaction in ELISA reader, the standard curve is plotted as a sigmoid curve, as peroxides is an allosteric enzyme.

3.2.16 Western Blot

Material — Electrophoresis chamber, semi-dry transfer unit, Sample buffer, SDS running buffer, transfer buffer

Method — Primary cells are purified and whole cell lysates are taken up in sample buffer containing 60mg/ml DTT. Samples are cooked for 10min at 95°C. The protein concentration is determined using the BioRad DC protein assay and equal amounts are used for separation on appropriate SDS/ polyacrylamide gels. Gels are then blotted onto a Nylon-P membrane. After transfer of protein onto the membrane, the protein is detected by a specific primary antibody in TBS/ Tween-20 (0.1%) milkpowder (5%) 1h at 20°C, the primary antibody is diluted as found to function best. The membrane is washed and primary antibody is detected by a secondary antibody which is conjugated to horseradish peroxidase (HRP). Chemiluminescence is detected on X-ray film. To demonstrate loading of equal amounts of protein, membranes are stripped of antibody using a stripping buffer and can then be stained for a ubiquitous protein such as actin.

3.2.17 Calculation of OVA clearance

Method

Amount of fluorescent ligand in cells at various time points was measured by flow cytometry and calculated as mean fluorescence intensity. For each one, the value at 55min was normalised to 100%. Then an exponential function was fitted to the data of all experiments using nonlinear least squares, i.e. the function $\sum\left(de^{\beta t} - x_i\right)^2$ was minimised with a numerical algorithm in the Matlab software by Mathworks.

3.2.18 Determination of cell numbers

Method

Cell suspensions are diluted by a factor of 10 in Trypan Blue solution and 10µl are applied to the Neubauer counting chamber. Determination of total cell number is performed by counting four large squares. Only viable cells (cells that are not stained by Trypan Blue) are counted. The total cell number is calculated by the formula:

$$total\ cell\ number\left[\frac{1}{ml}\right] = \frac{cell\ number\ [counted]}{4} \times 10[dilution] \times 10000[chamber\ factor]$$

3.2.19 Statistics

The Student's two tailed t test was used for the evaluation of both in vitro and in vivo experiments. Results are shown as mean + standard error of the mean (SEM), p-values <0.05 were considered significant, * = p<0.05, ** = p<0.01, *** = p<0.001.

4 Results

4.1 Rapid scavenging of circulating antigen by organ-resident LSEC

To investigate which cell population eliminated antigen circulating within the blood stream, cellular uptake of Alexa-647 labeled OVA in various organs following intravenous application was quantified. Blood-borne antigen was clearly present in hepatic cells, whereas only little antigen was taken up by cells in the spleen or lung and as expected almost no antigen uptake was observed into cells within peripheral lymph nodes or primary lymphatic tissues (Fig. 1a). Antigen-uptake by liver cells was far more efficient by at least two log steps compared to antigen uptake by cells in other organs (Fig. 1a). Confocal analysis of perfusion-fixed liver tissue revealed that hepatic cells lining the liver sinusoids were taking up circulating antigen (Fig. 1b, left).

Antigen uptake was stronger in the periportal field than in the perivenous field (Fig. 1b, right), indicating that cells lining the sinusoids in the periportal field are more efficient in antigen uptake. This phenomenon has been described before by the group of Barberá-Guillem who could show that increased antigen uptake in the periportal field was not due to better access to antigen, but a feature of periportal LSEC (Vidal-Vanaclocha et al., 1993a; Vidal-Vanaclocha et al., 1993b).

To further characterise this highly efficient hepatic scavenger cell population in the, liver cells were purified 1 hour after intravenous antigen administration and stained with specific antibody. Antigen-positive cells were not $CD11c^+$ (DC cell marker) or $CD11b^+$ (macrophage cell marker), but instead were $CD146^+$ (endothelial cell marker) (Schrage et al., 2008) (Fig. 1c). However, not all $CD146^+$ cells showed pronounced scavenger activity (Fig. 1c) suggesting that microvascular liver sinusoidal endothelial cells (LSEC) rather than macrovascular hepatic endothelial cells were responsible for antigen-uptake from the blood-stream. Direct comparison of scavenger activity of LSEC with DC from liver and spleen revealed that LSEC contained far more Alexa 647-OVA after intravenous application (Fig. 1d).

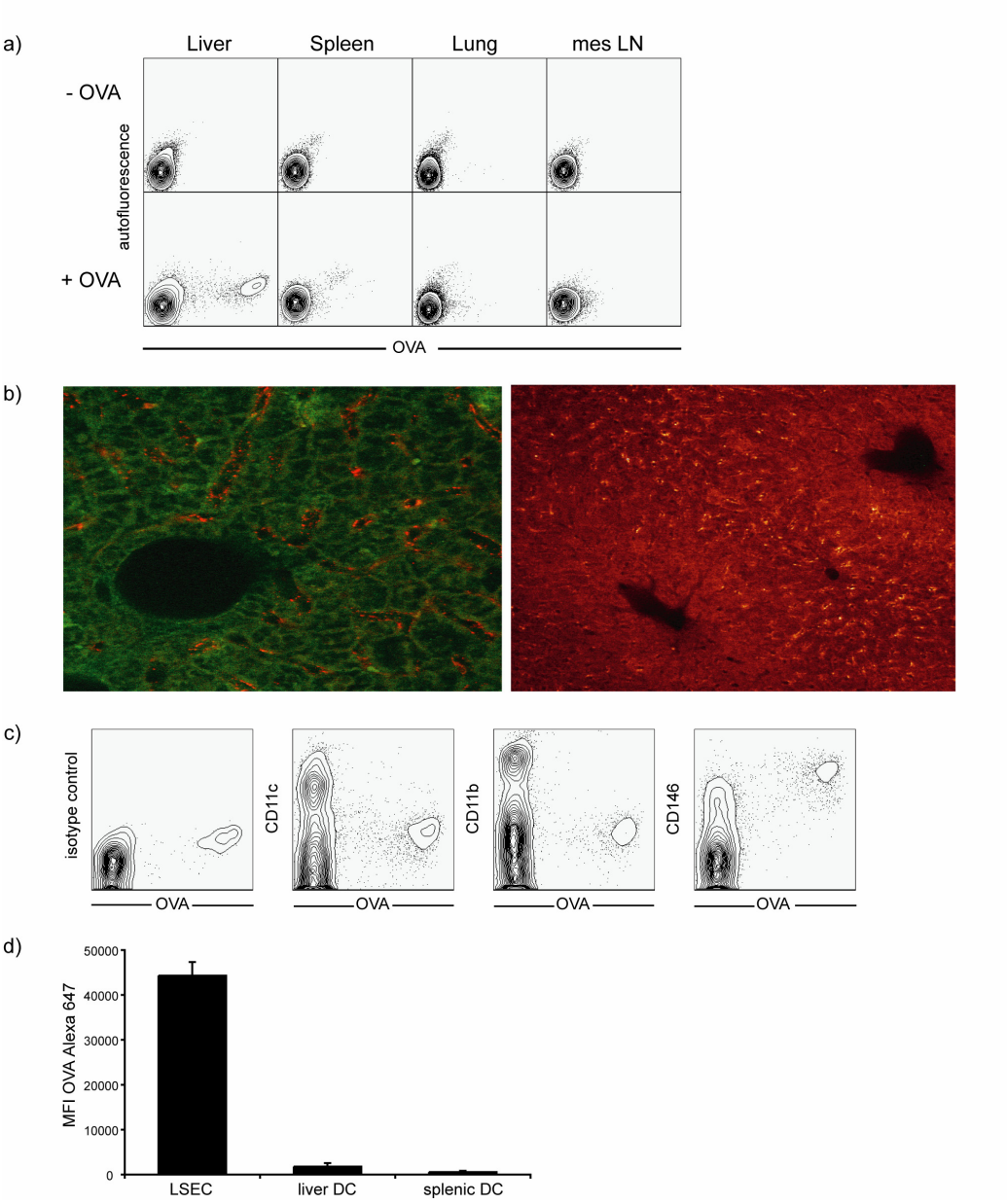

Figure 1. Preferential uptake of circulating soluble antigen by scavenger cells in the liver. *a) Alexa647-labeled OVA (4µg/mouse) was intravenously injected and cells from various organs were isolated after 1h for flow cytometric analysis (a and c) or livers were perfusion-fixed (4% PFA) and 80µm thick tissue slices were analyzed by confocal laser scanning microscopy, OVA Alexa 647 is stained in red, autofluorescent hepatocytes appear green in left figure (b). (c) Hepatic cells taking up OVA from the circulation were stained for various surface markers for phenotypical analysis by flow cytometry. (d) LSEC, splenic or hepatic DC were isolated from mice 1h after intravenous injection of Alexa647-labeled OVA (20 µg/mouse) and fluorescence was analysed by flow cytometry.*

4.2 More pronounced cross-presentation by LSEC compared to DC

The next question addressed was whether efficient antigen uptake also allowed LSEC to efficiently cross-present soluble antigen to CD8 T cells. Previously it has been shown, that LSEC cross-presented endocytosed OVA on $H2K^b$- MHC I molecules as demonstrated by positive staining with the $H2K^{b\text{-}SIINFEKL}$-specific antibody 25.D1-16 (Limmer et al., 2000). This finding indicated that scavenger LSEC constitute a homogenous cell population with respect to the ability to cross-present soluble antigens.

For the comparison of cross-presentation and priming capacity of LSEC and DC, an *in vitro* antigen dose titration experiment was performed. Similar numbers of DC and LSEC were pulsed with increasing concentrations of OVA protein and T cells of the B3Z hybridoma cell line or primary naïve CFSE labelled OT-I T cells were added, for the assessment of cross-presentation or priming, respectively. These experiments revealed that LSEC were more efficient than DC in cross-presenting soluble OVA to B3Z (Fig. 2a) and in priming naïve CD8 T cells for proliferation (Fig. 2b). The more prominent cross-presentation by LSEC was accompanied by more pronounced antigen-uptake *in vitro* compared to bone marrow derived dendritic cells (data not shown).

To further study the contribution of LSEC to cross-presentation of circulating antigen *in vivo*, a novel isolation procedure was developed, based on immuno-magnetic separation in combination with FACSorting of hepatic cells with high scavenging activity. This procedure yielded a purity of more than 99% for $CD146^+$ scavenger $receptor^+$ LSEC (Fig. 2c) and made it possible to unequivocally characterize cross-presentation mediated by LSEC in comparison to splenic $CD8\alpha^+$ DC *ex vivo*. $CD8\alpha^+$ DC have been shown to be the most important DC subset for cross-presentation of soluble antigen (den Haan et al., 2000; Pooley et al., 2001). Animals were challenged with 1mg OVA i.v. followed by a second injection 45 minutes later of a fluorochrome labelled scavenger ligand such as BSA or AcLDL. Mice were sacrificed 15 minutes after the second injection. $CD146^+$ LSEC and $CD11c^+$ splenic DC were isolated by AutoMACS positive selection. $CD11c^+$ cells were further stained for $CD8\alpha$ and CD3, to exclude $CD8\alpha^+$ T cells. LSEC with high uptake of scavenger ligand and $CD3^-$, $CD8\alpha^+$ and $CD3^-$, $CD8\alpha^-$ DC where directly sorted into 96 well plates and *ex vivo* cross-presentation to B3Z T cells was assessed.

Clearly, LSEC showed more pronounced cross-presentation of systemically circulating antigen than splenic $CD8\alpha^+$ dendritic cells (Fig. 2d). As expected, splenic $CD8\alpha^-$ dendritic cells did not show significant cross-presentation capacity (Fig. 2d).

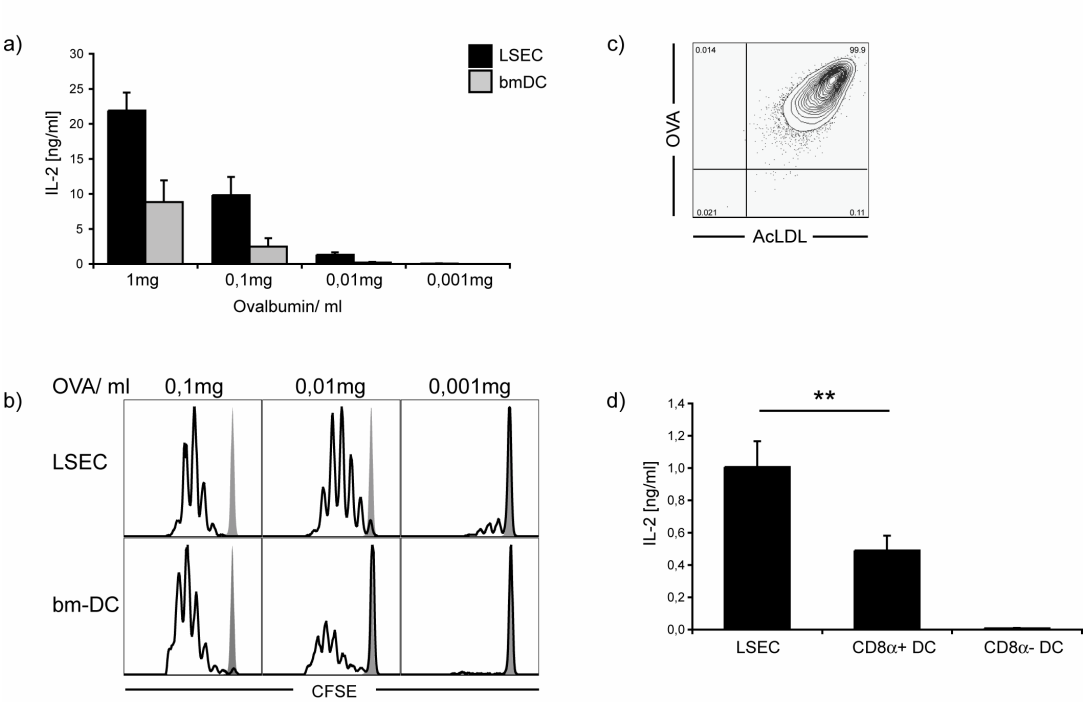

Figure 2. More pronounced early cross-presentation by LSEC compared to DC. *a) LSEC or bone marrow-derived DC (bmDC) were incubated with different concentrations of soluble OVA in vitro for 1h, cultured with H-2K$^{b\text{-}SIINFEKL}$-specific CD8 B3Z T cells over night and cross-presentation was determined by measuring IL-2 release into the cell culture supernatant. b) LSEC and bmDC pulsed with different concentrations of OVA were incubated with CFSE-labelled naïve OVA-specific OT-I CD8 T cells and proliferation was determined on day 3 by CFSE dilution. c) CD146^{+} LSEC were isolated from Alexa647-OVA iv injected mice. CD146^{+} LSEC were FACSorted according to high OVA uptake and purity of the sorted cell population was determined by further in vitro incubation with Alexa488-AcLDL (1µg/ml) followed by flow cytometric analysis for OVA and AcLDL-uptake. d) LSEC, CD8α^{+} or CD8a^{-} splenic CD11c^{+} DC were isolated from mice iv injected 1h before with OVA (1mg) and equal numbers of cells were FACSorted into 96 wells for direct ex vivo comparison of cross-presentation to B3Z cells.*

4.3

Elimination of ovalbumin from LSEC is rapid *in vivo*, limiting the duration of cross-presentation

To allow for efficient and continuous scavenging activity in LSEC, antigen uptake needed to be accompanied by rapid elimination. Therefore, I characterized how long LSEC would retain antigen that was given in a bolus injection via the intravenous route. Isolating cells at various time points after intravenous application of Alexa 647-labelled OVA, showed that fluorescence intensity in LSEC *in vivo* was maximal 1 hour post injection and then gradually declined with a half-life of approximately 6 hours (Fig. 3a). Detection of OVA by immuno-blotting from isolated LSEC confirmed the rapid uptake and turnover of antigen in this cell population (Fig. 3b). Importantly, rapid turnover of antigen in LSEC was accompanied by a decrease in cross-presentation 20 hours after the antigen-uptake *in vivo*, whereas no such decrease was observed for dendritic cells isolated from spleen (Fig. 3c). Taken together, these data demonstrated that LSEC are potent cross-presenting cells and that their scavenging activity with high antigen-turnover restricts the duration of cross-presentation.

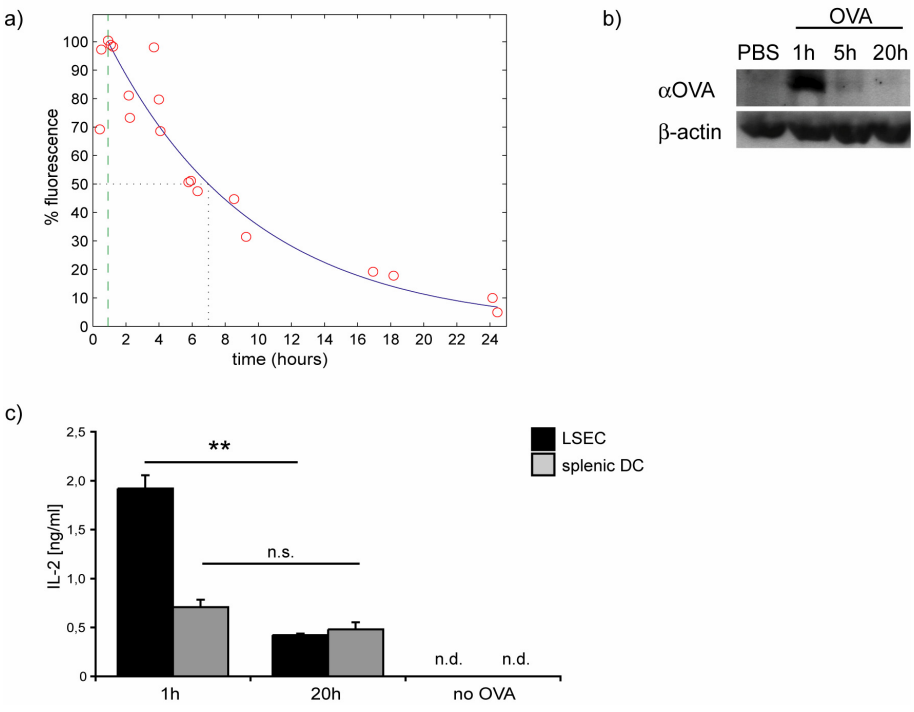

Figure 3. Rapid turn-over of endocytosed antigen in LSEC limits duration of cross-presentation in vivo. *a) Mice were i.v. injected with Alexa 647-OVA (4 µg) and LSEC were purified at various time points after injection and analysed by flow cytometry. Maximal uptake of OVA by LSEC determined as increase in mean fluorescence intensity (MFI) was observed 1h after injection and was set to 100%. Decreased MFI observed in LSEC at later time points is expressed in relation to maximal MFI. The non-linear correlation coefficient of antigen-clearance from LSEC is 0.967 and the t1/2 is calculated to 6h. b) LSEC were isolated at different time points after iv antigen injection and intracellular antigen*

concentrations were determined by western blot. c) Cross-presentation of LSEC ex vivo *to B3Z cells was determined at 1h or 20h after i.v. OVA injection. n.d. denotes not detected.*

4.4 The mannose receptor is redundant for cross-presentation of OVA in LSEC

Uptake of soluble antigen via the mannose receptor has been shown to determine cross-presentation in DC and macrophages (Burgdorf et al., 2007; Burgdorf et al., 2008). LSEC expressed the mannose receptor at high levels both at the cell surface and in intracellular compartments (Fig. 4a and b). The mannose receptor also colocalized with endocytosed fluorochrome-labelled OVA in endosomal compartments (Fig. 4c).

In contrast to DC, which strictly required expression of the mannose receptor for cross-presentation of soluble OVA, LSEC from mannose receptor-knockout animals retained their ability to cross-present OVA *in vitro* indicating that the mannose receptor was not essential (Fig. 4d).

However, dose titration experiments *in vitro* revealed that lack of mannose receptor expression diminished the ability of LSEC to initiate proliferation of naïve OT-1 T cells at low antigen concentrations (Fig. 4e). A contribution of the mannose receptor to cross-presentation was also observed under limiting antigen concentration *in vivo*, as LSEC from mannose receptor-knockout compared to wild-type animals showed less cross-presentation capacity *ex vivo* (Fig. 4f). Reduced cross-presentation was accompanied by reduced antigen uptake by mannose receptor deficient LSEC after intravenous OVA injection (Fig.4g).

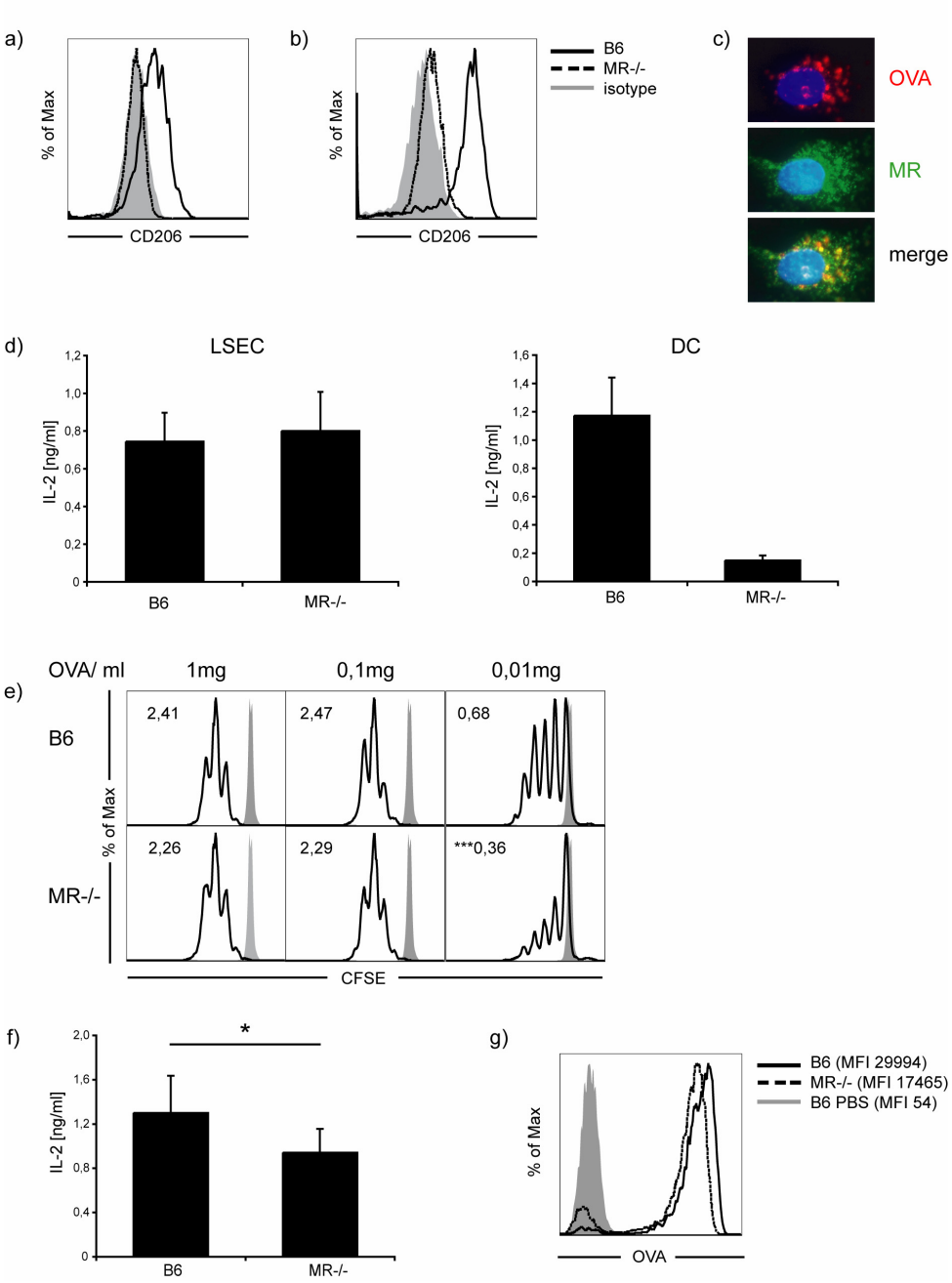

Figure 4. The mannose receptor is not essential for cross-presentation in LSEC. *a,b) LSEC were stained for surface (a) or intracellular expression (b) of the MR (CD206). c) Immunofluorescence microscopy of LSEC after endocytosis of fluorochrome-labeled OVA stained for the MR. d) LSEC from MR-/- and wt B6 were pulsed with OVA (1mg/ml) and cross-presentation to B3Z cells was measured by determination of IL-2 release by ELISA. e) Proliferation of CFSE-labeled naïve OT-I T cells 72h after priming by MR-/- or wt LSEC pulsed with different OVA concentrations; numbers denote division indices. f) Ex vivo cross-presentation by MR-/- and wt LSEC 1h after i.v. injection of OVA (1 mg); mean of 5 independent experiments is shown. g) Uptake of fluorochrome-labeled OVA by B6 or MR-/- or wt LSEC in vivo after i.v. injection of 4µg OVA, MFI is shown in brackets.*

4.5 Unique antigen shuttling after receptor-mediated endocytosis in LSEC

In macrophages and dendritic cells, uptake via different cell-surface receptors leads to delivery into distinct endosomal compartments that support either cross-presentation in case of the mannose receptor or MHC II-restricted presentation in case of the scavenger receptor (Burgdorf et al., 2007). LSEC apart from expressing the mannose receptor (Fig. 4a and b) also express other C-type lectin receptors like mSIGNR-1 and various scavenger receptors like the macrophage scavenger receptor-A (CD204) (Fig. 5a).

In LSEC, uptake through the scavenger receptor (for AcLDL and BSA), mannose receptor (for OVA) or transferrin receptor (for transferrin) all resulted in delivery of the respective ligands into the same early endosomal compartment within several minutes (Fig. 5b) indicating the existence of a common endosomal trafficking pathway for such receptor-mediated endocytosis. The endosomal compartment was characterized as an early endosomal one by expression of the marker early endosomal antigen 1 (EEA1) (Fig. 5c). Colocalization of endocytosed antigen with late endosomal ($Rab7^+$) or lysosomal compartments ($LAMP1^+$) could not be detected for up to 3 hours after antigen uptake (Fig. 5d). Macropinocytosis did not contribute to cross-presentation as amiloride failed to influence antigen-uptake and cross-presentation in LSEC (data not shown, (Limmer et al., 2005), while blocking all receptor mediated uptake by a high dose of polyI completely blocked cross-presentation (Fig. 5e).

In LSEC cross-presentation of OVA was rapidly achieved within 45 to 60 minutes after uptake (Fig. 5f) and (Limmer et al., 2000). Furtehrmore, OVA colocalized with $EEA1^+$ compartments only at early time-points after receptor-mediated uptake, already 60 minutes after antigen uptake, there was almost no colocalization of OVA and $EEA1^+$ compartments visible (Fig. 5g).

These findings suggested that the early endosomal compartment into which endocytosed OVA was initially delivered, was not stable over time. Further support for a continuous endosomal transport of endocytosed antigen in LSEC came from the observation that OVA taken up 45 minutes after a first OVA-challenge did not colocalize in early endosomal compartments with the OVA taken up earlier (Fig. 5h). Routing of antigen taken up by receptor-mediated endocytosis in LSEC is fundamentally different from that in dendritic cells or macrophages.

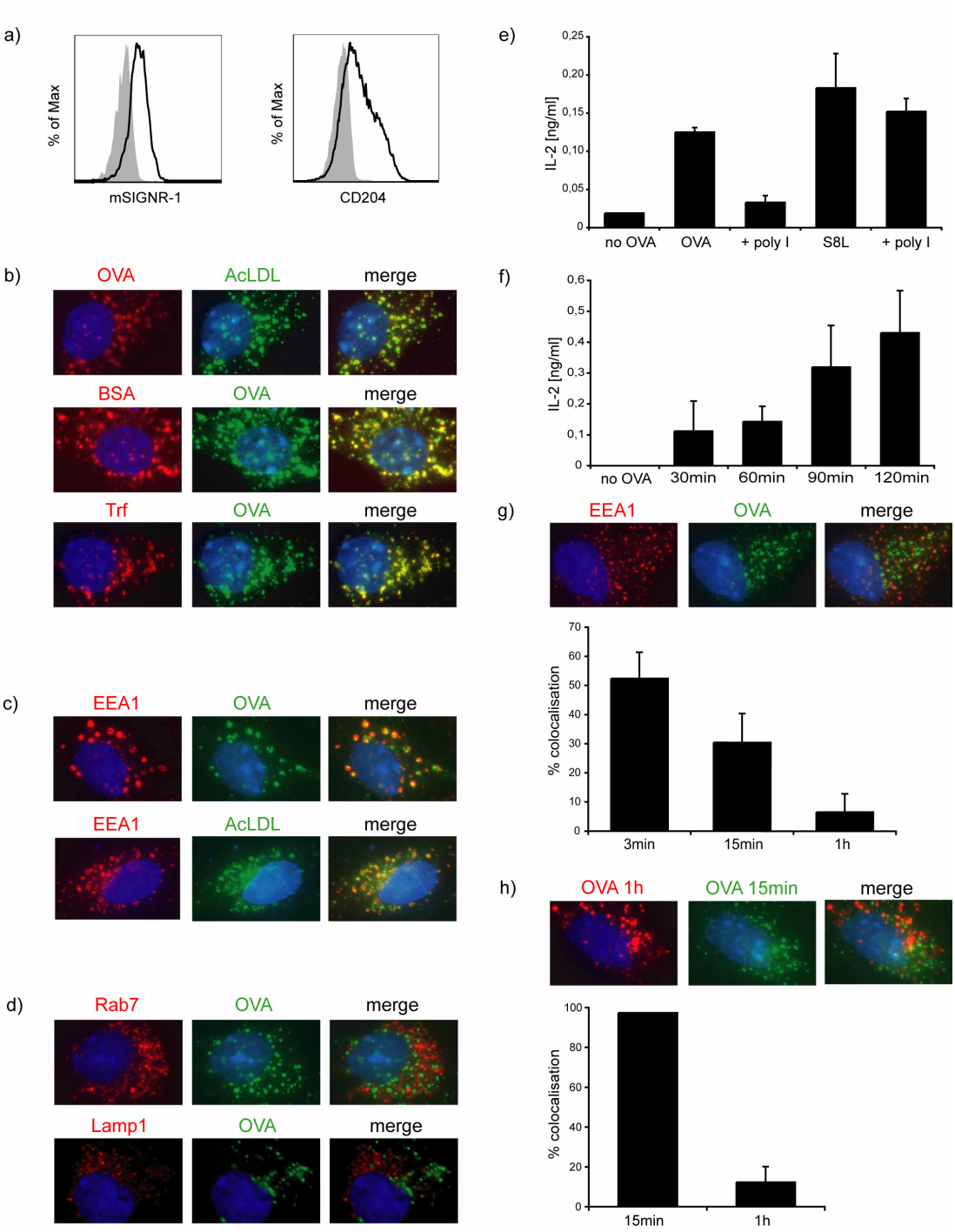

Figure 5. Unique routing of antigen in LSEC. *a) Surface expression of the receptors mSIGNR1 and CD204 on LSEC ex-vivo. b) Endocytosed antigens colocalise in an early endosomal compartment in LSEC. LSEC were simultaneously incubated with differentially fluorochrome-labelled ligands for 15 min and directly fixed with glutardialdehyde. c) Immunofluorescence microscopy of LSEC after endocytosis of fluorochrome-labelled OVA or AcLDL stained for EEA1 or d) stained for Rab7 and LAMP1 3h after endocytosis. e) Abrogation of cross-presentation by poly-inosinic acid (polyI) in vitro. f) Colocalisation of OVA and EEA1 declines within 1h of antigen uptake, immunofluorescence microscopy of LSEC stained for the EEA1 1h after endocytosis of fluorochrome-labelled OVA, at different*

time-points after endocytosis percent colocalisation was quantified. g) Immunofluorescence microscopy of LSEC after endocytosis of differentially labelled OVA given 45 minutes apart, percent colocalisation of differentially-labelled OVA is quantified for either simultaneous application or after separate applications.

4.6 Molecular mechanisms determining cross-presentation in LSEC

How do LSEC accommodate their scavenger and important clearance function with the ability to simultaneously cross-present soluble antigens? Firstly, endosomal acidification was required because cross-presentation in LSEC was prevented by drugs inhibiting vesicular ATPase such as bafilomycin (Fig. 6a) or chloroquine (data not shown).

Proteasomal processing was absolutely required for cross-presentation (Fig. 6b) as was already reported by our group before (Limmer et al., 2000). The need for proteasomal degradation indicated that cross-presentation required transport of antigen from presumably the early endosomal compartment into the cytosol. Functional transporter associated with antigen processing (TAP) was also required for cross-presentation as incubation of LSEC with a TAP-inhibitor derived from EBV (BNLF2) (Hislop et al., 2007) abrogated cross-presentation completely (Fig. 6c).

However, we could not detect TAP within $EEA1^+$ endosomes (Fig. 6d). This finding is in contrast to one made in DC, where the localisation of TAP to $EEA1^+$ endosomes confers cross-presentation competence to this particular compartment (Burgdorf et al., 2008). To further validate the independence of cross-presentation from peptide-loading of MHC I molecules within TAP containing endosomes, LSEC were pre-incubated with primaquine, a known inhibitor of endosomal transport to the cell surface, which abolished cross-presentation in dendritic cells (Burgdorf et al., 2007). Primaquine did not have a significant influence on cross-presentation in LSEC but inhibited cross-presentation in DC (Fig. 6e). These findings indicate that apart from showing very distinct routing of endocytosed antigen, LSEC furthermore did not employ the same endosomal compartment for cross-presentation as did DC.

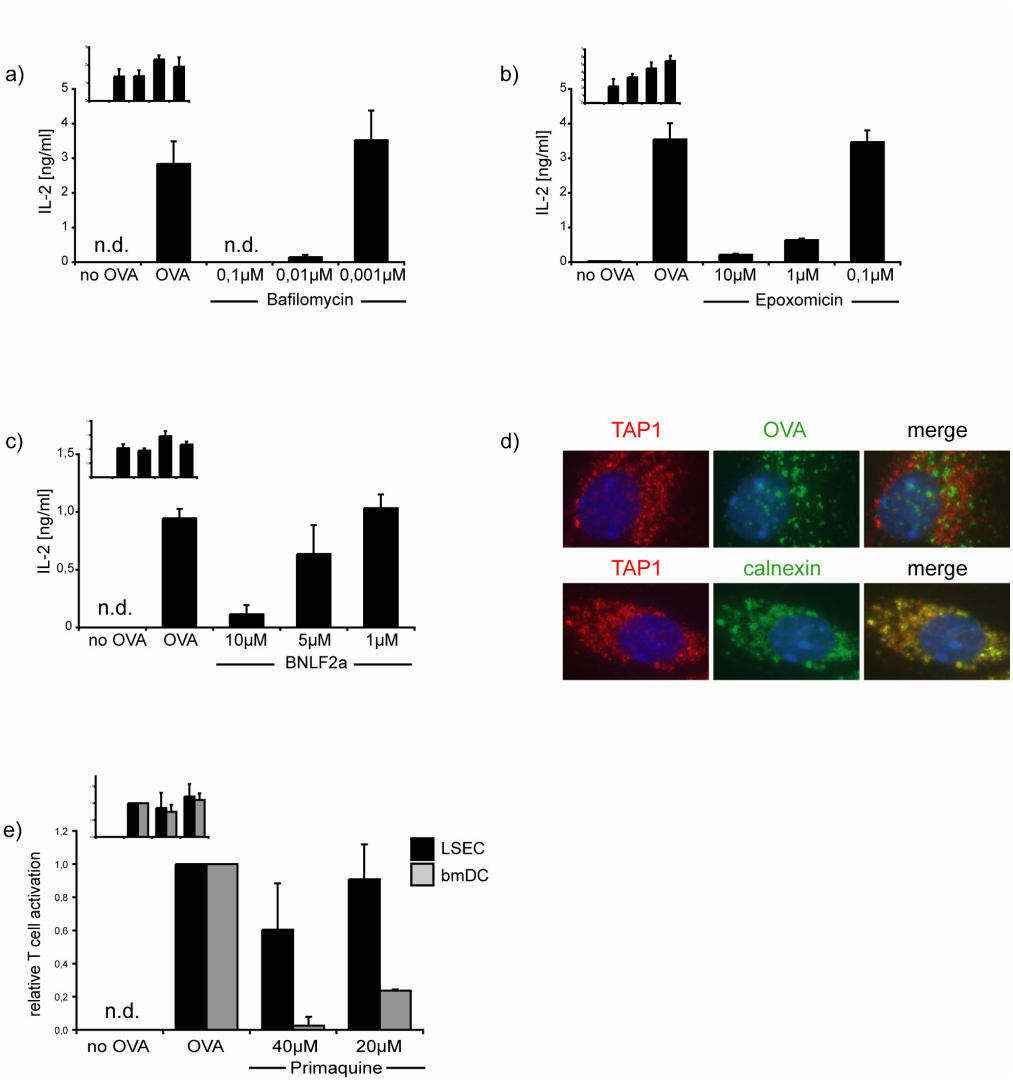

Figure 6. Mechanisms determining cross-presentation in LSEC. *ELISA of IL-2 in supernatant of B3Z cells incubated with OVA-pulsed LSEC treated with bafilomycin (a), epoxomicin (b) or a TAP-inhibiting peptide, derived from EBV BNLF2a (c). d) Immunfluorescence microscopy of LSEC staining for TAP1 and OVA (upper panel) or TAP1 and calnexin (lower panel). e) BmDC and LSEC treated with primaquine were pulsed with OVA and incubated with B3Z cells, % T cell activation was calculated from IL-2 release of T cells. For controls, LSEC were incubated with SIINFEKL-peptide and cross-presentation to B3Z cells was determined. Insets show peptide control (a, b and d) and solvent control (c).*

4.7 Immune complexed antigens are inefficiently cross-presented by LSEC

As cross-presentation was closely correlated to scavenging activity of LSEC, the possibility arose that antigen uptake through receptor-mediated endocytosis in general conferred the capacity of cross-presentation. To test this hypothesis, LSEC were pulsed with opsonized or immune complexed antigen. Immune complexed antigen is not taken up by scavenger receptors, but via Fcγ receptors (FcγR). Clearly, there was little uptake of fluorochrome-labelled antibodies compared to uptake of OVA (Fig. 7a) although LSEC expressed significant levels of FcγRII/III at the cell surface (Fig. 7b). Interestingly, FcγR-mediated antibody uptake was more pronounced in DC (data not shown) and has been reported to increase antigen-presentation (Nimmerjahn and Ravetch, 2008; Regnault et al., 1999).

These results demonstrated that FcγR-mediated uptake of antibodies in LSEC was slow compared to uptake through C-type lectin or scavenger receptors. Importantly, there was no significant colocalization of endocytosed OVA and antibody taken up by FcγRII in LSEC (Fig. 7c), which suggested distinct endosomal routing. To investigate whether antigen-uptake via FcγR altered the ability of LSEC for cross-presentation, LSEC were incubated with different ratios of OVA to anti-OVA antibodies. If OVA-specific antibodies were in excess over OVA, I observed a reduction in cross-presentation by LSEC *in vitro* (Fig. 7d). Collectively, these results demonstrated that FcγR-mediated antigen uptake occurred with low efficiency and that antigen complexed to immunoglobulins was cross-presented less efficiently by LSEC.

As LSEC are known to induce immune tolerance in naïve T cells, this observation could have important implications on the induction of a productive immune response in the presence of antibodies.

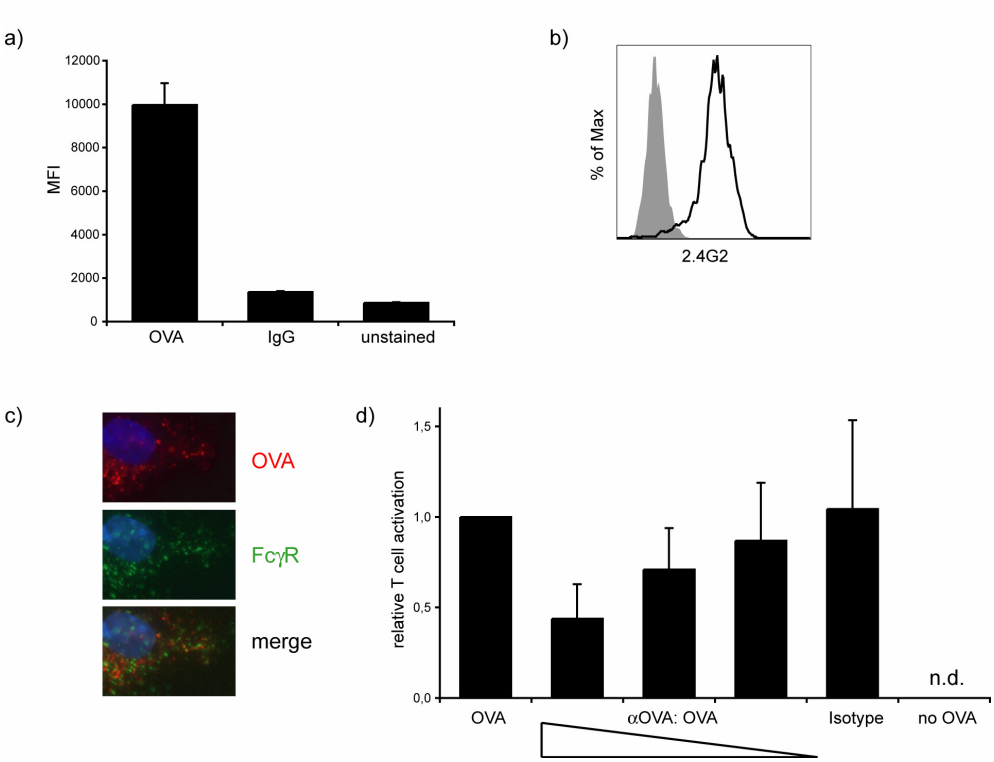

Figure 7. Reduced cross-presentation of immuncomplexed OVA. *a) Flow cytometric analysis of uptake of Alexa 647-labeled OVA or Alexa 488-labeled rat IgG at several time points, MFI values at 30min are shown. b) Immunostaining for FcγRII/III on LSEC. c) Fluorescence microscopy of LSEC after incubation with Alexa-647 OVA and Alexa 488-rat IgG for 15 min. d) ELISA of IL-2 from supernatant of B3Z cells incubated with LSEC pulsed with OVA in the presence of different concentrations of anti-OVA IgG (OVA 100µg complexed to 600µg, 300µg or 75µg specific mouse anti OVA IgG).*

So far I could show that LSEC are highly efficient scavenger cells for soluble antigen, which are capable of cross-presenting antigens to $CD8\alpha^+$ T cells, utilising molecular mechanisms and kinetics distinct from DC. It has been shown by our group before that rather than inducing immunity, LSEC can tolerize naïve T cells, suppressing immune reactions towards the respective antigens (Limmer et al., 2000; Limmer et al., 2005). In order to mediate immune functions LSEC can present antigen on MHC I and MHC II molecules and express co-stimulatory and co-inhibitory molecules usually found only on professional antigen presenting cells such as DC and macrophages. LSEC combine strong cross-presentation with low delivery of co-stimulation via CD80/ 86, but high co-inhibition via B7-H1 (PD-1 ligand) (Diehl et al., 2008). Thus LSEC can be classified as organ resident antigen presenting cells.

4.8 CD8α^+ T cells tolerized by LSEC show no cytotoxicity and have a distinct phenotype compared to those activated by DC

To investigate the functional outcome of antigen cross-presentation by LSEC to naïve CD8α^+ T cells in comparison to activation by DC, naïve OVA specific CD8α^+ OT-I T cells were cultured either with LSEC or DC cross-presenting OVA peptide. On day 1 the activation marker CD69 was up-regulated on all T cells, showing that they had recognised cognate antigen. L-selectin (CD62L) which mediates exclusion from lymph nodes, was down-regulated upon activation, this was more pronounced on T cells cultured with DC. The high affinity IL-2 receptor α-chain (CD25) was up-regulated on T cells primed by DC and to a lesser extent by those primed by LSEC (Fig. 8a and (Diehl et al., 2008)).

As priming by DC was always accompanied by the induction of IL-2 expression in naïve T cells, IL-2 release into the supernatant was measured at day 1. T cells cultured with DC had as expected released high amounts of IL-2. However, those cultured with LSEC did not release detectable IL-2 levels (Fig.8b). Albeit the quantitative difference in surface marker expression and IL-2 production, T cells primed by LSEC or DC showed no difference in proliferation on day 3 (Fig 8c). It has been described before that IL-2 has no direct effect on antigen driven T cell proliferation *in vivo* and *in vitro* (Kundig et al., 1993; Lantz et al., 2000).

On day 4 T cells were recovered from the cultures by using a lymphocyte separation gradient, excluding dead and non lymphoid cells. Cells were again stained for expression of various surface markers. The activation marker CD44 that is continuously expressed after initial activation was equally high up-regulated on both T cell groups. In contrast CD25 was completely down-regulated on LSEC primed T cells, but remained high on DC primed ones, while CD62L was high on the first and low on the latter group (Fig 8d and (Diehl et al., 2008)). To examine the functional properties of LSEC or DC primed T cells, T cells were seeded into αCD3 coated wells over night and cytokine release was measured by ELISA. In contrast to the strong cytokine release by DC primed T cells, those primed by LSEC showed strongly impaired IFNγ and IL-2 release (Fig 8e, and previously shown in (Diehl et al., 2008; Limmer et al., 2000)).

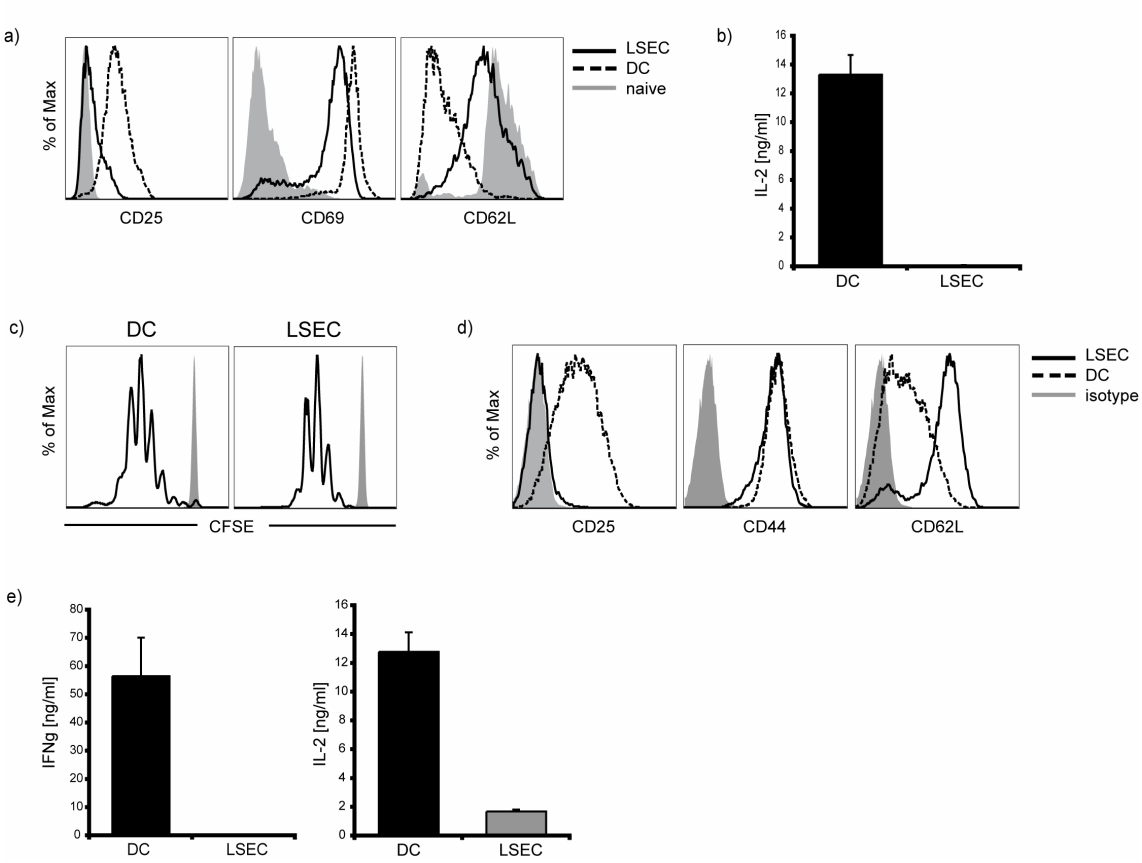

Figure 8. Phenotype of LSEC tolerized and DC activated T cells. *a) Surface marker expression on OT-I T cells at day 1 of co-culture with DC or LSEC, flow cytometric analysis and b) ELISA of IL-2 release. c) LSEC and DC are equally efficient in cross-priming of naïve CD8α$^+$ T cells. Proliferation profiles of CFSE labelled OT-I T cells on day 3 after priming, flow cytometric analysis of CFSE dilution. d) Expression of various surface markers on day 4 of culture. T cells were purified and stained for FACS analysis. e) Restimulation of OT-I T cells in αCD3 coated wells on day 4 shows abrogation of effector cytokine production by LSEC primed T cells. Representative experiments are shown.*

4.9 Exogenous IL-2 breaks tolerance induction by LSEC

One of the main differences during priming of naïve CD8α^+ T by LSEC was the absence of IL-2 production by the T cells (Fig 8b). It has been shown that IL-2 can overcome the induction of T cell anergy (Dure and Macian, 2009). To test whether *in vitro* addition of IL-2 to LSEC/ T cell culture would break the induction of LSEC mediated tolerance, exogenous IL-2 was added in different concentrations during onset of the culture. Addition of exogenous IL-2 slightly increased the surface expression of the high affinity IL-2 receptor α-chain, measured on day 1, although not to the expression level reached by T cells primed by DC (Fig 9a). T cells were recovered on day 4 and again restimulated in αCD3 coated wells. IFNγ production was then measured as an indicator for activation.

T cells cultured with LSEC in the presence of exogenous IL-2 showed IFNγ production upon restimulation which was comparable to T cells activated by DC (Fig 9b). To ascertain that T cells primed by LSEC in the presence of IL-2 had developed full effector functions an *in vitro* kill experiment was performed, measuring antigen specific kill of S8L pulsed target cells. T cells primed in the presence of IL-2 showed strong cytotoxic activity, which strength was directly correlated to the amount of IL-2 added during priming (Fig. 9c). Clearly these results show that IL-2 breaks tolerance induction by LSEC.

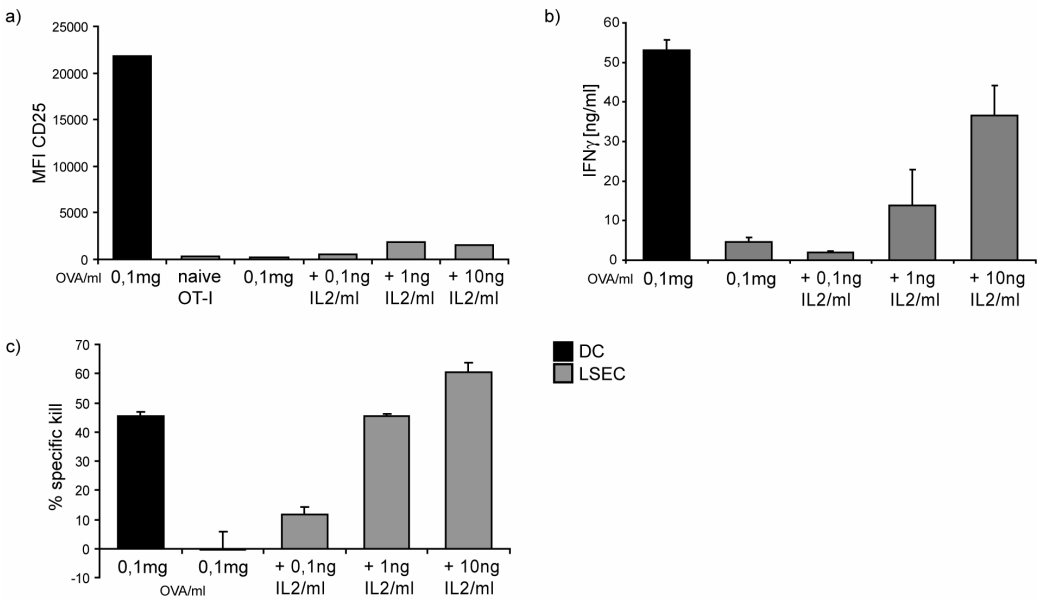

Figure 9. Influence of exogenous IL-2 on T cell priming by LSEC. *a) Surface expression of CD25 (day 1) on T cells primed in the presence or absence of exogenously added IL-2, flow cytometric analysis and b) restimulation on αCD3 (day 4). c) Representative in vitro kill experiment, T cells were purified from cultures on day 4 and specific lysis of S8L pulsed target cells was measured by FACS analysis and calculated as % kill.*

4.10 Intrinsic IL-2 production of T cells receiving a strong signal 1 through LSEC leads to effector cytokine production

While OT-I T cells primed by LSEC at 0,01mg- 0,1mg OVA/ml acquired a tolerant phenotype, I made the observation that T cells primed by LSEC at higher antigen concentrations (OVA 1mg/ml) were capable of producing comparable amounts of the effector cytokines IL-2 and IFNγ to DC activated T cells when restimulated at day 4 (Fig 10a). These findings suggested that a strong signal 1 delivered by the highly efficient cross-presentation mediated by LSEC could induce the production of IL-2 by T cells during priming, leading to the suppression of tolerance induction.

To test this hypothesis LSEC were pulsed with different OVA concentrations, starting at 1mg/ml and IL-2 release into the supernatant was measured at day 1. Indeed naïve OT-I T cells being primed by LSEC at high antigen concentrations produced significant amounts of IL-2 (Fig. 10b). When examining the surface expression of these LSEC activated T cells on day 4, T cells having received a very strong signal 1 maintained high CD25 expression in comparison to tolerized T cells (Fig 10c). However, in contrast to those activated by DC, LSEC primed T cells, showed no CD62L decrease (Fig 10c). Signalling via CD80/86 to CD28 on the T cells enhances the shedding of CD62L. This can be demonstrated through priming by CD80/86 deficient DC in which case CD62L surface expression on T cells remains high (data not shown).

To validate that the activation of T cells did indeed result from intrinsic IL-2 production upon strong activation via the TCR, an IL-2 blocking antibody was added to the LSEC/ T cell co-culture at 1mg/ml OVA. T cells which received a strong signal 1 but no longer a signal via IL-2, resembled tolerized T cells and failed to produce IFNγ or IL-2 upon restimulation (Fig.10d and data not shown).

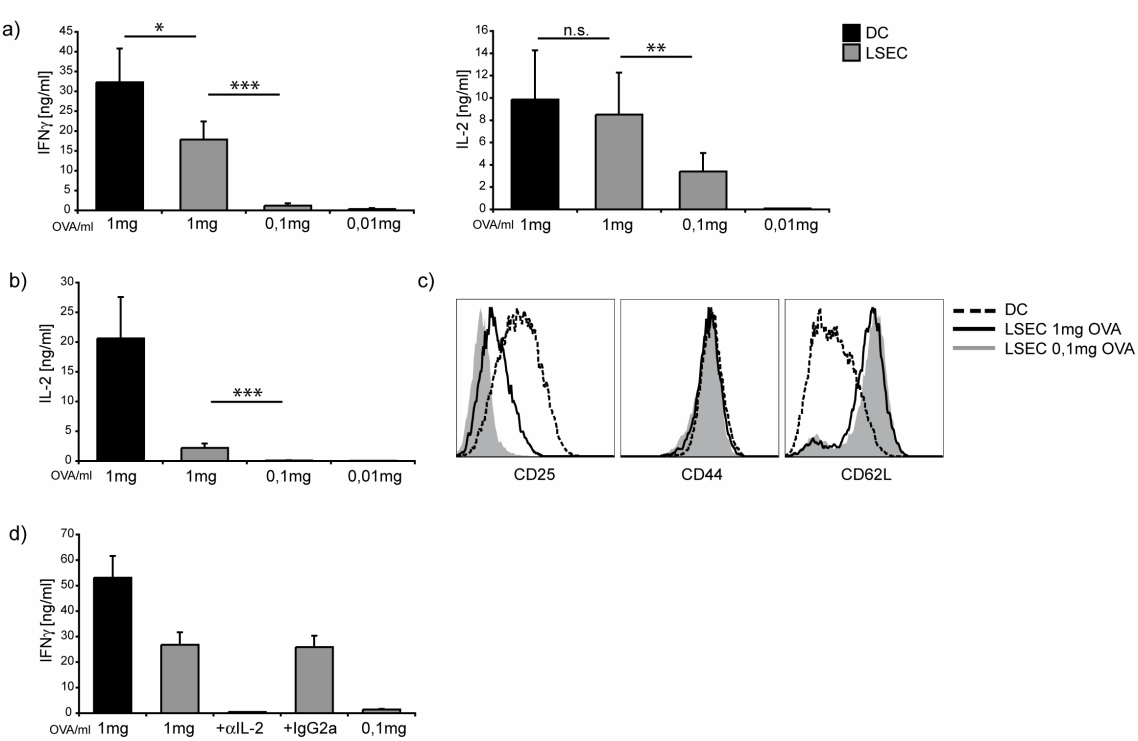

Figure 10. Influence of T cell derived IL-2 on tolerance induction by LSEC. *a) T cells were primed by LSEC at different OVA concentrations and restimulated at day 4. T cells primed at 1mg/ml OVA produced effector cytokines, measured by ELISA. b) Significant intrinsic IL-2 production by LSEC primed T cells at day1 at high OVA concentration (ELISA). c) Flow cytometric analysis of surface marker expression on day 4 after priming. d) Blocking IL-2 by a specific antibody rescues tolerance induction even at high OVA concentrations resulting in abrogation of IFNγ production, ELISA after restimulation on day 4.*

4.11 Susceptibility to activation via signal 1 depends on T cell receptor avidity

OT-I T cells are known to express a relatively high number of T cell receptor (TCR), conferring a high avidity towards their MHC I cognate antigen complex suggesting that they were readily activated. Would T cells that expressed only low amounts of TCR therefore be inert to reacting to a high number of peptide MHC class I molecules presented to them?

To this end LSEC or DC were cultured with naïve $CD8^+$ T cells derive from the St35 mouse strain, which recognise an adenovirus E1a protein derived peptide with the sequence SGPSNTPPEI (SGP) in the context of $H2K^b$. St35 T cells have approximately 5 fold reduced TCR expression compared to OT-I T cells (Fig 11a). DC and LSEC were pulsed with high concentrations of SGP and washed thoroughly before St35 T cells were added. However, St35 T cells could not be induced to produce IL-2 at day 1 when primed by LSEC even at high antigen concentrations (Fig 11b), which was easily achieved using OT-I T cells cultured with S8L pulsed LSEC (data not shown).

Furthermore, St35 T cells only produced IL-2 or IFNγ upon restimulation when cultured with DC but never after culture with LSEC. However if exogenous IL-2 was added to LSEC cultures during priming, St35 T cells were activated and produced effector cytokines when restimulated (Fig. 11c). The weak activatory TCR signal received due to the low avidity of St35 TCR was also evident in the overall reduced cytokine production compared to OT-I T cells.

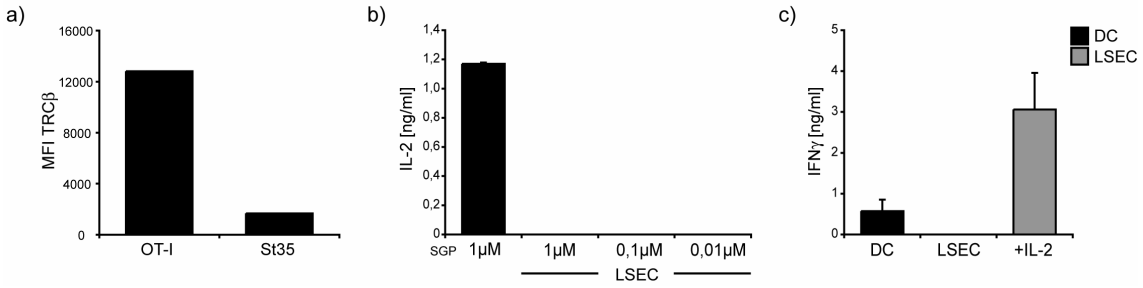

Figure 11. Influence of TCR avidity on T cell activation. *a) TCR surface expression was measured by staining with TCRβ specific antibody, flow cytometric analysis. b) IL-2 production by naive St35 CD8α$^+$ T cells on day 1 of co-culture of SGP pulsed DC and LSEC. c) Amount IFNγ produced by St35 T cells during restimulation (day 4) measured by ELISA.*

4.12 LSEC delivering a strong signal 1 to T cells do not induce cytotoxicity

Surprisingly, albeit the high amount of effector cytokines produced during restimulation by OT-I T cells which had received a strong signal 1 by LSEC, these T cells showed no cytotoxicity towards target cells carrying the cognate antigen (Fig 12a). The reason for this discrepancy between effector cytokine production especially IFNγ and cytotoxic function could result from the low signalling via co-stimulatory molecules (CD80/86) and absence of signal 3 as LSEC do not produce IL-12 in combination with high expression of the co-inhibitory molecule B7-H1 on LSEC (Diehl et al., 2008).

The co-inhibitory molecule B7-H1 is upregulated on LSEC in response to antigen specific interaction with T cells (Diehl et al., 2008). Furthermore the increase in B7-H1 expression on LSEC is directly correlated to the amount of antigen presented by LSEC and therefore to the strength of T cell/ LSEC interaction (Fig. 12b).

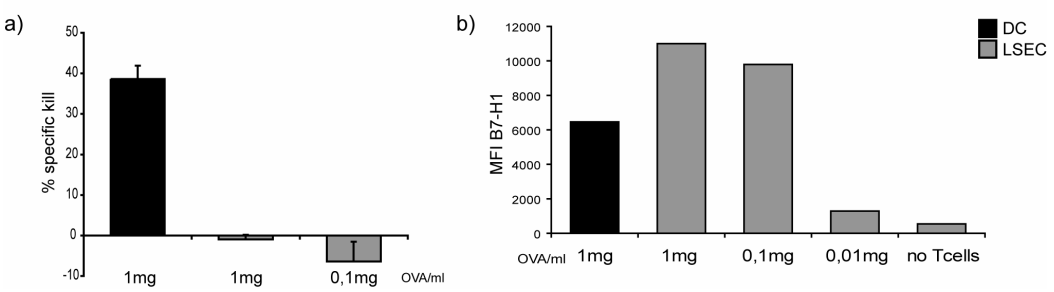

Figure 12. T cells primed by B7-H1 sufficient LSEC lack cytotoxicity. *a) Specific kill of S8L pulsed target cells on day 4 of culture. b) B7-H1 is upregulated on LSEC upon cognate interaction with OT-I T cells. LSEC were pulsed with different OVA concentrations, purified 1 day after culture onset and stained for B7-H1 surface expression for flow cytometric analysis.*

4.13 B7-H1 counteracts IL-2 production by T cells during priming, inhibiting full T cell activation

B7-H1 is vital in the induction of T cell tolerance by LSEC, as B7-H1 deficient LSEC did not induce tolerance, but on the contrary fully activated naïve CD8+ T cells (Diehl et al., 2008). During priming by B7-H1 deficient LSEC, IL-2 production by T cells was dramatically increased compared to wt primed T cells even at low antigen concentrations where the signal delivered via the TCR is weak (Fig 13a). However IL-2 production was not independent of TCR signal strength as reducing antigen concentrations led to reduced IL-2 production (Fig 13a).

T cells primed in the absence of B7-H1 showed strong expression of CD25 (Fig 13b) resulting in an increased sensitivity to IL-2, thereby further promoting activation. Upon restimulation these T cells produced high amounts of effector cytokines (Fig 13c and (Diehl et al., 2008)). Furthermore, in contrast to T cells which had been activated by wild type LSEC delivering a strong signal 1 but simultaneously co-inhibitory signals via B7-H1, T cells activated by LSEC in the absence of B7-H1 showed full cytotoxic activity (Fig 13d and (Diehl et al., 2008)).

However activation of T cells through B7-H1 deficient LSEC absolutely depended on the presence of IL-2. When IL-2 blocking antibodies were applied to the culture medium during priming, T cell activation was completely inhibited, resulting in abrogation of effector cytokine production and cytotoxicity (Fig 13e). These results strongly support the notion that B7-H1 supports the induction of tolerance in naïve $CD8^+$ T cells via the inhibition of IL-2.

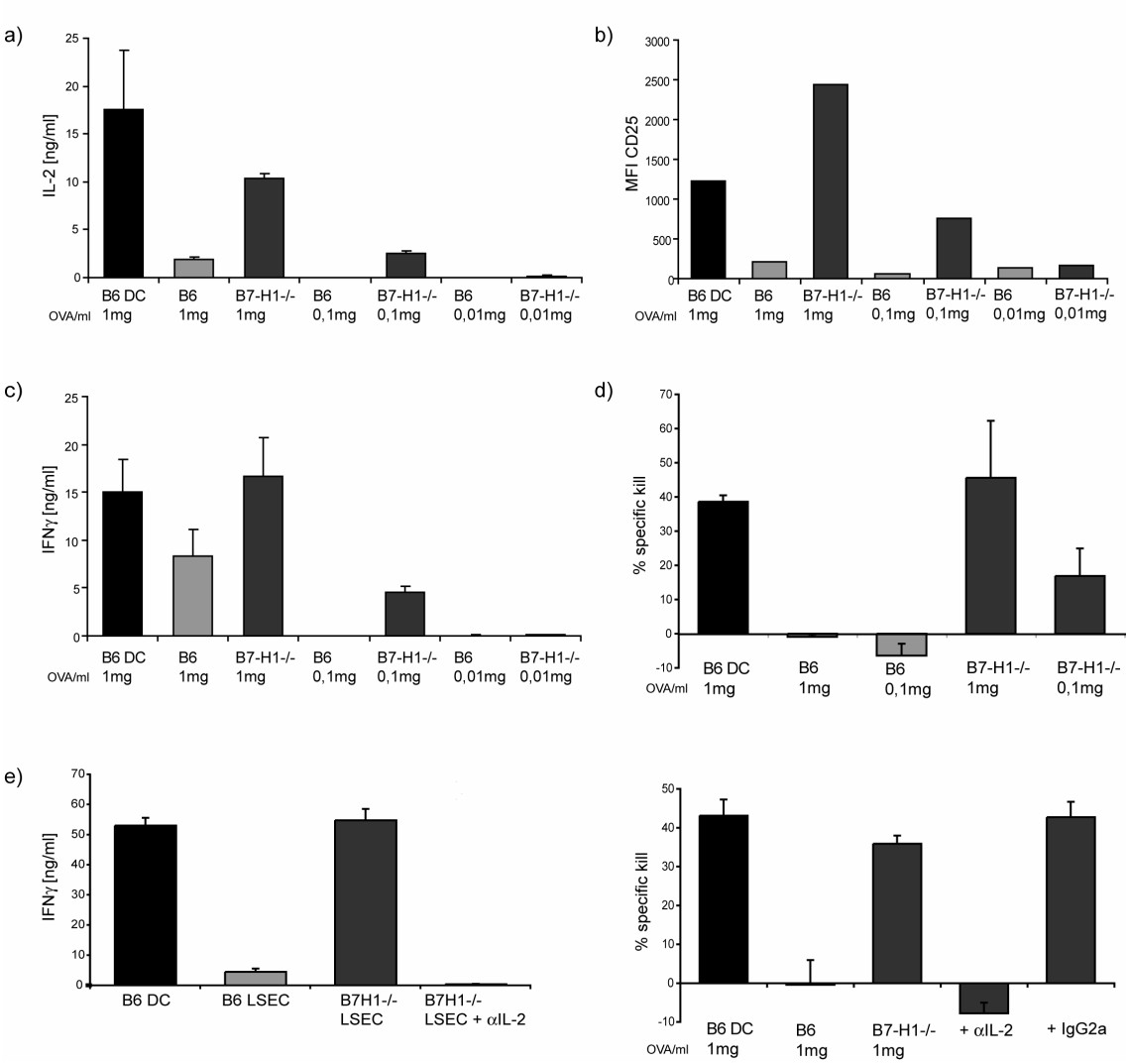

Figure 13. B7-H1 is vital for tolerance induction and mediates its effect via suppressing IL-2 production by T cells. *a) Priming by B7-H1 deficient LSEC leads to strong IL-2 production by T cells on day 1 as measured by ELISA. b) CD25 remains highly expressed on T cells primed by B7-H1 deficient LSEC, Flow cytometric analysis of surface stain on day 4. c) IFNγ release by T cells during restimulation is increased if B7-H1 is missing on LSEC during priming, day 4, ELISA. d) T cells primed by B7-H1 show strong cytotoxic activity. Evaluation of specific cytotoxicity on day 4. e) Induction of effector cytokine production and cytotoxicity by B7-H1 deficient LSEC is dependent on the presence of IL-2 during priming. Left diagramshows IFNγ production after restimulation and right diagram shows specific kill of target cells, ELISA and analysis by flow cytometry, respectively, both on day 4.*

5 Discussion

5.1 Hepatic immune functions

The liver has important clearance and metabolic functions. Portal venous blood reaching the liver is mainly derived from the gut (Knolle and Gerken, 2000) and is very rich in non self proteins derived from food and bacterial products mostly from commensal bacteria. The liver is endowed with the capacity to modulate immune responses in order to educate immune cells not to attack cells presenting harmless antigen in the periphery, therefore protection of hepatocytes is vital.

The hepatic microanatomy is formed to support this dual function. LSEC are located at the interface between cells of the immune system e.g. naïve but also activated lymphocytes and leukocytes travelling through the sinusoids and clearance and metabolism of antigen by hepatocytes. Therefore LSEC play a pivotal role in shielding hepatocytes from direct contact with passenger immune cells, inducing tolerance in naïve T cells, but also transferring antigen for metabolism and clearance by hepatocytes and finally fulfilling sentinel functions during infection of the liver (Billiar et al., 1992; Knolle et al., 1997). The clearance of host derived waste products like collagens (Malovic et al., 2007), bacterial degradation products and clearance of apoptotic cell fragments is an important function of the so called reticuloendothelial system that is comprised of Kupffer cells and LSEC (Knolle and Gerken, 2000). Antigen eliminated from LSEC is most likely transferred to hepatocytes for secretion via the bile in a process called transcytosis, because initially i.v. injected OVA could be detected in gallbladder bile at later time points (data not shown). Especially bacterial products contained within the venous blood from the gut would elicit strong immune responses if they reached the systemic circulation, therefore their clearance is vital to the organism's well-being (Knolle and Gerken, 2000). On the other hand such transcytotic transport across LSEC may for some viruses facilitate targeting of hepatocytes, as our group and others have recently reported (Breiner et al., 2001; Gardner et al., 2003).

5.2 Rapid uptake of blood borne antigen by LSEC

While performing their clearance function, LSEC simultaneously process endocytosed antigen for subsequent presentation on MHC class II to $CD4^+$ and cross-presentation on MHC class I to $CD8^+$ T cells for the induction of tolerance under homeostatic conditions (Limmer et al., 2000). Here I investigated the distribution of soluble systemic antigens. After i.v. injection of fluorochrome labelled OVA, cells of the liver sinusoids were found to show most prominent uptake (Fig 1 b). These cells were isolated and clearly identified as LSEC. No other cell population within the liver showed comparable uptake of soluble antigen. Furthermore no comparably efficient scavenger cell

population could be detected in other organs (Fig 1a), which strongly suggests that LSEC are the main scavenger cell population for clearance of soluble antigen in the body.

5.3 LSEC show more pronounced cross-presentation than DC

To investigate antigen presentation capacity of LSEC and splenic CD8α$^+$ DC from OVA injected mice were isolated. For unequivocal comparison of the *ex-vivo* cross-presentation capacity by the two cell populations, cells were highly purified by FACSorting. As only LSEC were found to efficiently take up soluble ligands in the liver, was i.v. injected. Highly scavenger ligand positive, CD146+ LSEC were then sorted, excluding contaminating DC, KC and macrovascular LEC which do not show scavenger activity (Knolle and Limmer, 2003). Splenic DC which were CD11c$^+$ CD8α$^+$ and CD3$^-$ to exclude CD8$^+$ T cells were also FACSorted.

CD8α$^+$ DC have been described to be the most efficient DC population responsible for cross-presentation of soluble antigens to CD8$^+$ T cells (den Haan et al., 2000; Pooley et al., 2001). However, I found that at a per cell basis LSEC showed better cross-presentation to CD8$^+$ T cells *ex vivo* than splenic CD8α$^+$ DC. In line with the *ex vivo* finding, antigen uptake and cross-presentation by LSEC *in vitro* was also superior to DC as revealed by antigen dose titration experiments. Even priming of naïve CD8$^+$ T cells and induction of proliferation, which requires additional signalling through costimulatory molecules (Frauwirth and Thompson, 2002; Nurieva et al., 2006), was more efficiently performed by LSEC than by DC at limiting antigen concentration. Rapid antigen-specific adhesion of naïve CD8$^+$ T cells, which is exclusively observed in the liver but not in secondary lymphatic tissue and is mediated by cross-presentation of circulating antigen by LSEC (von Oppen et al., 2008) supports the notion that cross-presentation of circulating antigens by LSEC *in vivo* is at least as prominent as cross-presentation by DC. Although cross-presentation by LSEC is stronger at early time points than cross-presentation by DC *ex vivo* and *in vitro*, it is important to note that LSEC take up far more antigen to achieve such strong presentation. It therefore seems that utilisation of antigen by DC is more efficient as in relation to the amount of antigen taken up cross-presentation was higher.

5.4 Distinct kinetics of antigen clearance and cross-presentation in LSEC

LSEC and DC show strong differences in their cross-presentation kinetics. As discussed before soluble antigen which was given in a bolus injection was found to be rapidly taken up by LSEC. The concentration of antigen in LSEC was found to be highest 1h post injection. In order to maintain continuous scavenger activity LSEC need to clear antigen efficiently. Indeed, endocytosed antigen was cleared with a half life of only 6hrs from LSEC *in vivo*, whereas antigen taken up by DC was observed to be maintained in these cells over a long period of time (Faure et al., 2009).

Rapid clearance of antigen from LSEC was accompanied by a decline in cross-presentation. Thus cross-presentation of the injected antigen was reduced in LSEC by more than 70% within 20h while in DC presentation remained stable during the same time period (Fig.3a). For DC one of the mayor functions is to sense the presence of pathogens and in response induce immunity in T lymphocytes. DC mature upon antigen encounter in combination with a danger signal from a tissue resident, strongly phagocytic cell with low antigen presenting capacity into a weakly phagocytic but efficient antigen presenting cell (Banchereau and Steinman, 1998; Palm and Medzhitov, 2009). After having taken up antigen and thereupon being activated, DC travel to LN in order to meet cognate T cells in a specialized microenvironment facilitating contact between antigen-presenting DC and T cells. It has recently been shown that DC did not prime T cells when in contact with tissue cells like LSEC or fibroblasts (Schildberg et al., 2008). This suggeststhat activation of T cells by DC can only take place in lymphatic organs. Once reaching the lymph nodes DC might have to persist for some time until encountering T cells expressing receptors recognizing the presented antigen, as this presumably might take some time, the time window for cross-presentation must be extended to facilitate induction of T cell responses.

In contrast, LSEC do not need to undergo maturation in order to efficiently present antigen (Knolle and Limmer, 2003). Furthermore as sessile cells they do not move to lymph nodes to interact with lymphocytes, but can prime T cells within the hepatic microenvironment. However, the entire blood volume moves through the liver more than 300 times a day (Knolle and Limmer, 2003) carrying about 10^8 peripheral lymphocytes (Racanelli and Rehermann, 2006), facilitating fast encounter of LSEC and potentially reactive T cells present in the circulation. Presumably most circulating antigens taken up by LSEC are also presented. As the duration for presentation of a given antigen is short, it can be hypothesized that tolerance is induced only towards antigens which are continuously circulating. Foreign antigens which are only present for a short time or at low concentrations might not be presented, therefore, T cells most likely remain ignorant.

Antigens which are present for prolonged time could either be derived from self in which case peripheral tolerance is induced or derived from a persistent infection, in

which case tolerance induction by LSEC would contribute to manifestation of chronic disease. Indeed many pathogens with liver tropism can cause chronic infections e.g. HBV and HCV.

5.5 The mannose receptor is not essential for cross-presentation in LSEC

Recently, it has been published by Burgdorf et al that in DC and macrophages cross-presentation of soluble OVA is dependent on the expression of the mannose receptor. The mannose receptor facilitates cross-presentation by delivery of OVA into a stable early endosomal antigen 1 (EEA1) positive compartment where antigen loading onto MHC I molecules occurs (Burgdorf et al., 2007). Mannose receptor negative DC and macrophages were not able to cross-present OVA.

LSEC express higher amounts of the mannose receptor on the surface and intracellularily than do DC, therefore the importance of the mannose receptor for cross-presentation in LSEC was investigated. In contrast to the situation in DC and macrophages in LSEC the mannose receptor is redundant for cross-presentation. Mannose receptor deficient LSEC could cross-present soluble OVA *ex vivo* and *in vitro*. However, the mannose receptor does seem to contribute to cross-presentation as became apparent at limiting antigen concentrations, where antigen presentation was reduced in the absence of the mannose receptor.

5.6 LSEC show distinct routing of antigen for cross-presentation

The observation that the mannose receptor on LSEC was not essential for cross-presentation of soluble antigen suggested the utilization of additional receptors. Blood passing through the liver is rich in foreign antigens, derived from food and bacteria and it might therefore not be sufficient to rely on a single receptor for cross-presentation and cross-tolerance induction. LSEC express a whole array of different scavenger and C-type lectin receptors such as mSIGNR1, the murine functional homologue of human L-SIGN (Koppel et al., 2005), which can also bind OVA. All of these receptors have been shown to efficiently internalize bound cargo (Lovdal et al., 2000).The employment of several receptors for cross-presentation could allow the induction of tolerance towards a greater range of antigens.

To investigate the involvement of different endocytic receptors in cross-presentation in LSEC, intracellular routing of several ligands was analyzed. Burgdorf et al. could show a strict spatial separation for mannose-receptor mediated delivery into an $EEA1^+$ compartment for cross-presentation or pinocytosis and scavenger-receptor-mediated delivery into lysosomal compartments for MHC class II restricted presentation in DC and macrophages, respectively (Burgdorf et al., 2007; Burgdorf et al., 2008). In LSEC, however, ligands of the mannose receptor, scavenger receptor and transferrin receptor were all rapidly delivered into a common endosomal compartment. This compartment

was also EEA1+. Clearly, in LSEC receptor mediated uptake is mandatory for cross-presentation, as cross-presentation of soluble antigen was completely abrogated when blocking all receptor mediated uptake by poly-inosinic acid (polyI). PolyI has no influence on pinocytosis (data not shown), confirming the finding that pinocytosis is not involved in cross-presentation in LSEC (Limmer et al., 2005). This is in line with the finding, that antigen taken up via pinocytosis DC is located to lysosoms (Burgdorf et al., 2007).

In DC the early endosomal compartment dedicated to cross-presentation has been reported to be stable for several hours (Burgdorf unpublished observation). In contrast the early endosomal compartment in LSEC was not stable, but nearly completely lost the early endosomal marker EEA1 within 1h. Furthermore if two ligands were given at different time points they did not colocalize within the early endosomal compartment. These findings suggest that development of endosomes in LSEC is highly dynamic and might indicate endosomal movement through the cell. Endosomes in LSEC possibly carry cargo for transcytosis, quickly eliminating antigen.

DC on the other hand have been reported to cross-present antigen upon maturation by a danger signal, which was taken up 48h previously, clearly showing that DC can sequester antigen for later presentation (Delamarre et al., 2003).

5.7 Molecular mechanisms of cross-presentation in LSEC

My studies showed that in contrast to DC, LSEC employ the mannose and scavenger receptors to shuttle antigen into early endosomal compartments for subsequent cross-presentation. Acidification of this endosomal compartment is required, as the inhibitors of vacuolar H+ ATPase bafilomycin and chloroquine abrogate cross-presentation in LSEC. Possibly acidification is only mild, as endosomes have been described to have a weakly acidic pH (Burgdorf and Kurts, 2008). Endocytosed antigen does not localize to lysosomal compartments for at least 3h in LSEC, while cross-presentation can be detected as early as 30min after antigen uptake. These observations are in line with the finding that fusion of endosomes with lysosomes destroys antigenic peptides for MHC class I cross-presentation (Burgdorf and Kurts, 2008). Cross-presentation in LSEC is most likely initialized from the early endosomal compartment as antigens not routed into this compartment are badly cross-presented as will be discussed later.

From endosomes antigen is exported into the cytosol by an unknown mechanism. Recently it has been shown, that transport of antigen into the cytosol involves components of the ER-associated degradation system (Ackerman et al., 2006; Wiertz et al., 1996) which might also be operative in LSEC. After delivery into the cytosol, antigen is processed by the proteasome, as shown by inhibition of the proteasome with epoxomicin, leading to complete abrogation of cross-presentation (shown here and by (Limmer et al., 2005)). In myeloid DC cross-presentation of exogenous soluble antigen is also dependent on the proteasome (Burgdorf et al., 2008).

Subsequent to proteasomal processing in the cytosol, antigen-derived peptides are reintroduced into early endosomes in DC. To this end the transporter for antigen processing (TAP), a usually ER-associated heterodimeric peptide transporter (Neefjes and Momburg, 1993) is recruited to endosomes for re-import of peptides from cytosol and loading onto MHC class I molecules within the endosome. I found no evidence in LSEC that peptides are reintroduced into the original early endosome after processing by the proteasome. TAP was detected in the ER where it colocalized with calnexin but not in endosomes colocalizing with either EEA1 or endocytosed OVA. As endosomes in LSEC were only transiently EEA1+ and not stable, this highly dynamic development of vesicles might not support loading within the endosomal compartment.

TAP recruitment to stable early endosomes in DC occurs in response to danger signals such as LPS (Burgdorf et al., 2008) and thus reinforces cross-presentation of antigens that are associated with danger signals. In LSEC no TAP recruitment to endosomes even after exogenous addition of LPS could be observed (data not shown). However TAP is involved in cross-presentation of OVA in LSEC as blocking TAP with an inhibitor derived from the Ebstein-Barr virus (EBV) BNLF2a protein prevented cross-presentation. BNLF2a binds to the cytosolic portion of TAP (Hislop et al., 2007) and therefore binding to TAP located in the ER or any other membrane cannot be distinguished.

The ER distribution in LSEC is extensive (data not shown) which potentially could facilitate fast peptide import. The half-life of peptides within the cytosol is very short as they are quickly broken down by peptidases (Reits et al., 2003). Transport of soluble protein into the ER and MHC I loading at this site has been observed in DC (Ackerman et al., 2005). Another hint for the involvement of the ER or ER-derived components comes from the observation that cross-presentation in LSEC can be blocked by brefeldin A, which inhibits transport from the ER to the Golgi. Furthermore primaquine which inhibits endosomal recycling abrogates cross-presentation in DC, but has only little influence on cross-presentation in LSEC. The sensitivity to brefeldin A but not primaquine strongly suggests that there is no endosomal cross-presentation compartment in LSEC. However, it should be noted that primaquine is toxic to LSEC at higher concentrations (data not shown) indicating that endosomal recycling is a very important mechanism in these cells, even if not involved in cross-presentation.

It has been shown that presentation of antigen on MHC class I molecules is highly inefficient. Only 0,1% of specific peptides, approximately, survive intracellular processing and can be loaded on MHC class I molecules (Yewdell et al., 2003). The extraordinary scavenging activity of LSEC could compensate for the lack of a specialized endosomal compartment incorporating antigen loading for cross-presentation, but instead allow the utilisation of a less selective molecular mechanisms such as loading in the ER. However, even if antigen loading is not achieved within the early endosomal compartment in LSEC, receptor mediated shuttling towards it still

determines entry of antigens into the cross-presentation pathway as will be discussed in the following.

5.8 LSEC cross-presented immune complexed antigen only weakly

To investigate whether all receptor mediated uptake resulted in routing of the ligand into the early endosomal compartment targeted by scavenger, mannose and transferrin receptor, antigen was incubated with specific antibody to allow uptake via FcγR. Antigens taken up by FcγR in LSEC did not colocalize with scavenger or mannose receptor ligands. Furthermore, they were not transferred into an EEA1 or transferrin positive compartment, showing that they were not localized to an early endosomal compartment. Although LSEC strongly expressed FcγRII and III on the surface, FcγR-mediated uptake of antibodies was much lower compared to uptake of OVA or AcLDL. Most importantly in the presence of excess antibodies to OVA cross-presentation was reduced. In addition to demonstrating that most efficient endocytosis and cross-presentation is not a uniform feature of all endocytic receptors in LSEC these findings also suggest that antigens, which have already elicited an CD4 T cell and B cell response leading to antibody production, will not be cross-presented by LSEC.

As LSEC are known to induce tolerance towards their cross-presented antigen in T cells, reduced or absent cross-presentation of opsonized antigen is an intriguing possible mechanism to avoid the induction of tolerance towards an antigen which is targeted by an ongoing immune response. Lack of cross-presentation by LSEC may instead passively support induction of CD8 T cell immunity, helping to combat infection. On the other hand the strong bias of LSEC to induce tolerance could facilitate evasion mechanisms of pathogens. Hepatitis B virus is known to produce extensive amounts of virus antigens (Lopes et al., 2008) which if not opsonized sufficiently could lead to the induction of chronic hepatitis supported by tolerance inducing LSEC.

5.9 Functional outcome of LSEC mediated cross-presentation

As the functional outcome of efficient cross-presentation by LSEC is of major importance in health and disease, it's study has been further pursued. The phenotype of $CD8^+$ T cells having been primed by immunogenic DC or tolerogenic LSEC *in vitro* is distinct. Early during priming both groups show similar up-regulation of the high affinity IL-2 receptor (CD25) and the activation marker CD69, which allows retention of T cells within LN (Shiow et al., 2006). T cells primed by either, DC or LSEC, show proliferation undergoing several rounds of division indistinguishable in magnitude. However, those primed by LSEC cease to proliferate after approximately 72h (Diehl et al., 2008). The halt in proliferation is not followed by induction of apoptosis. T cells primed by LSEC have been shown to be positive for the anti-apoptotic molecule Bcl-2 and additionally they expressed the low affinity IL-2 receptor (CD122) and the IL-7 receptor (CD127) further aiding the survival of tolerant T cells (Diehl et al., 2008). This

indicates that tolerized T cells can persist. The IL-7 receptor is implicated in the survival of virus specific T cells during chronic hepatitis B infection (Lopes et al., 2008).

T cells acquiring a tolerant phenotype will eventually express low CD69 (not shown) and CD25 and high CD44 and L-selectin (CD62L) excluding them from leaving the blood to home to LN (Henrickson et al., 2008). When restimulated by triggering TCR signalling via targeting CD3 with a specific antibody, T cells tolerized by LSEC will not respond with effector cytokine production. This is in contrast to DC activated T cells which produce high amounts of IL-2 and IFNγ. Furthermore, T cells primed by LSEC do not exhibit cytotoxicity towards antigen presenting target cells (Diehl et al., 2008; Limmer et al., 2000). The lack of cytokine production and specific cytotoxicity is not caused by insufficient priming through LSEC, as continued high expression of CD44 on the whole population confirms that all T cells have encountered their cognate antigen (Diehl et al., 2008)

5.10 IL-2 can break tolerance induction in naïve $CD8^+$ T cells by LSEC

IL-2 has been shown to mediate important immune functions, supporting clonal expansion and sustained T cell responses as well as functioning as a survival signal for regulatory T cells (D'Souza and Lefrancois, 2003). Furthermore IL-2 can overcome the induction and reverse established T cell anergy (Dure and Macian, 2009). Therefore IL-2 can play a dual role supporting immune activation and termination.

To investigate the influence of IL-2 during priming by LSEC, cultures were supplemented with exogenous IL-2. Addition of the exogenous cytokine broke tolerance induction by LSEC and led to full T cell activation *in vitro*. However, the strength of activation absolutely depended on the amount of exogenously added IL-2. While low IL-2 concentrations resulted in mild cytokine production and weak cytotoxic activity, both features could be increased with increasing IL-2 concentrations. This shows that IL-2 signalling is dynamic resulting in different levels of T cell activation, depending on the original signal strength. Could tolerance induction therefore be a result of low amounts or absent IL-2 during priming?

Indeed, a clear difference during the first 24h of priming by LSEC or DC is seen in the production of IL-2 by T cells. T cells primed by DC secreted significant amounts of IL-2, while when primed by LSEC IL-2 was undetectable in supernatant (Fig10b).

The production of IL-2 and CD25 in T cells is induced by signalling via the TCR (Acuto and Michel, 2003) and supported by co-stimulation via CD80/86 to CD28, which lowers the number of TCR that need to be triggered (Viola and Lanzavecchia, 1996). Presumably, the low IL-2 production by LSEC primed T cells was the result of insufficient co-stimulation, as LSEC only express low numbers of CD80/86. Furthermore, CD80/86 is not upregulated by LSEC upon antigen specific T cell

interaction as is the case for DC (Diehl et al., 2008; Sharpe and Freeman, 2002). Cross-presentation in DC which was found to be lower at a given antigen concentration than in LSEC (Fig 2), could still induce robust IL-2 production by naïve T cells. Most likely DC achieve this strong activation by enhancing TCR signalling via co-stimulation.

It is important to note that naïve T cells albeit the lack of intrinsic IL-2 production, upon priming by LSEC, initially upregulated CD25. This finding clearly shows that T cells were able to respond to IL-2 but limited in its production. This implies that LSEC do not inhibit IL-2 responsiveness in T cells, but do at low antigen concentrations not support itsexpression.

To evaluate if LSEC principally were capable of promoting IL-2 production in T cells, the concentration of antigen given to co-cultures was increased to promote stronger cross-presentation by LSEC. Increased cross-presentation should result in enhanced TCR triggering. Indeed these experiments revealed that the efficient cross-presentation mediated by LSEC could at high antigen concentrations (OVA 1mg/ml) trigger the TCR sufficiently to induce IL-2 production by T cells. The IL-2 released during priming led to an activated state in T cells, which produced levels of IL-2 and IFNγ upon restimulation comparable to T cells primed by DC. This indicates that IL-2 can function as a mediator of co-stimulation complementing co-stimulation by LSEC.

Furthermore, strong TCR signalling caused increased expression of CD25 on T cells which remained up-regulated, demonstrating a prolonged sensitivity to IL-2. Presumably, IL-2 was partially responsible for the upregulation of it's own receptor (Goebel et al., 2006).

However, even strong priming by LSEC did not induce the shedding of CD62L which would allow T cells to home to LN. Again, this observation could be attributed to the low amount of CD80/86 expressed by LSEC being insufficient to mediate CD62L shedding. The same observation can be made for CD80/86 deficient DC (data not shown). Subsequent to an initial downregulation of CD62L during priming, no significant difference in CD62L expression in LSEC activated or tolerized T cells could be seen at later time points (data not shown), while DC activated T cells remained CD62L low. This finding indicates that CD80/86 signalling is essential for CD62L shedding while IL-2 is not, as T cell secreted or even exogenously added IL-2 did not mediate a significant reduction (Fig. 10c and data not shown). In support of this notion is the finding that CD62L downregulation could be induced in rats treated with a CD28 specific antibody (Muller et al., 2008).

My findings show that LSEC can mediate a strong TCR signal (signal 1) due to a high amount of cross-presented antigen and activate T cells. The strong TCR signal could induce IL-2 production augmenting the low CD80/86 expression.

In this system IL-2 seemed to be the most relevant influence on overcoming tolerance induction by LSEC. To ascertain that T cell activation by LSEC was indeed induced solely by IL-2, IL-2 was blocked by a functional antibody added to the culture.

Blocking IL-2 signalling to T cells during priming rescued tolerance induction by LSEC.

5.11 TCR triggering by LSEC is responsible for IL-2 induction

As LSEC deliver little co-stimulation, strong TCR signalling must be primarily responsible for T cell intrinsic IL-2 production. It has been described that TCR signalling alone can trigger IL-2 production and proliferation if the TCR occupancy is high enough (Acuto and Michel, 2003). A high TCR occupancy could be facilitated by high numbers of TCR expressed on OT-I T cells resulting in high T cell avidity.

For further validation of the importance of TCR signalling strength for activation of T cells by intrinsic IL-2 production, peptide pulsed LSEC or DC were cultured with naïve T cells derived from the transgenic St35 line. St35 $CD8^+$ T cells recognize a peptide derived from adenovirus E1a protein SGPSNTPPEI on $H2K^b$. In contrast to OT-I T cells they show low avidity due to approximately 5 fold lower TCR expression (Fig 11a). St35 T cells should therefore not be activated by LSEC if TCR signalling was the essential trigger for T cell stimulation by IL-2 production. Indeed this was observed; St35 T cells could not be induced to produce detectable amounts of IL-2 during priming even at high antigen concentrations presented on LSEC (Fig.11b). This implies that in case of low avidity and low co-stimulation, TCR signalling is not sufficient to activate intrinsic IL-2 production. Although overall cytokine production was strongly reduced compared to OT-I T cells, St35 T cells were not unresponsive per se. DC could activate St35 T cells by delivering TCR signals in conjunction with co-stimulation via CD80/86. Furthermore St35 T cells showed full activation if exogenous IL-2 was added to LSEC culture during priming, supporting the hypothesis that IL-2 mediates the decision between tolerance induction and activation of naïve $CD8^+$ T cells by LSEC.

T cells with high avidity receptors are usually not reactive with self, as they are depleted during negative selection in the thymus (Palmer, 2003). It is therefore likely that high avidity T cells are specific for foreign antigen. During infection their responsiveness might favour activation by cross-presenting LSEC, over tolerance induction. Furthermore, IL-2 production by naïve high avidity and possibly memory T cells upon antigen encounter could potentially further promote T cell activation within the liver. Indeed it has been observed that memory T cells are able to produce sufficient amounts of IL-2 to inhibit induction of T cell tolerance by LSEC *in vitro* (Limmer unpublished data).

5.12 T cells primed by LSEC in the presence of co-stimulation do not exhibit cytotoxicity

Surprisingly, T cells which had been activated by LSEC and showed strong IFNγ and IL-2 production upon restimulation, displayed no cytotoxicity. This discrepancy in cytokine production and missing cytotoxicity could result from: Firstly, an insufficient

amount of IL-2 produced by T cells during priming to cause full activation, as the strength of T cell activation was directly correlated to the amount of IL-2 present during priming (Fig 9a). Secondly, an insufficient amount of co-stimulation given by LSEC in combination with completely missing signal 3 e.g. IL-12 could not induce cytotoxicity.

Thirdly, missing cytotoxicity could result from actively co-inhibiting T cells via programmed death-1 (PD-1) B7-H1 interaction, the latter of which is constitutively expressed on LSEC. B7-H1 is a member of the B7 family delivering co-inhibitory signals (Collins et al., 2005).

B7-H1 interaction with PD-1 has been shown to be mandatory and sufficient for tolerance induction by LSEC. LSEC deficient in B7-H1 induce T cell immunity (Diehl et al., 2008). In another study blocking B7-H1/ PD-1 interaction during chronic hepatitis B was shown to lead to the recovery of some virus specific T cell functions (Lopes et al., 2008), which could indicate the involvement of LSEC in tolerance induction towards hepatic viruses. Importantly, B7-H1 was found to be upregulated on LSEC but not on DC in response to interaction with T cells (Diehl et al., 2008). The increase in B7-H1 surface expression on LSEC was directly correlated to the strength of interaction between LSEC and T cells (Fig. 13b). Therefore the amount of peptide cross-presented in combination with the T cell avidity determines the strength of inhibitory signal delivered. Thus T cells receiving a strong signal 1 will also receive a strong inhibitory signal by LSEC which could counteract full activation. This finding suggests a fine balance between TCR, co-stimulatory and co-inhibitory signalling delivered to T cells by LSEC.

5.13 B7-H1 mediated co-inhibition counteracts IL-2 production by T cells

PD-1 on T cells is transiently upregulated during priming which has recently been reported to be initiated by NFATc1 (Oestreich et al., 2008). NFATc1 is upregulated in response to TCR and co-stimulatory signals and promotes also IL-2 expression (Macian, 2005; Serfling et al., 2006).

Upon interaction with LSEC T cells upregulate PD-1 which can then bind to B7-H1 on LSEC promoting tolerance induction in T cells (Diehl et al., 2008).

The upregulation of PD-1 on T cells, was correlated to the amount of antigen presented by LSEC (data not shown). This observation indicates that increased TCR signalling led to upregulation of PD-1, presumably via NFAT activation. As B7-H1 was also increased in proportion to the amount of cross-presented antigen, strong cross-presentation can lead to strong co-inhibition.

To test the hypothesis that co-inhibition through B7-H1 on LSEC is mediated by negatively influencing IL-2 production by T cells, IL-2 production during priming by B7-H1 deficient LSEC was examined.

Priming by B7-H1 deficient LSEC led to significantly increased IL-2 production by T cells compared to those primed by wild type LSEC. It is interesting to speculate that B7-H1 signalling inhibits IL-2 production, possibly via PD-1 delivering a negative feedback signal for NFATc1 activation.

Furthermore, priming by B7-H1 deficient LSEC resulted in an up to 10 fold increase in surface expression of CD25 on T cells, thereby additionally increasing sensitivity to IL-2 mediated signalling. In line with the observation made in naïve T cells primed by wild type LSEC the amount of IL-2 produced upon priming in the absence of B7-H1 was directly proportional to the amount of presented antigen and therefore to the strength of signal 1. In case of presentation of very low amounts of antigen T cells primed by B7-H1 deficient LSEC did not produce IL-2 and were not activated. The same was true when applying an IL-2 blocking antibody to the culture at high amounts of antigen. T cells receiving a strong signal 1 by B7-H1 deficient LSEC in the absence of IL-2 neither showed cytokine production upon restimulation, nor any cytotoxicity (Fig 13e). This finding indicates that full activation of T cells by LSEC lacking B7-H1 is mediated by the increased IL-2 production induced by missing co-inhibition.

5.14 Conclusion

My results show that LSEC are the hepatic cell population which is most efficient in the uptake of soluble antigen from the circulation. Strong scavenger activity in LSEC is accompanied by robust cross-presentation which is even superior to that seen in DC. In contrast to DC, LSEC utilise several receptors to take up antigen for cross-presentation. Scavenger and mannose receptor shuttle into the same early endosomal compartment, potentially increasing the quantity of internalized antigen. The efficiency of antigen uptake might compensate for the apparent lack of a compartment dedicated exclusively to cross-presentation.

The early endosomes in LSEC are not stable, but rapidly develop and are most likely not the site for antigen loading. The compartment in which peptides for cross-presentation are loaded in LSEC remains elusive.

The onset of cross-presentation in LSEC is very fast. However, antigen is not sequestered in LSEC, but cleared with a half life of only 6 hours. Antigen clearance is accompanied by rapid decline in cross-presentation. In DC, cross-presentation remains unaffected during the same time period. Clearly, LSEC and DC employ cross-presentation mechanisms with very different dynamics and kinetics.

The short duration of cross-presentation in LSEC suggests that only antigens which are present in the circulation for prolonged time are sufficiently presented. Furthermore, immune complexed antigens were only weakly presented by LSEC, indicating a mechanism for the prevention of tolerance induction towards antigens targeted by an immune response.

LSEC induce T cell tolerance towards soluble antigens (Limmer et al., 2000). However, I could show that LSEC can also induce activation of naïve $CD8^+$ T cells when delivering a strong signal 1. The highly efficient cross-presentation mediated by LSEC in combination with naïve T cells expressing receptors of high avidity leads to the production of T cell intrinsic IL-2 during priming albeit low co-stimulation. IL-2 can overcome tolerance induction by LSEC indicating that IL-2 functions as a co-stimulatory molecule. Furthermore, the presence or absence of IL-2 during priming by LSEC ultimately decides whether immunity or tolerance is induced.

The co-inhibitory molecule B7-H1 is constitutively expressed on LSEC, but upregulated in response to T cell interaction (Diehl et al., 2008). This upregulation is directly proportional to the strength of MHC-TCR interaction. Thus T cells receiving a strong signal 1 will at the same time receive strong inhibitory signals. When LSEC are deficient in B7-H1, T cells are activated to become cytotoxic T cells. This activation is caused by robust IL-2 production by T cells during priming. When IL-2 is blocked, tolerance induction in the absence of B7-H1 commences. These findings clearly suggest a role for B7-H1 in counteracting IL-2 production.

Abbreviations

°C	degree Celsius
µ	micro
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AcLDL	Acetylated low density protein
AGE	Advanced glycation end products
AICD	activation induced cell death
AIRE	Autoimmune regulator
APC	antigen presenting cell, allophycocyanin
B7-H1	B7-Homolog 1
Bcl-2	B cell lymphoma 2
BSA	bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxy Fluorescein Succinimidyl Ester
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T-lymphocyte antigen 4
DC	dendritic cells
DesTCR	Desiré TCR
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
e.g.	*lat.:* exempli gratia (for example)
EDTA	ethylenediaminetetraacetic acid
EEA1	Early endosomal antigen 1
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
et al.	*lat.:* Et alteres (and others)

FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FcγR	Receptor recognizing the constant region of immunoglobulin G
Fig.	Figure
FITC	Fluorescein isothiocyanat
g	gravity, gram
GBSS	Gey's Balanced Salt Solution
h	Hour
H-2	Histocompatibility-2
HDC	Hepatic dendritic cell
HSC	Hepatic stellate cell
i.v.	Intra venous
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
iTreg	induced regulatory T cells
KC	Kupffer cell
l	Litre
LPS	Lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
LN	Lymph node
m	meter, milli
M	Molar
MACS	magnetic cell separation
MHC	Major Histocompatibility complex
min	minute

MR	Mannose receptor
mTEC	medullary thymic epithelial cells
MW	Molecular weight
n	nano
NFAT	Nuclear factor of activated T cells
NK	natural killer -
NKT	natural killer T -
nTreg	natural regulatory T cells
OD	optical densitiy
OVA	ovalbumin
OxLDL	Oxidised low density protein
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PD-1	Programmed death 1
PE	phycoerythrin
PGE_2	prostaglandin E2
pH	*lat.:* potential hydrogenii
poly I	Polyinosinic potassium acid
PRR	Pattern recognition receptor
RT	room temperature
SA	streptavidin
SD	Standard deviation
sec	second
SEM	Standard error of the mean
S8L	ovalbumin peptide
SR	Scavenger receptor
TAE	Tris-acetate-EDTA

TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	transforming growth factor β
T_h	helper T -
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	tumour necrosis factor α
U	Unit
V	Volt
v/v	volume per volume
VCAM	Vascular adhesion molecule
w/v	weight per volume

Figures

References

Ackerman, A.L., and Cresswell, P. (2004). Cellular mechanisms governing cross-presentation of exogenous antigens. Nat Immunol *5*, 678-684.

Ackerman, A.L., Giodini, A., and Cresswell, P. (2006). A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. Immunity *25*, 607-617.

Ackerman, A.L., Kyritsis, C., Tampe, R., and Cresswell, P. (2005). Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. Nat Immunol *6*, 107-113.

Acuto, O., and Michel, F. (2003). CD28-mediated co-stimulation: a quantitative support for TCR signalling. Nat Rev Immunol *3*, 939-951.

Alferink, J., Tafuri, A., Vestweber, D., Hallmann, R., Hammerling, G.J., and Arnold, B. (1998). Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking. Science *282*, 1338-1341.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. Annu Rev Immunol *18*, 767-811.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Bevan, M.J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med *143*, 1283-1288.

Bevan, M.J. (2006). Cross-priming. Nat Immunol *7*, 363-365.

Billiar, T.R., Curran, R.D., Williams, D.L., and Kispert, P.H. (1992). Liver nonparenchymal cells are stimulated to provide interleukin 6 for induction of the hepatic acute-phase response in endotoxemia but not in remote localized inflammation. Arch Surg *127*, 31-36; discussion 36-37.

Bissell, D.M., Wang, S.S., Jarnagin, W.R., and Roll, F.J. (1995). Cell-specific expression of transforming growth factor-beta in rat liver. Evidence for autocrine regulation of hepatocyte proliferation. J Clin Invest *96*, 447-455.

Boskovic, J., Arnold, J.N., Stilion, R., Gordon, S., Sim, R.B., Rivera-Calzada, A., Wienke, D., Isacke, C.M., Martinez-Pomares, L., and Llorca, O. (2006). Structural model for the mannose receptor family uncovered by electron microscopy of Endo180 and the mannose receptor. J Biol Chem *281*, 8780-8787.

Breiner, K.M., Schaller, H., and Knolle, P.A. (2001). Endothelial cell-mediated uptake of a hepatitis B virus: a new concept of liver targeting of hepatotropic microorganisms. Hepatology *34*, 803-808.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P.A., and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. Science *316*, 612-616.

Burgdorf, S., and Kurts, C. (2008). Endocytosis mechanisms and the cell biology of antigen presentation. Curr Opin Immunol *20*, 89-95.

Burgdorf, S., Lukacs-Kornek, V., and Kurts, C. (2006). The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. J Immunol *176*, 6770-6776.

Burgdorf, S., Scholz, C., Kautz, A., Tampe, R., and Kurts, C. (2008). Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat Immunol *9*, 558-566.

Collins, M., Ling, V., and Carreno, B.M. (2005). The B7 family of immune-regulatory ligands. Genome Biol *6*, 223.

Cormier, E.G., Durso, R.J., Tsamis, F., Boussemart, L., Manix, C., Olson, W.C., Gardner, J.P., and Dragic, T. (2004). L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. Proc Natl Acad Sci U S A *101*, 14067-14072.

Crispe, I.N., Dao, T., Klugewitz, K., Mehal, W.Z., and Metz, D.P. (2000). The liver as a site of T-cell apoptosis: graveyard, or killing field? Immunol Rev *174*, 47-62.

Crispe, I.N., Giannandrea, M., Klein, I., John, B., Sampson, B., and Wuensch, S. (2006). Cellular and molecular mechanisms of liver tolerance. Immunol Rev *213*, 101-118.

Curtsinger, J.M., Lins, D.C., and Mescher, M.F. (2003). Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J Exp Med *197*, 1141-1151.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K., and Mescher, M.F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol *162*, 3256-3262.

Curtsinger, J.M., Valenzuela, J.O., Agarwal, P., Lins, D., and Mescher, M.F. (2005). Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol *174*, 4465-4469.

D'Souza, W.N., and Lefrancois, L. (2003). IL-2 is not required for the initiation of CD8 T cell cycling but sustains expansion. J Immunol *171*, 5727-5735.

Davey, G.M., Kurts, C., Miller, J.F., Bouillet, P., Strasser, A., Brooks, A.G., Carbone, F.R., and Heath, W.R. (2002). Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. J Exp Med *196*, 947-955.

de la Rosa, M., Rutz, S., Dorninger, H., and Scheffold, A. (2004). Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. Eur J Immunol *34*, 2480-2488.

Delamarre, L., Holcombe, H., and Mellman, I. (2003). Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. J Exp Med *198*, 111-122.

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med *192*, 1685-1696.

Dhein, J., Walczak, H., Baumler, C., Debatin, K.M., and Krammer, P.H. (1995). Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature *373*, 438-441.

Di Pucchio, T., Chatterjee, B., Smed-Sorensen, A., Clayton, S., Palazzo, A., Montes, M., Xue, Y., Mellman, I., Banchereau, J., and Connolly, J.E. (2008). Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. Nat Immunol *9*, 551-557.

Diehl, L., Schurich, A., Grochtmann, R., Hegenbarth, S., Chen, L., and Knolle, P.A. (2008). Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. Hepatology (Baltimore, Md *47*, 296-305.

Dong, H., Zhu, G., Tamada, K., and Chen, L. (1999). B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med *5*, 1365-1369.

Dure, M., and Macian, F. (2009). IL-2 signaling prevents T cell anergy by inhibiting the expression of anergy-inducing genes. Mol Immunol *46*, 999-1006.

Elvevold, K., Simon-Santamaria, J., Hasvold, H., McCourt, P., Smedsrod, B., and Sorensen, K.K. (2008a). Liver sinusoidal endothelial cells depend on mannose receptor-mediated recruitment of lysosomal enzymes for normal degradation capacity. Hepatology *48*, 2007-2015.

Elvevold, K., Smedsrod, B., and Martinez, I. (2008b). The liver sinusoidal endothelial cell: a cell type of controversial and confusing identity. Am J Physiol Gastrointest Liver Physiol *294*, G391-400.

Faure, F., Mantegazza, A., Sadaka, C., Sedlik, C., Jotereau, F., and Amigorena, S. (2009). Long-lasting cross-presentation of tumor antigen in human DC. Eur J Immunol.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. (2005). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity *22*, 329-341.

Frauwirth, K.A., and Thompson, C.B. (2002). Activation and inhibition of lymphocytes by costimulation. J Clin Invest *109*, 295-299.

Freeman, G.J., Long, A.J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L.J., Malenkovich, N., Okazaki, T., Byrne, M.C*., et al.* (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med *192*, 1027-1034.

Gallegos, A.M., and Bevan, M.J. (2006). Central tolerance: good but imperfect. Immunol Rev *209*, 290-296.

Gardner, J.P., Durso, R.J., Arrigale, R.R., Donovan, G.P., Maddon, P.J., Dragic, T., and Olson, W.C. (2003). L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc Natl Acad Sci U S A *100*, 4498-4503.

Geerts, A. (2007). The simple truth is seldom true and never simple: dual role for p75(NTR) in transdifferentation and cell death of hepatic stellate cells. Hepatology *46*, 600-601.

Goddard, S., Youster, J., Morgan, E., and Adams, D.H. (2004). Interleukin-10 secretion differentiates dendritic cells from human liver and skin. Am J Pathol *164*, 511-519.

Godfrey, D.I., MacDonald, H.R., Kronenberg, M., Smyth, M.J., and Van Kaer, L. (2004). NKT cells: what's in a name? Nat Rev Immunol *4*, 231-237.

Goebel, J., Forrest, K., Wills-Karp, M., and Roszman, T.L. (2006). Tubulin polymerization modulates interleukin-2 receptor signal transduction in human T cells. J Recept Signal Transduct Res *26*, 87-106.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 family revisited. Annu Rev Immunol *23*, 515-548.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., and Roncarolo, M.G. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature *389*, 737-742.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature *425*, 397-402.

Heath, B.M., Xia, J., Dong, E., An, R.H., Brooks, A., Liang, C., Federoff, H.J., and Kass, R.S. (1998). Overexpression of nerve growth factor in the heart alters ion channel activity and beta-adrenergic signalling in an adult transgenic mouse. J Physiol *512 (Pt 3)*, 779-791.

Heath, W.R., Belz, G.T., Behrens, G.M., Smith, C.M., Forehan, S.P., Parish, I.A., Davey, G.M., Wilson, N.S., Carbone, F.R., and Villadangos, J.A. (2004). Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. Immunol Rev *199*, 9-26.

Hellevik, T., Martinez, I., Olsen, R., Toh, B.H., Webster, P., and Smedsrod, B. (1998). Transport of residual endocytosed products into terminal lysosomes occurs slowly in rat liver endothelial cells. Hepatology *28*, 1378-1389.

Henrickson, S.E., Mempel, T.R., Mazo, I.B., Liu, B., Artyomov, M.N., Zheng, H., Peixoto, A., Flynn, M.P., Senman, B., Junt, T.*, et al.* (2008). T cell sensing of antigen dose governs interactive behavior with dendritic cells and sets a threshold for T cell activation. Nat Immunol *9*, 282-291.

Hernandez, J., Aung, S., Redmond, W.L., and Sherman, L.A. (2001). Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. J Exp Med *194*, 707-717.

Hislop, A.D., Ressing, M.E., van Leeuwen, D., Pudney, V.A., Horst, D., Koppers-Lalic, D., Croft, N.P., Neefjes, J.J., Rickinson, A.B., and Wiertz, E.J. (2007). A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. J Exp Med *204*, 1863-1873.

Huang, L., Soldevila, G., Leeker, M., Flavell, R., and Crispe, I.N. (1994). The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. Immunity *1*, 741-749.

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nat Immunol *5*, 987-995.

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol *54 Pt 1*, 1-13.

Jenkins, M.K., Pardoll, D.M., Mizuguchi, J., Quill, H., and Schwartz, R.H. (1987). T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. Immunol Rev *95*, 113-135.

Jensen, P.E. (2007). Recent advances in antigen processing and presentation. Nat Immunol *8*, 1041-1048.

Kempka, G., and Kolb-Bachofen, V. (1988). Binding, uptake, and transcytosis of ligands for mannose-specific receptors in rat liver: an electron microscopic study. Exp Cell Res *176*, 38-48.

Khor, B., and Makar, R.S. (2008). Toward a molecular explanation for cross-presentation of antigens to the immune system. Transfus Med Rev *22*, 188-201.

Knolle, P.A., and Gerken, G. (2000). Local control of the immune response in the liver. Immunol Rev *174*, 21-34.

Knolle, P.A., and Limmer, A. (2003). Control of immune responses by savenger liver endothelial cells. Swiss Med Wkly *133*, 501-506.

Knolle, P.A., Loser, E., Protzer, U., Duchmann, R., Schmitt, E., zum Buschenfelde, K.H., Rose-John, S., and Gerken, G. (1997). Regulation of endotoxin-induced IL-6 production in liver sinusoidal endothelial cells and Kupffer cells by IL-10. Clin Exp Immunol *107*, 555-561.

Knolle, P.A., Schmitt, E., Jin, S., Germann, T., Duchmann, R., Hegenbarth, S., Gerken, G., and Lohse, A.W. (1999). Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells. Gastroenterology *116*, 1428-1440.

Koppel, E.A., van Gisbergen, K.P., Geijtenbeek, T.B., and van Kooyk, Y. (2005). Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. Cell Microbiol *7*, 157-165.

Kudo, S., Matsuno, K., Ezaki, T., and Ogawa, M. (1997). A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. J Exp Med *185*, 777-784.

Kundig, T.M., Schorle, H., Bachmann, M.F., Hengartner, H., Zinkernagel, R.M., and Horak, I. (1993). Immune responses in interleukin-2-deficient mice. Science *262*, 1059-1061.

Kurts, C., Heath, W.R., Carbone, F.R., Allison, J., Miller, J.F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. J Exp Med *184*, 923-930.

Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F., and Heath, W.R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J Exp Med *186*, 239-245.

Kurts, C., Miller, J.F., Subramaniam, R.M., Carbone, F.R., and Heath, W.R. (1998). Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. J Exp Med *188*, 409-414.

Lan, R.Y., Selmi, C., and Gershwin, M.E. (2008). The regulatory, inflammatory, and T cell programming roles of interleukin-2 (IL-2). J Autoimmun *31*, 7-12.

Lantz, O., Grandjean, I., Matzinger, P., and Di Santo, J.P. (2000). Gamma chain required for naive CD4+ T cell survival but not for antigen proliferation. Nat Immunol *1*, 54-58.

Leclercq, I.A., Da Silva Morais, A., Schroyen, B., Van Hul, N., and Geerts, A. (2007). Insulin resistance in hepatocytes and sinusoidal liver cells: mechanisms and consequences. J Hepatol *47*, 142-156.

Limmer, A., Ohl, J., Kurts, C., Ljunggren, H.G., Reiss, Y., Groettrup, M., Momburg, F., Arnold, B., and Knolle, P.A. (2000). Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. Nat Med *6*, 1348-1354.

Limmer, A., Ohl, J., Wingender, G., Berg, M., Jungerkes, F., Schumak, B., Djandji, D., Scholz, K., Klevenz, A., Hegenbarth, S.*, et al.* (2005). Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. Eur J Immunol *35*, 2970-2981.

Limmer, A., Sacher, T., Alferink, J., Nichterlein, T., Arnold, B., and Hammerling, G.J. (1998). A two-step model for the induction of organ-specific autoimmunity. Novartis Found Symp *215*, 159-167; discussion 167-171, 186-190.

Lin, J., and Weiss, A. (2001). T cell receptor signalling. J Cell Sci *114*, 243-244.

Liu, G.Y., Fairchild, P.J., Smith, R.M., Prowle, J.R., Kioussis, D., and Wraith, D.C. (1995). Low avidity recognition of self-antigen by T cells permits escape from central tolerance. Immunity *3*, 407-415.

Lopes, A.R., Kellam, P., Das, A., Dunn, C., Kwan, A., Turner, J., Peppa, D., Gilson, R.J., Gehring, A., Bertoletti, A., and Maini, M.K. (2008). Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. J Clin Invest *118*, 1835-1845.

Lovdal, T., Andersen, E., Brech, A., and Berg, T. (2000). Fc receptor mediated endocytosis of small soluble immunoglobulin G immune complexes in Kupffer and endothelial cells from rat liver. J Cell Sci *113 (Pt 18)*, 3255-3266.

Luckashenak, N., Schroeder, S., Endt, K., Schmidt, D., Mahnke, K., Bachmann, M.F., Marconi, P., Deeg, C.A., and Brocker, T. (2008). Constitutive crosspresentation of tissue antigens by dendritic cells controls CD8+ T cell tolerance in vivo. Immunity *28*, 521-532.

Macian, F. (2005). NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol *5*, 472-484.

MacPhee, P.J., Schmidt, E.E., and Groom, A.C. (1992). Evidence for Kupffer cell migration along liver sinusoids, from high-resolution in vivo microscopy. Am J Physiol *263*, G17-23.

MacPhee, P.J., Schmidt, E.E., and Groom, A.C. (1995). Intermittence of blood flow in liver sinusoids, studied by high-resolution in vivo microscopy. Am J Physiol *269*, G692-698.

Magnusson, S., and Berg, T. (1989). Extremely rapid endocytosis mediated by the mannose receptor of sinusoidal endothelial rat liver cells. Biochem J *257*, 651-656.

Malek, T.R. (2002). T helper cells, IL-2 and the generation of cytotoxic T-cell responses. Trends Immunol *23*, 465-467.

Malek, T.R., and Bayer, A.L. (2004). Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol *4*, 665-674.

Malovic, I., Sorensen, K.K., Elvevold, K.H., Nedredal, G.I., Paulsen, S., Erofeev, A.V., Smedsrod, B.H., and McCourt, P.A. (2007). The mannose receptor on murine liver

sinusoidal endothelial cells is the main denatured collagen clearance receptor. Hepatology *45*, 1454-1461.

Medzhitov, R., and Janeway, C.A., Jr. (1997a). Innate immunity: impact on the adaptive immune response. Curr Opin Immunol *9*, 4-9.

Medzhitov, R., and Janeway, C.A., Jr. (1997b). Innate immunity: the virtues of a nonclonal system of recognition. Cell *91*, 295-298.

Mehal, W.Z., Juedes, A.E., and Crispe, I.N. (1999). Selective retention of activated CD8+ T cells by the normal liver. J Immunol *163*, 3202-3210.

Mescher, M.F., Curtsinger, J.M., Agarwal, P., Casey, K.A., Gerner, M., Hammerbeck, C.D., Popescu, F., and Xiao, Z. (2006). Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev *211*, 81-92.

Muller, N., van den Brandt, J., Odoardi, F., Tischner, D., Herath, J., Flugel, A., and Reichardt, H.M. (2008). A CD28 superagonistic antibody elicits 2 functionally distinct waves of T cell activation in rats. J Clin Invest *118*, 1405-1416.

Neefjes, J.J., and Momburg, F. (1993). Cell biology of antigen presentation. Curr Opin Immunol *5*, 27-34.

Neijssen, J., Herberts, C., Drijfhout, J.W., Reits, E., Janssen, L., and Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. Nature *434*, 83-88.

Nicholson, A.C., Frieda, S., Pearce, A., and Silverstein, R.L. (1995). Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell lines. Evidence implicating the lipid moiety of the lipoprotein as the binding site. Arterioscler Thromb Vasc Biol *15*, 269-275.

Nimmerjahn, F., and Ravetch, J.V. (2008). Fcgamma receptors as regulators of immune responses. Nat Rev Immunol *8*, 34-47.

Nurieva, R., Thomas, S., Nguyen, T., Martin-Orozco, N., Wang, Y., Kaja, M.K., Yu, X.Z., and Dong, C. (2006). T-cell tolerance or function is determined by combinatorial costimulatory signals. The EMBO journal *25*, 2623-2633.

O'Connell, J. (2000). Immune privilege or inflammation? The paradoxical effects of Fas ligand. Arch Immunol Ther Exp (Warsz) *48*, 73-79.

Oda, M., Han, J.Y., and Yokomori, H. (2000). Local regulators of hepatic sinusoidal microcirculation: recent advances. Clin Hemorheol Microcirc *23*, 85-94.

Oestreich, K.J., Yoon, H., Ahmed, R., and Boss, J.M. (2008). NFATc1 regulates PD-1 expression upon T cell activation. J Immunol *181*, 4832-4839.

Oldstone, M.B., Nerenberg, M., Southern, P., Price, J., and Lewicki, H. (1991). Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell *65*, 319-331.

Oquendo, P., Hundt, E., Lawler, J., and Seed, B. (1989). CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. Cell *58*, 95-101.

Palm, N.W., and Medzhitov, R. (2009). Pattern recognition receptors and control of adaptive immunity. Immunol Rev *227*, 221-233.

Palmer, E. (2003). Negative selection--clearing out the bad apples from the T-cell repertoire. Nat Rev Immunol *3*, 383-391.

Pei, Y., Zhu, P., Dang, Y., Wu, J., Yang, X., Wan, B., Liu, J.O., Yi, Q., and Yu, L. (2008). Nuclear export of NF90 to stabilize IL-2 mRNA is mediated by AKT-dependent phosphorylation at Ser647 in response to CD28 costimulation. J Immunol *180*, 222-229.

Pooley, J.L., Heath, W.R., and Shortman, K. (2001). Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells. J Immunol *166*, 5327-5330.

Probst, H.C., McCoy, K., Okazaki, T., Honjo, T., and van den Broek, M. (2005). Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol *6*, 280-286.

Racanelli, V., and Rehermann, B. (2006). The liver as an immunological organ. Hepatology *43*, S54-62.

Redmond, W.L., Hernandez, J., and Sherman, L.A. (2003). Deletion of naive CD8 T cells requires persistent antigen and is not programmed by an initial signal from the tolerogenic APC. J Immunol *171*, 6349-6354.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med *189*, 371-380.

Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J.W., and Neefjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. Immunity *18*, 97-108.

Rock, K.L., York, I.A., and Goldberg, A.L. (2004). Post-proteasomal antigen processing for major histocompatibility complex class I presentation. Nat Immunol *5*, 670-677.

Sadlack, B., Kuhn, R., Schorle, H., Rajewsky, K., Muller, W., and Horak, I. (1994). Development and proliferation of lymphocytes in mice deficient for both interleukins-2 and -4. Eur J Immunol *24*, 281-284.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. Cell *133*, 775-787.

Savina, A., and Amigorena, S. (2007). Phagocytosis and antigen presentation in dendritic cells. Immunol Rev *219*, 143-156.

Scheffold, A., Huhn, J., and Hofer, T. (2005). Regulation of CD4+CD25+ regulatory T cell activity: it takes (IL-)two to tango. Eur J Immunol *35*, 1336-1341.

Schildberg, F.A., Hegenbarth, S.I., Schumak, B., Scholz, K., Limmer, A., and Knolle, P.A. (2008). Liver sinusoidal endothelial cells veto CD8 T cell activation by antigen-presenting dendritic cells. Eur J Immunol *38*, 957-967.

Schrage, A., Loddenkemper, C., Erben, U., Lauer, U., Hausdorf, G., Jungblut, P.R., Johnson, J., Knolle, P.A., Zeitz, M., Hamann, A., and Klugewitz, K. (2008). Murine CD146 is widely expressed on endothelial cells and is recognized by the monoclonal antibody ME-9F1. Histochem Cell Biol *129*, 441-451.

Schwartz, R.H. (2005). Natural regulatory T cells and self-tolerance. Nat Immunol *6*, 327-330.

Serfling, E., Klein-Hessling, S., Palmetshofer, A., Bopp, T., Stassen, M., and Schmitt, E. (2006). NFAT transcription factors in control of peripheral T cell tolerance. Eur J Immunol *36*, 2837-2843.

Sharpe, A.H., and Freeman, G.J. (2002). The B7-CD28 superfamily. Nat Rev Immunol *2*, 116-126.

Shen, L., and Rock, K.L. (2006). Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. Current opinion in immunology *18*, 85-91.

Shimaoka, T., Kume, N., Minami, M., Hayashida, K., Sawamura, T., Kita, T., and Yonehara, S. (2001). LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. J Immunol *166*, 5108-5114.

Shiow, L.R., Rosen, D.B., Brdickova, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G., and Matloubian, M. (2006). CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature *440*, 540-544.

Shortman, K., and Liu, Y.J. (2002). Mouse and human dendritic cell subtypes. Nat Rev Immunol *2*, 151-161.

Singer, G.G., Carrera, A.C., Marshak-Rothstein, A., Martinez, C., and Abbas, A.K. (1994). Apoptosis, Fas and systemic autoimmunity: the MRL-lpr/lpr model. Curr Opin Immunol *6*, 913-920.

Smedsrod, B. (2004). Clearance function of scavenger endothelial cells. Comp Hepatol *3 Suppl 1*, S22.

Smedsrod, B., De Bleser, P.J., Braet, F., Lovisetti, P., Vanderkerken, K., Wisse, E., and Geerts, A. (1994). Cell biology of liver endothelial and Kupffer cells. Gut *35*, 1509-1516.

Smedsrod, B., Pertoft, H., Gustafson, S., and Laurent, T.C. (1990). Scavenger functions of the liver endothelial cell. Biochem J *266*, 313-327.

Song, J., Lei, F.T., Xiong, X., and Haque, R. (2008). Intracellular signals of T cell costimulation. Cell Mol Immunol *5*, 239-247.

Takahashi, T., and Sakaguchi, S. (2003). Naturally arising CD25+CD4+ regulatory T cells in maintaining immunologic self-tolerance and preventing autoimmune disease. Curr Mol Med *3*, 693-706.

Tandon, N.N., Kralisz, U., and Jamieson, G.A. (1989). Identification of glycoprotein IV (CD36) as a primary receptor for platelet-collagen adhesion. J Biol Chem *264*, 7576-7583.

Tavassoli, M., Kishimoto, T., and Kataoka, M. (1986a). Liver endothelium mediates the hepatocyte's uptake of ceruloplasmin. J Cell Biol *102*, 1298-1303.

Tavassoli, M., Kishimoto, T., Soda, R., Kataoka, M., and Harjes, K. (1986b). Liver endothelium mediates the uptake of iron-transferrin complex by hepatocytes. Exp Cell Res *165*, 369-379.

Vidal-Vanaclocha, F., Rocha, M., Asumendi, A., and Barbera-Guillem, E. (1993a). Isolation and enrichment of two sublobular compartment-specific endothelial cell subpopulations from liver sinusoids. Hepatology *18*, 328-339.

Vidal-Vanaclocha, F., Rocha, M.A., Asumendi, A., and Barbera-Guillem, E. (1993b). Role of periportal and perivenous sinusoidal endothelial cells in hepatic homing of blood and metastatic cancer cells. Semin Liver Dis *13*, 60-71.

Villadangos, J.A., and Schnorrer, P. (2007). Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol *7*, 543-555.

Viola, A., and Lanzavecchia, A. (1996). T cell activation determined by T cell receptor number and tunable thresholds. Science *273*, 104-106.

von Oppen, N., Schurich, A., Hegenbarth, S., Stabenow, D., Tolba, R., Weiskirchen, R., Geerts, A., Kolanus, W., Knolle, P., and Diehl, L. (2008). Systemic antigen cross-presented by liver sinusoidal endothelial cells induces liver-specific CD8 T-cell retention and tolerization. Hepatology.

Warren, A., Le Couteur, D.G., Fraser, R., Bowen, D.G., McCaughan, G.W., and Bertolino, P. (2006). T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. Hepatology *44*, 1182-1190.

Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science *270*, 985-988.

Watts, C. (1997). Immunology. Inside the gearbox of the dendritic cell. Nature *388*, 724-725.

Wiertz, E.J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A., and Ploegh, H.L. (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature *384*, 432-438.

Winau, F., Hegasy, G., Weiskirchen, R., Weber, S., Cassan, C., Sieling, P.A., Modlin, R.L., Liblau, R.S., Gressner, A.M., and Kaufmann, S.H. (2007). Ito cells are liver-resident antigen-presenting cells for activating T cell responses. Immunity *26*, 117-129.

Wisse, E. (1970). An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. J Ultrastruct Res *31*, 125-150.

Wisse, E., De Zanger, R.B., Charels, K., Van Der Smissen, P., and McCuskey, R.S. (1985). The liver sieve: considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. Hepatology *5*, 683-692.

Wolf, P.R., and Ploegh, H.L. (1995). How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. Annu Rev Cell Dev Biol *11*, 267-306.

Yewdell, J.W., Reits, E., and Neefjes, J. (2003). Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat Rev Immunol *3*, 952-961.

Zehn, D., and Bevan, M.J. (2006). T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. Immunity *25*, 261-270.

Zinkernagel, R.M., Moskophidis, D., Kundig, T., Oehen, S., Pircher, H., and Hengartner, H. (1993). Effector T-cell induction and T-cell memory versus peripheral deletion of T cells. Immunol Rev *133*, 199-223.