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NKT cells crosstalk with liver sinusoidal endothelial cells is triggering induction of polyclonal T-cell and NK cell immunity

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I dedicate this thesis to

my Parents, Brothers, Sisters, Wife, my whole Family,

and every sacrifice to make people's life better

for their constant support, inspiration and unconditional love.

I love you all dearly.

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Aufgrund ihrer integralen Funktion als Stoffwechsel und Biofilter, der die Darmmetaboliten und das zirkulierende Blut verarbeitet, beherbergt die Leber ihr lokales Immunsystem, dass das Gleichgewicht zwischen Immunogenität und Immuntoleranz gegen die Entzündungshinweise aufrechterhält, die ständig durch die Lebergefäße dringen. Es wurde gefunden, dass die Lebermikroumgebung, d.h. leberdendritische Zellen, Kupffer Zellen, sinusförmige Leberendothelzellen (LSECs), hepatische Sternzellen und Hepatozyten, als lokale Antigen-präsentierende Zellen fungieren. Dadurch tragen diese Antigen-präsentierenden Zellen zur lokalen Immunität bei, entweder durch Aktivierung der T Zell-abhängigen Immunität gegen Infektion oder durch Verschiebung der Antigen-spezifischen Immunantworten in Richtung Toleranz, was die Persistenz von Viren erleichtert die Hepatozyten infizieren. Die Fähigkeit dieser Leberantigen-präsentierenden Zellen, sich an Antigen-spezifischen Wechselwirkungen zu beteiligen, wurde intensiv untersucht, wobei der Schwerpunkt auf der MHC-beschränkten Aktivierung von CD4 und CD8 T-Zellen lag. Allerdings die Leber Häfen eine große Anzahl von angeborenen Lymphozyten, die die Leber lokale Immunregulation beeinflussen können in MHC-unabhängiger Stimulation wie die unveränderlichen natürlichen Killer T (iNKT) Zellen. Leber NKT Zellen machen den größten Teil der NKT Zellen im gesamten Körper aus und machen 35 bis 40% der gesamten hepatischen Lymphozyten aus. In mehreren *In-vivo*- Berichten wurde die Rolle von hervorgehoben NKT Zellen erkennen sowohl endogene als auch exogene Lipidantigene in einem CD1-beschränkten Prozess. Es ist jedoch weitgehend unklar geblieben, welche lokalen Zellpopulationen in der Leber in der Lage sind, NKT-Zellen auf CD1-spezifische Weise zu aktivieren, und ob eine solche Antigenaktivierung von NKT-Zellen an der Orchestrierung der Immunität von CD8-T-Zellen und NK-Zellen in der Leber beteiligt ist.

Hier haben wir gezeigt, dass die CD1d-vermittelte Antigenpräsentation durch LSECs in der Lage ist, die NKT-Zellaktivierung im Vergleich zu anderen hepatischen Antigen-präsentierenden Zellen schnell und robust zu induzieren. In der Zwischenzeit zeigten unsere Ergebnisse, dass auf diese verwandte Wechselwirkung die Expression von Entzündungsmediatoren folgte, die indirekt die polyklonale Aktivierung von CD8 T-Zellen und NK Zellen über eine feste Adhäsionsplattform von LSECs induzierten. Darüber hinaus fanden wir auch, dass die Antigen-unabhängige

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Aktivierung von NK Zellen und CD8 T-Zellen als Reaktion auf eine verwandte LSEC / NKT-Wechselwirkung auf einen in der Leber residenten Phänotyp von CD8 und NK Zellen beschränkt war. Diese Doppelfunktion von LSECs bei der Aktivierung von NKT Zellen in einer Antigen-spezifischen Form und der Förderung einer gleichzeitigen polyklonalen Aktivierung von CD8 und NK Zellen war einzigartig und lieferte den Anstoß für ein besseres Verständnis der Kreuzreaktion von NKT Zellen mit LSECs für die Entwicklung von NKT-basierten Zellen Immuntherapien gegen Leberinfektionen und Krebs.

SUMMARY

Due to its integral function as a metabolic and bio-filter processing the gut metabolites and circulating blood, the liver harbors its local immune system which maintains the balance between immunogenicity and immune tolerance against the inflammatory cues constantly raiding through the hepatic vasculatures. The liver micro-environment, i.e. liver dendritic cells, Kupffer cells, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells and hepatocyte, has been found to function as local antigen presenting cells. Thereby, these antigen presenting cells contribute to local immunity either through activation of T-cell dependent immunity against infection or skewing antigen-specific immune responses toward tolerance, which facilitates persistence of viruses that infect hepatocytes. The ability of these liver antigen presenting cells to engage in antigen-specific interaction has been intensively studied with a focus on MHC-restricted activation of CD4 and CD8 T cells. However, the liver harbors a large number of innate lymphocytes that may influence the liver local immune regulation in MHC-independent stimulation such as the invariant natural killer T (iNKT) cells. Liver NKT cells constitute for the majority of NKT cell in total body and represent 35 to 40% of the total hepatic lymphocytes. Several *in vivo* reports highlighted the role of NKT cells in recognizing both endogenous and exogenous lipid-antigens in a CD1-restricted process. However, it has remained largely unclear which local cell populations in the liver are capable of activating NKT cells in a CD1-specific fashion and whether such antigen-activation of NKT cells is involved in the orchestration of hepatic CD8 T cell and NK cell immunity.

Here we showed that CD1d mediated antigen presentation by LSECs was capable of inducing NKT cell activation in a rapid and robust fashion compared to other hepatic antigen presenting cells. Meanwhile, our findings demonstrated that this cognate interaction was followed by expression of inflammatory mediators, which indirectly induced polyclonal activation of CD8 T-cell and NK cell through a firm adhesion platform of LSECs. Furthermore, we also found that the antigen-independent activation of NK cells and CD8 T-cells in response to LSEC/NKT cognate interaction was restricted to a liver-resident phenotype of CD8 and NK cells. This dual function of LSECs in activating NKT cells in an antigen-specific form and promoting a concomitant polyclonal activation of CD8 and NK cells was unique, providing the

impetus for further understanding of NKT cells cross-reaction with LSECs for the development of NKT-based immunotherapies against hepatic infections and cancer.

1. INTRODUCTION

1.1 Liver structure and metabolic function

The liver is the biggest organ in the mammalian body with complex and highly variable functions. As a bidirectional bio-filter receiving both portal and systemic blood, the liver maintains the homeostasis of the organism through the rapid metabolism of most nutritional materials, neutralization and removal of both exogenous and endogenous toxic materials emerging through the gastrointestinal tract or the systemic blood, respectively. Due to these biologic functions, the liver is always raided by enormous microbiological and antigenic cues, which require a highly functional immune defense within the liver microenvironment. Of the unique angio-architecture of the liver, only hepatocytes and cholangiocytes are the only two unique cell types. Hepatocytes comprise 60 to 80% of total hepatic cells with a highly polarized morphology depending on their location, surface receptors and proteins. Cholangiocytes represent the biliary channels, which sustain the regular flow of the bile and drainage from the parenchyma (Arias et al., 2020).

Along with these cells, several cell types with a highly developed morphology to function characteristics support the multiple functions of the liver. The endothelial cells are the lining of the sinusoid with a unique fenestrated structure. Hepatic myofibroblasts play also different functions such as the storage of vitamins, growth factors and cytokines. In addition to filtering the blood, kupffer cells (KCs) which are the liver-resident macrophages play an integral role in the local immune balance together with the different lymphocytes and dendritic cells, which confers the liver as the largest organ of the immune system. In addition, the mesothelial cells of the Gilsson capsule contributes as an important reservoir of lymph production and other hepatic cells. The portal vein and the hepatic artery represent the unique vasculature in liver. The portal vein maintains approximately 80% of the liver's blood supply, enriched with nutrients and toxins from the stomach, gut, pancreas and spleen (Crispe, 2009; Gao et al., 2008; Kubes and Jenne, 2018; Racanelli and Rehermann, 2006). The hepatic artery supplies the hepatic capsule and other vasculatures with 40-50% oxygenated blood from the aorta. All the blood in the liver is collected by the central hepatic veins to end up to the circulation via vena cava. Between the afferent and efferent vessels lies a very special vascular system of sinusoids, which guarantee the perfusion of hepatic capsules with the blood at low pressure and flow-

rate. These mechanistic conditions confer the efficient communication between the blood and hepatocytes for optimum metabolism, storage, neutralization, detoxification and excretion. Hepatocytes would not accomplish these functions without the biliary system. Together with the gut microbiota, hepatocytes develop the 16 different enzymes required for the bile acids production. The produced bile acids move later in a centrifugal motion in the bile canalicules, emulsifying the gut-derived elements of the food, converting the lipid bilayers into micelles and excreting the waste products from the blood.

Within this paradigm, Liver is constantly exposed to a wide range of inflammatory cues harbored by the bacterial products, endotoxins and food antigens. To maintain a rapid protection from these potential toxins in a regulated fashion, the liver harbors its own immune system that balances between immunogenicity and immune tolerance.

1.2 Hepatoimmunology

As the largest solid organ maintaining the digestive and metabolic functions, Liver exhibits a high degree of vascularization that maintains a slow blood flow across the hepatic tissues (Mackay, 2002). This blood flow through a highly permeable vascular sinusoid keeps the liver persistently exposed to a wide range of bacterial endotoxins, endogenous and exogenous antigens (Orlando et al., 2009). To efficiently maintain its physiological functions, the liver harbors a unique immune system, which can efficiently exert anti-inflammatory and immune-tolerant functions. This immune hemostasis renders a rapid and robust immune response to the harmful antigens without eliciting immunopathology. Such immune balance is attributed to a sophisticated interaction between liver microenvironment including hepatocytes, non-parenchymal cells (NPCs) and hepatic stellate cells (HSCs), and numerous of intrahepatic lymphocytes (IHLs) (Figure 1) (Kubes and Jenne, 2018).

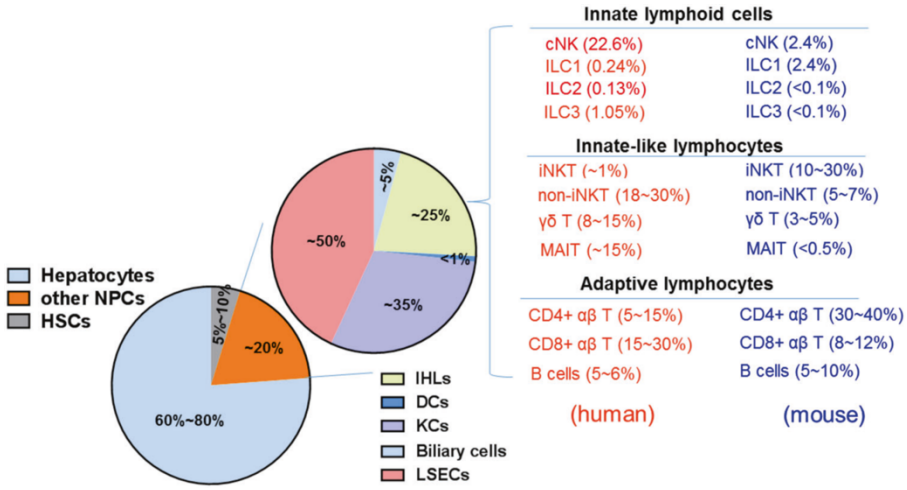

Figure 1. Cell composition of healthy liver
Parenchymal hepatocytes represent the dominant part of hepatic tissue. Nonparenchymal cells (NPCs) include hepatic stellate cells (HSCs), KCs, LSECs, DCs and intrahepatic lymphocytes (IHLs). IHLs are divided into three main groups including innate lymphoid cells, innate-like lymphocytes and adaptive lymphocytes. Each of these groups constitutes different subtests of cells with percentage related to the total IHLs. REF. (Chen and Tian, 2020)

1.2.1 Local antigen presentation in the liver

1.2.1.1 Hepatocytes

Beside its role as the main parenchymal cells in the liver representing nearly 60% to 80% of the total liver cells, Hepatocytes are contributing in the liver immune function by their ability to present antigens via major histocompatibility complex (MHC) I and MHC-II, using Collectrin-specific mechanism of antigen processing and presentation (Dolina et al., 2017; Jenne and Kubes, 2013). Although hepatocytes do not have a direct contact with the vascular sinusoid, lymphocytes use their protruding filopodia through the endothelium fenestration to scan the surface of hepatocytes for the presented antigens (Warren et al., 2007; Warren et al., 2006).

This antigen presentation by hepatocytes is playing an integral role in the immune homeostasis as well as priming, activation and proliferation of naïve CD8+ T-cells (Ebrahimkhani et al., 2011). However, due to the lack of co-stimulatory factors such as CD80, CD86 and IL-2, hepatocytes are not able to maintain the survivability of activated CD8+ T-cells (Bertolino et al., 1998).

During viral infection, the activated CD8+ T-cells are able to eliminate the infected hepatocytes in a cytolytic and non-cytolytic fashion (Wohlleber et al., 2012). However, this cytotoxic effect of CD8 T-cells is inversely proportional to the number of virus-infected hepatocytes. Studies showed that the more antigen presentation by

infected hepatocytes, the less efficient elimination of the infected cells due to the exhaustion of CD8+ T-cells (Ochel et al., 2016; Tay et al., 2014a). On the other hand, hepatocytes, which do not express MHC-II molecules under the steady state condition, are able to acquire the MHC-II antigen presentation capacity during inflammation. Studies showed that the hepatic micro-environment is independently able to activate naïve CD4+ T-cells (Herkel et al., 2003). This activation is elicited by liver resident phagocytic cells, mostly kupffer cells (KCs), which are able to cross-present the hepatocyte-derived materials loaded on MHC-II molecule (Tay et al., 2014b).

1.2.1.2 Hepatic stellate cells (HSCs)

HSCs express MHC-II molecule together with co-stimulatory molecules such as CD40 and CD80, the potential of antigen presentation by HSCs is still controversial due to their weak capacity of antigen uptake (Ebrahimkhani et al., 2011; Viñas et al., 2003; Winau et al., 2007). Several studies tried to address the *in vitro* uptake and processing of soluble antigens by HSCs. In these studies, they reported a potent capacity of HSCs to recognize soluble antigens and thereby activate naïve CD4+ and CD8+ T cells (Winau et al., 2007). Contrastingly, other studies showed the ability of HSCs to hamper the T-cell response through B7-H1 and B7-H4 signaling, skewing the T-cells toward apoptosis (Charles et al., 2013; Chinnadurai and Grakoui, 2010; Yu et al., 2004).

Away from the capacity of antigen presentation by HSCs, considerable studies confirmed their integral role in the immune regulation in the liver. Upon antigen presentation and crosstalk with monocytes in a cell contact dependent manner, HSCs trigger formation of indoleamine 2,3-dioxy-genase (IDO)+ DCs with tolerogenic capacity and drift the monocyte differentiation toward the myeloid derived suppressor cells (MDSCs) formation (Höchst et al., 2013; Sumpter et al., 2012). This immune-modulatory capacity was found to also inhibit the activation of CD8+ T-cells and modulate naïve CD4+ T-cell activation towards T-regulatory (T_{reg}) cells differentiation in a metabolism-dependent manner using retinoic acid (Dunham et al., 2013; Schildberg et al., 2011). Accordingly, these studies supported the understating of HSCs as an integral part of the tolerogenic milieu in the liver.

1.2.1.3 Macrophages

In addition to the blood monocytes patrolling the liver, the liver harbors 80% of the total macrophages in the body (Ju and Tacke, 2016). Hepatic macrophages are remarkably heterogeneous concerning their versatile phenotypic profiles, which define their immune functions during liver diseases. Based on the origin, the general classification of hepatic macrophages can be divided into two main subtypes: the resident macrophages and bone marrow-derived macrophages (Perdiguero et al., 2015). The liver resident macrophages are known as kupffer cells (KCs), account for 20% of the total liver cells and 80-90% of the total tissue-resident macrophages in the body (Vollmar and Menger, 2009). During the embryonic development, KCs are developed with a self-renewable characteristic and they persist in the liver independent of the blood monocytes (Perdiguero et al., 2015; Ramachandran et al., 2012). Bone marrow derived monocytes gives rise in the liver to monocyte-derived hepatic macrophages, and are highly connected to the recruitment of monocytes during liver injury (Blériot et al., 2015). Liver macrophages are contributing inevitably in liver homeostasis.

During steady state conditions, liver macrophages are using their phagocytosis machinery through C3 and Fc receptors to eliminate bacterial particles, and weakened cells from the sinusoidal blood (Smedsrød et al., 1994). Together with the liver sinusoidal cells, macrophages guarantee the removing of worn-out cells, toxins and inflammatory cues from the perfusing blood (Smedsrød, 2004). During hepatic infection, monocyte derived macrophages are recruited to the inflammatory sites in an IFN-γ dependent manner (Blériot et al., 2015). Upon activation, macrophages produce a wide range of inflammatory mediators (chemokines and cytokines), which balance between acute-phase reactions and immune-tolerance (Parker and Picut, 2005).

1.2.1.4 Liver sinusoidal endothelial cells (LSECs)

Sinusoidal endothelial cells are lining the liver vasculature, which receives and transport the blood from portal vein and hepatic artery across the hepatic capsule. Due to their direct exposure to the blood-flow including bacterial product, endotoxins and food antigens, LSECs in combination with KCs are considered the main filtering machinery of large particles and harmful ingredients from the perfusing blood in the liver (Sørensen et al., 2011). These activities of LSECs are attributed to their high

fenestration, lack of a classical basement membrane, high expression of scavenger receptors and possessing the most potent endocytosis capacity in the body (Poisson et al., 2017). Accordingly, LSECs are considered to have a decisive immune function as a rheostat of immunogenicity and immune-tolerance in the liver (Li et al., 2011).

1.2.1.4.1 Role of LSECs in balancing immune response and tolerance

The initial step in the immune response starts with the pattern recognition receptors (PRRs), which recognize and update foreign antigens. These PPRs are evolutionary conserved and comprise the Toll-like receptor (TLR) family and scavenger receptors (Chen and Nuñez, 2010). Lipopolysaccharide (LPS) sensing by KCs and LSECs through TLR4 is one of the examples of bacterial products recognition in the liver, which followed by inflammatory signals. However, chronic exposure of hepatic environment to LPS was reported to induce a refractory state, especially by LSECs to prevent liver damage by the constantly activated immune response against the regularly raiding gut-derived bacterial products. This mechanism allows LSECs to dampen the leukocyte adhesion by reducing the nuclear translocation of the nuclear factor-κB (NF-κB), which in turn downstream the chemokines and cytokine production (Uhrig et al., 2005). Meanwhile, LSECs were reported to also respond to other TLRs signals including TLR1, TLR4, TLR6, TLR8 and TLR9 in an organ specific pathways that guarantee the tolerogenic milieu in the liver (Wu et al., 2010).

The high expression of scavenger receptors by LSECs compared with the conventional endothelium is one of their unique characteristics within the hepatic structure. Similarly, scavenger receptors play an integral role in antigen sensing as part of the pattern recognition receptor family. Despite their evolutionary conserved structure, scavenger receptors were believed to govern an unspecific uptake of ligands. However, recent evidences showed their ability to respond to antigens in a cell-specific manner, inducing either pro-inflammatory or anti-inflammatory response (Canton et al., 2013). Membrane-bound scavenger receptors internalize their recognized ligands via endocytosis, which transfer extracellular ligands to intracellular compartments such as endosomes (Elvevold et al., 2008). The high and diverse expression of scavenger receptors on the surface of LSECs including mannose receptors (MR), stabilin-1, stabilin-2 and C-type lectins, confers a high capacity of endocytosis by liver endothelium (Malovic et al., 2007; Politz et al., 2002; Sørensen et al., 2011).

During innate response to viral infections, LSECs use their surface-expressed scavenger receptors to bind and internalize the viral particles such as hepatitis C virus (HCV) and sever acute respiratory syndrome coronavirus (SARS) (Gramberg et al., 2005; Lai et al., 2006). Studies using mouse models of adenovirus infections showed the capacity of LSECs to uptake 90% of the viral load compared to only 10% by KCs after a minute of intravenous viral infection. This rapid and robust endocytic-capacity of LSECs toward different viruses confers its inevitable role in viral clearance, especially blood-borne viruses (Ganesan et al., 2011). However, the rapid uptake of viruses by LSECs was suggested to be part of a local redistribution of viral particles to the other cells (Cormier et al., 2004). Studies showed that during HBV infection, the viral particles are transferred to the underlying hepatocytes after a first internalization by LSECs (Breiner et al., 2001). This balance in the innate response to viral infection would be critical to determine the further response by the adaptive immunity and the later clearance or persistence of the viral infection.

1.2.1.4.2 Role of LSECs in the adaptive immunity

LSECs not only collaborate with the innate immune response but also play a crucial role in the adaptive immune response through antigen presentation to T-cells (Figure 2). Knolle's group demonstrated that antigen uptake by scavenger receptors, processing and presentation on LSECs surface via MHC-I molecules results in antigen-recognition by CD8+ T-cells (Limmer et al., 2000). In steady state conditions, antigen presentation in such mechanism by LSECs to naïve CD8+ T-cells induced a tolerogenic response through the up-regulation of co-inhibitory molecules such as programmed cell death-1 ligand (PDL-1) on LSECs surface (Diehl et al., 2008; Limmer et al., 2005). However, this immune tolerance was shown to skew towards effector T-cell differentiation based on other co-stimulatory factors such as the antigen concentration and the local IL-2 secretion (Schurich et al., 2010). Meanwhile, antigen presentation to CD8+ T-cells by LSECs found to induce a robust IL-6 *trans*-signaling, eliciting differentiation and activation of effector CD8+ T-cells (Böttcher et al., 2014).

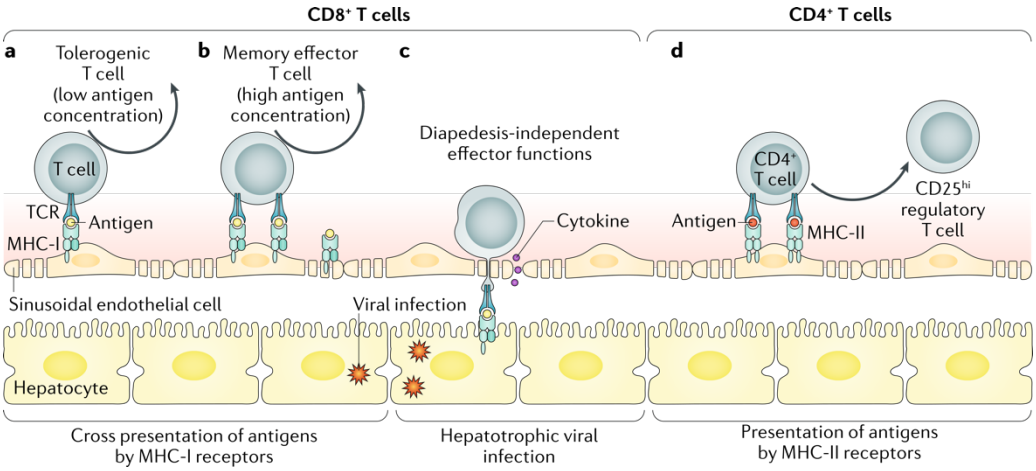

Figure 2. LSECs function as non-professional antigen presenting cell
(a) At low antigen levels, LSECs can present antigens on MHC-I to CD8+ T-cells, leading to tolerance and deletion of cytotoxic CD8 T-cells. (b) At high antigen presentation level, LSECs circumvent the tolerance state to induce development of effector memory T-cells. (c) LSECs arrest CD8+ T-cells during hepatotropic infections such as Hepatitis B virus in a platelet-dependent process triggering the recognition of infected hepatocytes by cytotoxic CD8 T-cells and inducing hepatocyte killing. (d) Antigen presentation by LSECs via MHC-II to CD4+ T-cells leads to formation of a suppressor T-cell phenotype (T-reg). REF. (Shetty et al., 2018)

In addition to MHC-I, LSECs are able to present antigens to CD4+ T-cells via MHC-II molecule (Lohse et al., 1996). However, the weak expression of co-stimulatory molecules on LSECs favors differentiation of naïve CD4+ T-cells into T-$_{reg}$ rather than T-helper (T_h) formation (Arias et al., 2020; Carambia et al., 2014; Knolle et al., 1999). This tolerogenic paradigm of LSECs was found to maintain the immune tolerance in liver milieu by inhibiting circulating inflammatory CD4+ T-cells (T_H-1 and T_H-17 cells) and suppressing their inflammatory-cytokine secretion (Carambia et al., 2013).

1.2.1.4.3 Role of LSECs in inflammatory liver diseases

From the fact that LSECs are the lining of the hepatic vasculature with different capacities to sense and present antigens to other immune cells, LSECs play a decisive role in the recruitment and direction of leukocytes toward the infected tissues. During liver injury, the balance in leukocytes recruitment from the circulations determines the progress of hepatitis toward either resolved injury, or chronic hepatitis or cirrhosis and liver failure (Lalor et al., 2002). In liver microenvironment, the activated LSECs harboring chemo-attractants and chemokines in the endothelial glycocalyx, induce the leukocyte recruitment to the site of infection through a multistep process includes paracrine-signaling, adhesion, crawling and transmigration (Figure 3) (Nourshargh and Alon, 2014; Tanaka et al., 1993).

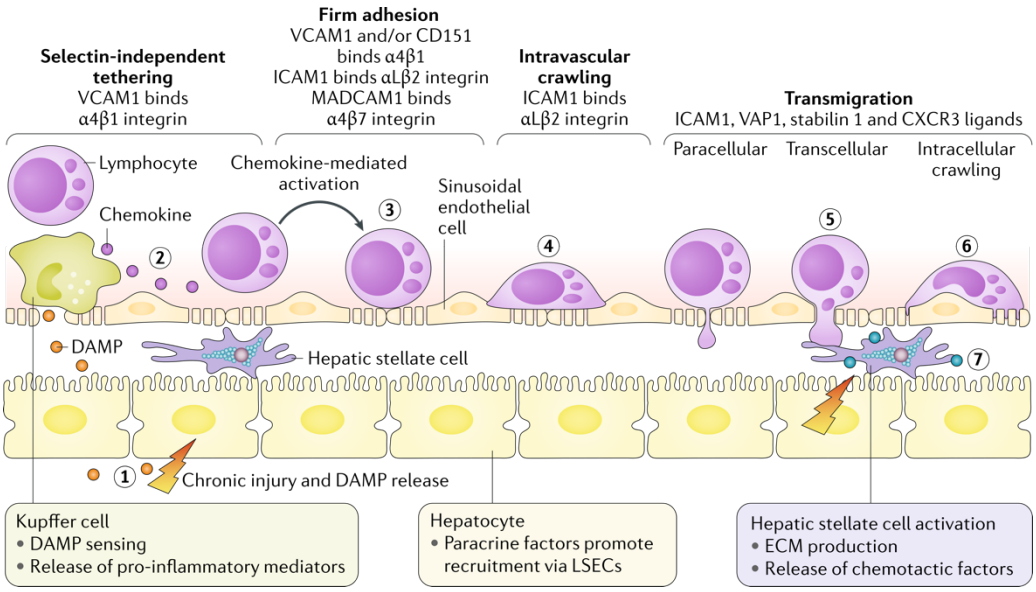

Figure 3. Lymphocyte recruitment and activation of LSEC surface
LSECs sensitization to the danger-associated molecular patterns (DAMPs) and pro-inflammatory mediators by Kupffer cells and stellate cells leads to increased expression of adhesion molecules on LSECs surface (step1). Lymphocyte recruitment to the vascular endothelium through tethering process (step2), which end up with crawling and adhesion of lymphocytes to the surface of LSECs using integrin-mediated adhesion (step3). Lymphocyte crawling over the endothelium (step4) facilitates recognition of cell damage-signals from hepatocytes and induces lymphocytes transmigration through LSECs (step5 and step6). Subsequent activation signals from HSCs increases the magnitude of migration and adhesion of lymphocytes to the liver tissues (step7). REF. (Shetty et al., 2018)

Firstly, during rolling step, the liver patrolling leukocytes receive a paracrine signaling or membrane-bound selectins, leading to the rolling of leukocytes on the surface of LSECs and followed by activation of integrins on the surface of leukocytes (McEver, 2015). Integrins activation induces their binding with immunoglobulin superfamily on the surface of LSECs, which provide the firm adhesion platform to the activated leukocytes (Campbell et al., 1998). Next, the adherent leukocytes crawl on the endothelium before it starts trafficking through the endothelium into the underlying parenchyma, without disrupting the vascular barrier (Muller, 2016). The architecture of the sinusoids with a very narrow size in some places and a low shear stress entitles LSECs to regulate the leukocytes recruitment via its adhesive machinery.

These unique characteristics highlighted the integral role of LSECs and its mediators in controlling the liver immunity and opened the door for new therapeutic targets against viral hepatitis and autoimmune diseases.

1.2.2 Adaptive-mediated immune response to liver infections

1.2.2.1 B cell responses

B cells play a key part of the adaptive immune response to pathogens via their ability to produce highly specific antibodies. Unlike the healthy liver that harbors small numbers of B cells, hepatic viruses such as hepatitis C virus (HCV) infection was found to induce the proliferation and differentiation of B cells within the germinal centers of intraportal lymphoid follicles (Burrows and Cooper, 1997). These germinal centers harbor Ig-M, Ig-D and Ig-G-positive B cells, characterized by the expression of Ki-67, CD23, bcl-2 and bcl-6, and suggested to play a central role in the resolved HCV infection (Murakami et al., 1999).

In addition to the virus-derived peptides, a subtype of B cells was found to recognize the glycolipid antigens via CD1d molecule (Barral et al., 2008; Kinjo et al., 2011; Leadbetter et al., 2008). However, studies showed that the early activation and production of neutralizing antibodies required B cells stimulation through the early IL-4 secretion by natural killer T (NKT) cells (Gaya et al., 2018).

1.2.2.2 T-cell responses

CD4 and CD8 T-cells are fundamentally responsible for the liver-immune response against pathogens. Thanks to the predominant expression of MHC-I and MHC-II molecules on all nucleated cells, effector CD4+ and CD8+ T-cells are able to recognize and discriminate between numerous of antigens, conferring a great degree of accuracy to eliminate the harmful pathogens. However, to allow a robust activation and differentiation of naïve T-cells, second signal is needed through the professional antigen-presenting cells that express the required co-stimulatory molecules such CD40, CD80 and IL-2 (Gonzalo et al., 2001). In inflammatory conditions, LSECs and KCs represent the front line in recognizing and passively taking up the inflammatory cues to be presented to naïve T-cells. These cells further patrol the liver sinusoid or reside forming lymphoid aggregates in the portal tract (Bertolino et al., 2002). However, in steady-state conditions, antigen presentation by LSECs was found to drift naïve T-cells toward immune-tolerance rather than immunogenicity (Limmer et al., 2000). For instance, in non-inflammatory condition, LSEC-mediated antigen-presentation to naïve CD4 T-cells results in a cytokine drift from IL-2 and IFN-γ to IL-4 and IL-10 secretion (Accapezzato et al., 2004; Knolle et al., 1999). This skewed

cytokine milieu down regulates the migration-capacity of dendritic cells to the draining lymph-nodes and induce the priming of T-cells, which are prone to lose their effector functions (Groux et al., 1996; Takayama et al., 2001).

Similarly, LSEC-primed CD8 T-cells in non-inflammatory conditions were reported to have reduced proliferative capacity and cytokine productions (Limmer et al., 2000). Meanwhile, reduced cytokine production by CD8+ T-cells, especially IFN-γ and IL-2 was found to alter the expression of programmed death-1 (PD-1) receptor on CD8+ T-cells, inducing more apoptosis via the crosstalk with PD-1 ligand (PD-L1)-expressing LSECs and KCs (Latchman et al., 2001). On the other hand, studies showed that addition of exogenous IL-2 to the LSEC/CD8+ cell co-culture was able to circumvent the tolerogenic capacity of LSECs and alter CD8+ T-cell effector functions and cytokine production (Knolle et al., 1999).

Of note, CD8+ T-cell can be still activated even in presence of inhibited antigen-presenting cells using the cross-priming pathway (Bevan, 1976a; Bevan, 1976b). In this scenario, the taken-up antigen by a weak or inhibited antigen-presenting cell is further taken up via endocytosis by another cell. Endocytosis and processing of antigens or cell-associated particulates using the cross-priming pathway, results in antigen presentation via MHC-I molecule directly to CD8+ T-cells (Heath and Carbone, 2001; Yewdell et al., 1999). The effectiveness of antigen-presentation to CD4 and CD8 T-cell depends on different environmental factors in the liver including the number of infected cells, their viability, the amount of surface expressed antigen, the number and types of liver patrolling lymphocytes and the cytokine milieu (Heller and Rehermann, 2005; Racanelli and Rehermann, 2003). The efficiency to induce a balanced immune response to hepatic infections determines the clinical outcome of the hepatic diseases between, resolved hepatitis to chronic infection and cirrhosis (Thimme et al., 2002).

1.2.3 Innate-mediated immune response to liver infections

1.2.3.1 Natural killer (NK) cells and innate lymphoid cells (ILCs)

Over the last decades, NK cells have been classified into two different subsets based on their CD56 expression and their cytolytic activities (Zimmermann et al., 2013). Unlike the conventional NK cells, liver-resident NK cells were found to have a robust and homeostatic response to hepatic viral infections based on the TLRs signaling (Tu

et al., 2008). Meanwhile, liver-resident NK cells were found to share many characteristics of the innate lymphoid cells (ILCs) (Peng and Tian, 2015). ILCs is a wide group of cells with no antigen-specific receptor and plays a vital role in the innate immune response to pathogens and viral infections using their high sensing capacity to TLRs and cytokine signaling (Chen and Tian, 2021). According to the International Union of Immunological Societies (IUIS), ILCs are classified into five subsets including: NK cells, ILC1 (Group 1), ILC2 (Group 2), ILC3 and lymphoid-tissue inducers (LTis) cells (Group 3) (Vivier et al., 2018). This classification is based on the surface phenotype, functionality and transcriptional regulation (Gasteiger et al., 2015; Spits et al., 2016; Watarai et al., 2008), whereas studies showed a controlled plasticity between these subtypes depending on the cytokines milieu and transcriptional levels (Bernink et al., 2015; Ohne et al., 2016).

1.2.3.2 Innate-like lymphoid T-cells (ILTCs)

Over the last decades, Innate-like T lymphocytes is a group of unconventional T cells with diverse repertoire, discovered in both mouse and human. By definition as T-cells, ILTCs are characterized of carrying diverse but conserved T-cell antigen receptors (TCRs), which are able to recognize a wide range of protein, metabolite and lipid antigens, presented on MHC-I like molecules (Godfrey et al., 2018; Strid et al., 2011). Meanwhile, the rapid ILTCs' response to pathogens independently from the TCRs and carrying of surface molecules similar to those of innate lymphocytes confirm their contribution in the innate response. ILTCs were shown to respond to infections via secretion of a wide range of cytokines, which imperatively balance the immune response (Godfrey et al., 2018). Classification of ILTCs is mainly divided into 3 subsets including: γδ T-cells, mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells (Godfrey et al., 2018), based on the TCRs-chain and the specific-agonists (Wencker et al., 2014). Comprising up to 40% of the intrahepatic lymphocytes (IHLs), NKT cells represent the biggest fraction of ILTCs in the liver (Watarai et al., 2008).

1.2.3.3 NKT cells: An essential component of liver immunity

NKT cells belong to a specialized subset of T-cells. Unlike the conventional T-cells which recognize peptide antigen presented by MHC-I molecules, NKT cells identify and react to the self and foreign lipid-antigens presented by a MHC-I like molecule known as CD1d through invariant Vα14-Jα18-Vβ11 TCR (Brigl and Brenner, 2004).

NKT cells have been shown to exert either protective or harmful immune response during several diseases including microbial and viral infections, cancer, autoimmune and allergic diseases (Brigl and Brenner, 2010; Meyer et al., 2007; Novak and Lehuen, 2011; Tupin et al., 2007; Vivier et al., 2012). Due to their shared immunological features between the adaptive and the innate immune systems, NKT cells were considered as a bridge maintains the rapid and robust innate response to pathogens, with a classical selection features in the thymus (Godfrey and Berzins, 2007; Stetson et al., 2003). Upon its activation, NKT cells are deemed to produce a wide range of pro-inflammatory cytokines, which exert its effector or regulatory functions (Brigl et al., 2003; Nagarajan and Kronenberg, 2007).

1.2.3.3.1 NKT cell subsets

In both mice and human, NKT cells were identified as CD4+ and CD4- NKT cells with different percentages varying among individuals (Gumperz et al., 2002; Lee et al., 2002). Although several studies have divided CD4+ and CD4- NKT cells based on the cytokine secretion into T_h1-type and T_h2-type respectively, recent studies showed the ability of both NKT subsets to secret T_h1-type and T_h2-type cytokines including IFNγ, tumor necrosis factor (TNF), IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IL-21 and GM-CSF (Coquet et al., 2008; Gumperz et al., 2002; Lee et al., 2002). In addition to CD4+ subset, CD4- subset of NKT cells was shown to harbor a substantial portion of CD8+ NKT with more cytotoxic activities than CD4+ NKT cells (O'Reilly et al., 2011; Takahashi et al., 2002).

Due to the absence of a selective group of markers to identify the different subsets of NKT cells, NKT have been classified into three main subsets: T_h1-like, T_h2-like T_h17-like NKT cells, based on their transcriptional regulation during the thymic development (Figure 4) (Watarai et al., 2012).

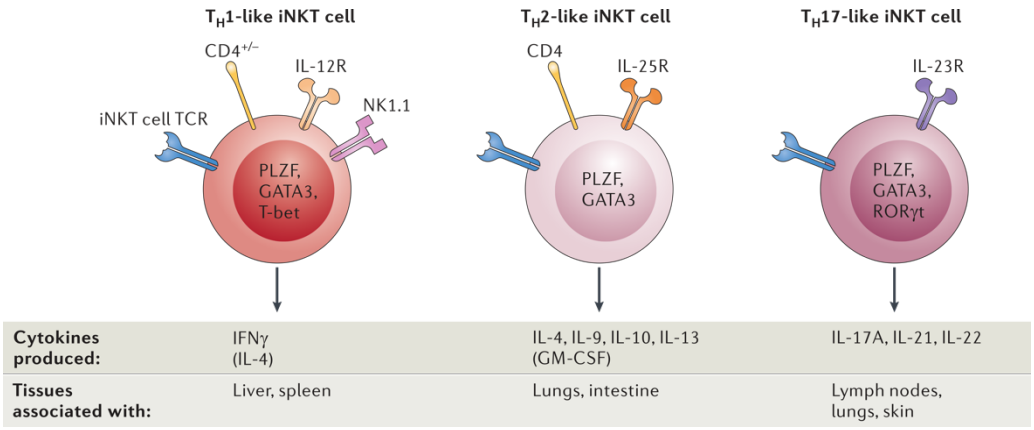

Figure 4. iNKT cell subsets
Comparison between the three known subsets of iNKT cells; T_h1-like, T_h2-like T_h17-like NKT cells in term of their transcriptional regulation, surface markers and the constitutive production of cytokines. REF. (Brennan et al., 2013)

Of note, the plasticity between the different subsets of iNKT cells has not been clearly investigates yet.

1.2.3.3.1.1 Th1-like NKT cells (NKT1)

The development of NKT1 cell in thymus is transcriptionally regulated by PLZF, T-bet and GATA3 transcription factors (Watarai et al., 2012). NKT1 represents the majority of iNKT cells in the liver with either CD4+ or CD4- surface phenotype (Terashima et al., 2008). NK1.1+ NKT cells constitute the majority of IFN-γ secreting NKT1 (Coquet et al., 2008). Studies have shown the capacity of IFN-γ secreting NKT1 cells to further produce some of T_h2 or T_h1 cytokines depending on the activation pathway through TCR stimuli or IL-12, respectively (Watarai et al., 2012).

1.2.3.3.1.2 T_h2-like NKT cells (NKT2)

NKT2 cells constitute the majority of iNKT cells in the lung and airways. Transcriptionally, NKT2 does not have specific transcription factors but it lacks expression of both T-bet and retinoic acid receptor-related orphan receptor-γt (RORγt) (Watarai et al., 2012). The production of T_h2-type cytokines by NKT2 in response for IL-25 signaling was shown to be dependent on GATA3 and E4 promoter-binding protein 4 (E4BP4) transcription factors (Stock et al., 2009; Terashima et al., 2008). T_h2-type cytokines production including IL-4, IL-9, IL-10 and IL-13 by NKT2 cells was shown to contribute in the airways hyper-reactivity (Watarai et al., 2012).

1.2.3.3.1.3 T_h17-like NKT cells (NKT17)

NKT17 cells were found to mostly reside in the peripheral lymph nodes, lungs and skin, and their development in thymus depends on RORγt transcription factor (Coquet et al., 2008). Similar to MHC-restricted TH17 cells, NKT17 cells mostly belong to the CD4-NK1.1- subpopulation and mainly producing IL-17 after activation (Michel et al., 2007). NKT17 cells were suggested to be the main source of IL-21 and IL-22 production (Coquet et al., 2008; Paget et al., 2012). Activation of NKT17 cells takes place in presence of IL-23, which induces their CCR6 expression and IL-17 production in response for the microbial infections (Pichavant et al., 2008).

1.2.3.3.2 Patrolling NKT cells in the liver sinusoid

The microscopic screening showed that iNKT cells, especially NKT1 constitutes up to 40% of total hepatic lymphocytes in mice compared to 3-5 % in human liver (Matsuda et al., 2000; Syn et al., 2010). At steady state condition, maintenance and long-term residency of NKT in the liver rely on several factors including surface molecules, cytokines, transcription factors, adhesion molecules and chemo-attractants (Liang et al., 2012; Mackay et al., 2016; Monticelli et al., 2009). The intravital microscopic imaging showed that NKT cells patrol the liver sinusoid in a random and crawling motion independent from the direction of blood flow (Geissmann et al., 2005) (Figure 5a).

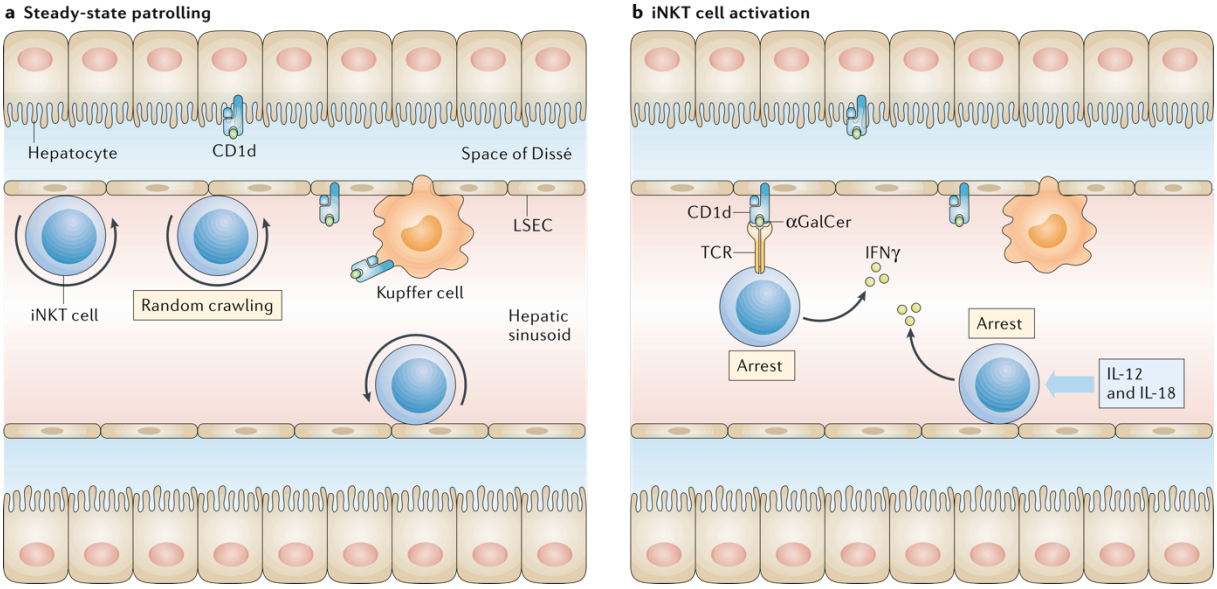

Figure 5. Liver-sinusoid patrolling iNKT cells
(a) At steady state condition, iNKT patrol the hepatic sinusoid in a random motion with and against the blood flow. (b) In response to antigen recognition via CD1d or cytokine stimulation such as IL-12 or IL-18, iNKT cell slows down its speed and adhere to LSECs, leading to IFN-γ production. REF. (Crosby and Kronenberg, 2018)

Meanwhile, expression of the NKT-specific antigen-presenting CD1d molecule was found to be distributed between different cells including LSECs, KCs, DCs, HSCs and hepatocytes (Geissmann et al., 2005; Zeissig et al., 2017). Upon antigen presentation by the surrounding CD1d molecules in liver tissues or exposure to inflammatory cytokines, NKT cells were seen to dampen their patrolling motion, be arrested on the sinusoidal wall and secret inflammatory mediators such as IFN-γ, which induced hepatitis (Lee et al., 2010; Velázquez et al., 2008) (Figure 5b).

1.2.3.3.3 NKT cells activation-pathways in the liver

In vivo studies showed that NKT cells activation in the liver relies on two different pathways. The first pathway is TCR signaling-dependent via recognition of self or foreign antigens presented by CD1d molecule (Brigl et al., 2011). Depending on antigen concentration and the predominant subset of NKT cells, NKT cells produce a wide range of cytokines, which would elicit an immune response by the other hematopoietic cells including T-cells, NK cells, B cells, DCs and macrophages (Engel et al., 2016). In case of weak antigen presentation by CD1d molecule, NKT cells rapidly respond to the inflammatory cytokines as a second activation pathway. For instance, IL-12 and IL-18 activate NKT cells in a more cytotoxic manner compared to TCR signaling, inducing more IFN-γ secretion by NKT cells (Velázquez et al., 2008).

Thanks to their activation capacity and prevalence in the liver, NKT cells were suggested to play crucial roles in liver immunity against infections.

1.2.3.3.4 NKT cell function in liver diseases

1.2.3.3.4.1 Bacterial and viral infections

Due to their unique ability to recognize both endogenous and exogenous glycolipid-antigens, the role of liver NKT cells in recognizing bacterial infections has been extensively studied. Lyme disease is one of the inflammatory diseases known to target skin, joints, heart and nervous system causing a multi-systemic disorder. *Borrelia burgdorferi* is a bacterial species belongs to the spirochete family and considered as the etiologic agent of Lyme disease (Tupin et al., 2008a). During the infection, *Borrelia burgdorferi* secrets a glycolipid-antigen, which is taken up by liver KCs and presented to the liver NKT cells via CD1d molecule (Kinjo et al., 2006; Lee et al., 2010). After 8 hours of antigen recognition, CXCR3 positive NKT cells become stationery and form clusters with KCs in the liver sinusoid in response for antigen recognition and the chemokine ligand-9 (CXCL9) expression by KCs. The clustered NKT cells showed a high expression of CD69 and increased IFN-γ production that neutralizes the pathogen and hamper the bacterial emigration toward other organs (Lee et al., 2010; Tupin et al., 2008b) (Figure 6a). In vivo studies of *Borrelia burgdorferi* infection using NKT cell-deficient mice showed a more intense joint inflammation and an extensive accumulation of the spirochetes in the bladder, joints and heart (Olson et al., 2009).

Similarly, several studies tried to address the contribution of NKT cells during viral hepatitis. Studies comparing the number of NKT cells in the liver and peripheral blood between healthy individuals and chronic hepatitis C virus (HCV) patients, showed a dramatic reduction in NKT cells-numbers during the chronic infection (Deignan et al., 2002). Other studies showed that the acute-resolved HCV infection was attributed to the inhibition of HCV replication in the liver by IFN-γ secreting NKT cells (Miyaki et al., 2017).

Furthermore, mouse models of hepatitis B virus (HBV) showed that the infected hepatocytes produce endogenous antigenic-lipids including HBV-generated lysophospholipids. Then, hepatocyte uses the microsomal triglyceride transfer protein (MTP) to transfer the lipid-antigens from the endoplasmic reticulum via CD1d to the surface expression (Zeissig et al., 2017). Thereafter, patrolling NKT cells recognize

the presented antigens and contribute in viral inhibition and clearance through a robust IFN-γ secretion. Absence of liver NKT cells or loss of MTP, were associated with a delayed or diminished HBV clearance (Jiang et al., 2011; Zeissig et al., 2012).

1.2.3.3.4.2 Liver inflammation

Several studies suggested a strong contribution of NKT cells in chronic inflammation. *In vivo* studies using different mouse models reported a lethal inflammation accompanied with a serious hepatocyte death after the injection of NKT cell agonist α-galactosyl ceramide (α-GalCer). This tissue damage in response for α-GalCer injection was attributed to NKT cell cytotoxicity against hepatocytes (Santodomingo-Garzon and Swain, 2011). Activated NKT cells were found to induce a non-cytolytic killing of hepatocytes through their rapid secretion of IFN-γ, which directly neutralize the infected cells and stimulate the recruitment of T_h1 T-cells to the sinusoid. Additionally, NKT cells up-regulate the co-stimulatory molecules ligand such CD40L, which in turn interact with CD40+ DCs and induce the later to secret more inflammatory cytokines such as IL-12 (Santodomingo-Garzon and Swain, 2011) (Figure 6b). Meanwhile, activated NKT cells elicit a direct cytolytic effect on hepatocytes through the FAS-FAS ligand (FASL) interaction, secretion of perforins and granzyme B (Takeda et al., 2000).

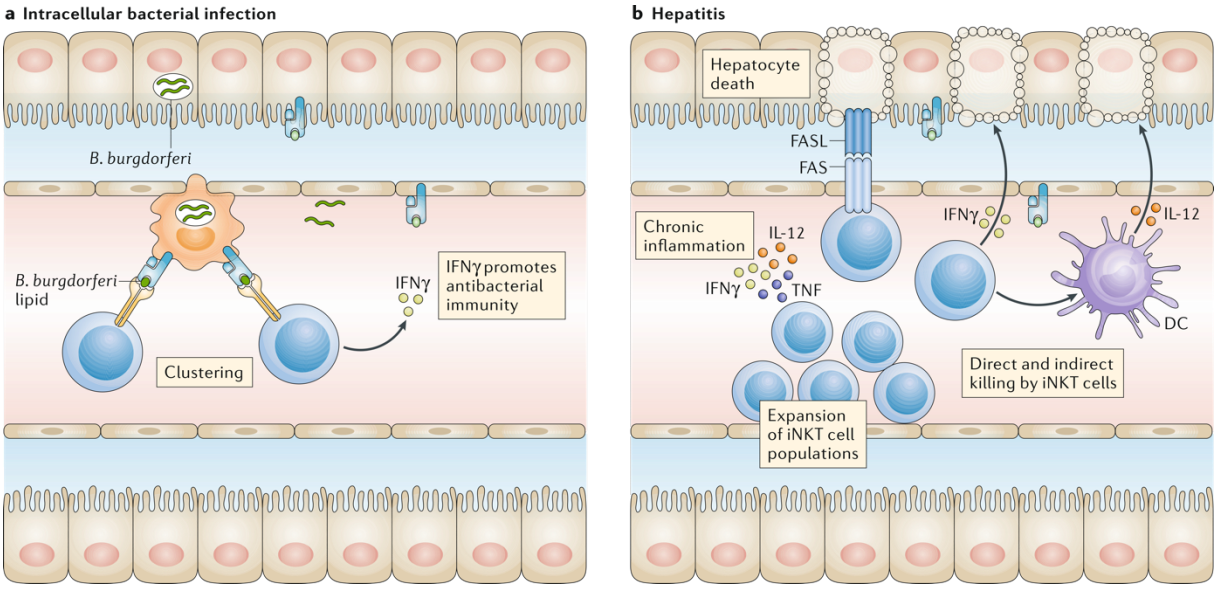

Figure 6. iNKT cell response to microbial infection and hepatic inflammation
(a) Presentation of bacterial particles, taken up by KCs or hepatocytes to iNKT cells in a CD1d restricted process leads to expansion, differential and cytokine production by NKT cells. (b) During chronic hepatic inflammation, NKT cell sensitizes the pro-inflammatory mediators secreted by other cell types, leading to more production of IFN-γ, IL-12 and TNF. Meanwhile, NKT cell exerts a direct killing of hepatocytes via the apoptosis mediating surface antigen Fas-FasL axis. REF. (Crosby and Kronenberg, 2018)

In fatty liver diseases such as the non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), activation and homeostasis of the immune system plays an inevitable role in the disease progression. Due to the clinical manifestation of these diseases, represented by the accumulation of fatty tissues within the hepatic structure, the role of NKT cells as the main lipid-antigen specific immune cells in progression or resolving of these diseases was intensively investigated. In mouse models, high-fat high-cholesterol diets were used to induce NASH like liver pathology (Bhattacharjee et al., 2017; Wolf et al., 2014). Several parameters associated with NKT cell immunology such as the increased expression of lipid antigens through CD1d molecules, increase in CD69+ NKT cells, accumulation and formation of intrahepatic NKT colonies, increased alanine aminotransferase (ALT) levels, increased recruitment of CD8+ T-cells, increased production of IL-12, TNF-α and IFN-γ were investigated (Hines et al., 2018a; Jiang et al., 2009; Jin et al., 2011). These elevated parameters were clinically associated with increased fibrinogenesis and higher score of the disease (Syn et al., 2012). Contrastingly, studies carried on using NKT deficient mice or NAFLD human patients showed a strong correlation between the score of the disease progression and presence of NKT cells (Syn et al., 2012; Tajiri et al., 2009).

1.2.3.3.5 NKT cells – as a promising immunotherapeutic approach

NKT cells play important roles not just in host defence against pathogens, viral infections and malignant cells, but also in eliciting inflammation and progressing tissue damage. Their shared characteristics from innate and adaptive immunity confer the rapid and robust activation of NKT cells in response for different activation pathways recognizing unconventional lipid-antigens, inflammatory cytokines, and cellular stress. To maintain its potent cytotoxicity, NKT cells express a wide range of molecules such as Vα14-Jα18-Vβ11 TCR, NK1.1, NKG2D and CD69, which became all potential therapeutic-targets for immunotherapy (Godfrey et al., 2018). For instance, targeting CD1d molecule through α-GalCer injection activates NKT cells, which in turn eliminate the senescence hepatocytes (Mossanen et al., 2019). This elimination prevents hepatocarcinogenesis, especially in the context of NAFLD and NASH (Wang and Gao, 2020). Contrastingly, inhibiting the NKT associated hedgehog pathway or the LIGIT-LTβR interaction curb the crosstalk between NKT cells and hepatocytes, preventing liver damage and hepatocellular carcinoma (HCC) (Syn et al., 2012; Wolf et al., 2014). Another approach used peroxisome proliferator

activated-receptor α (PPARα) inhibitors to prevent the NKT cell-mediated hepatitis in response to Concanavalin A (Con A) toxicity (Hines et al., 2018b).

In term of cancer immunotherapy, several clinical trials for treatment of solid tumors using either intravenous injection of NKT cell-agonists or *ex vivo* expansion of patient-derived primary NKT cells were applied (Exley et al., 2017; Giaccone et al., 2002; Imataki et al., 2008; Kunii et al., 2009; Motohashi et al., 2006; Osada et al., 2005; Yamasaki et al., 2011). Simultaneously, other clinical trials using chimeric-antigen receptor-NKT (CAR-NKT) cells were investigated to treat neuroblastoma through targeting the high expression of GD2 ganglioside (CAR.GD2) on cancer cells (Heczey et al., 2014; Simon et al., 2018).

Despite this progress achieved in understating the immune capacity of NKT cells, a number of important questions regarding the thymic selection, stability in the periphery, migration and residency remains unanswered. First, whether the transcriptional programming of NKT cells in the thymus is distinct enough to differentiate between the different subsets of NKT cells including NKT1, NKT2 and NKT17? It is also important to understand how significant the second differentiation of NKT cells in the periphery, to which extent does it change the pre-differentiated subsets, and which factors dictate the homing and residency of the different NKT cell subsets in different tissues?

To sufficiently establish a deep understating of NKT functions in different tissues, it is essential to know the inter-conversion capacity between the different NKT cell subsets. What are the different factors in the tissue milieu that regulate their functions? For instance, several in vivo studies tried to investigate the NKT crosstalk with the liver micro-environment, and they were successful to unleash following studies of NKT cells in the context of hepatic infections and HCC. However, it is still extremely substantial to define separately the underlying mechanisms that exclusively regulate the crosstalk of NKT cells with the different cell-populations in the liver. How specific and different they are? How the various antigen-presenting cells direct the NKT cell responses? Moreover, do they exert additional influence on NKT cell cross talk with the other cells?

A deeper knowledge of NKT cells cognate-interactions combined with comprehensive insight into the immune reaction of the surrounding cells within the tissue-milieu will markedly help to properly use combinatory therapies against diseases.

1.3 Research questions, aims and objectives

This research project aimed to study the crosstalk of iNKT cells with CD1 expressing antigen-presenting cells in the liver. For that, we employed intensive *in vitro* studies where individual populations of primary cells were separately cultured under different conditions with isolated primary NKT cells from liver. The protocols used in this study for isolation of cells, maintenance and mimic of the sophisticated biological conditions in the hepatic milieu were successfully established over the last years by Prof. Knolle group at the Institute of Molecular Immunology (IMI). Over the last 30 years, studies were successful to unveil different characteristics of iNKT cells and document their immune contribution during pathogenic / viral infections, cancer and autoimmune diseases. In Liver, which harbors the majority of iNKT, many *in vivo* reports highlighted the role of iNKT cells in recognizing both endogenous and exogenous lipid-antigens presented by CD1 molecule. However, understating of iNKT cells strength of interaction with the different CD1 expressing antigen presenting cells in hepatic capsules remains unclearly investigated. It has been profoundly explained by Prof. Knolle how the liver sinusoidal endothelial cells (LSECs) act as the rheostat between immunogenicity and immune tolerance due to their location as the lining of liver vasculature.

The main hypothesis of my research project is that LSECs function as a platform for adhesion of various lymphocytes and elicit antigen cross-presentation with naïve CD8+ T-cells, inducing their differentiation into memory T-cells (Knolle and Wohlleber, 2016). Many factors such as lipid antigens, cytokines, CD1, co-stimulatory, adhesion and chemokine molecules would dictate the NKT cell interaction with LSECs. Accordingly, my aim was to study the outcome of this interaction and the contribution of these different factors in modulating NKT cell response.

Firstly, we investigated the expression of CD1 by different cell populations and the prevalence of viable NKT cell subsets in the liver to determine the strength of CD1-dependent activation of NKT cells. Secondly, we synchronized the lipid-antigen expression in the liver through LSECs and DCs by using the synthetic glycolipid α-galactosylceramide (α-GalCer) to investigate NKT cell response to their antigen-presentation. Then, we characterized the LSECs-induced NKT cell activation and its role to boost a concomitant CD8 T-cell and NK cell activation. Next, we determined

the principle of CD8 T-cell and NK cell polyclonal activation in response for LSECs-NKT cognate interaction. Thereafter, we investigated the potential of LSEC metabolites such as ATP and Calcium to elicit a polyclonal activation of the immune response. Meanwhile, we measured the production of inflammatory mediators including interleukins, cytokines and chemokines in response for LSEC-NKT cell interaction, and we determined their role in evoking CD8 T-cell and NK cell immune response. Eventually, we studied the role of CXCR6-CXCL16 axis in LSECs-induced activation of CD8 T-cell and NK cell.

This *ex vivo* system where the phenotypical changes, mechanisms and post-transcriptional cytokine-production defining the outcome of LSEC-NKT crosstalk can be studied in detail, giving us the determining insight into this immune-crosstalk and its influence on the surrounding tissue milieu will help us to optimally circumvent the immune tolerance in liver diseases.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Mouse facility

C57BL/ 6 (B6) mice were ordered from Janvier Labs (Le Genest-Saint-Isle, France), raised, and housed under pathogen-free hygienic conditions and according to FELASA[1]-Guidelines. All mice of the same sex were used 6 to 12 weeks of age and littermates of the same sex were used for the same experimental groups.

2.1.2 Mouse Lines

Mouse line	Description	Manufacturer
C57BL / 6	Wild type	Janvier

2.1.3 Equipments

Equipment	Manufacturer
AutoMACS separator	Miltenyi Biotech, Bergisch Gladbach, Germany
Balance	Kern 572, Bailingen-Frommingen, Germany
Benchtop centrifuge Hereus Fresco 17	Thermo Scientific, Waltham, USA
BioPlex Pro II microplate Wash station	Bio-Rad, Hercules, USA
Centrifuge Multifuge X3R	Thermo Scientific, Waltham, USA
Counting chamber Neubauer improved	Hermann-Herenz, Hamburg, Germany
EasyPet pipet controller	Eppendorf, Hamburg, Germany
Implen P330 nanophotometer	SERVA, Heidelberg, Germany
Incubator Heracell 150i	Thermo Scientific, Waltham, USA
Infinite M1 1000 PRO	Tecan GmbH, Crailsheim, Germany
LightCycler® 480 II	Roche, Basel, Switzerland
Microscope Axio Vert A1	Zeiss, Oberkochen, Germany
PCR Cycler (ProFlex PCR System)	Applied Biosystems, Foster City, CA / USA
Perfusion pump	Cole-Parmer GmbH, Wertheim, Germany

[1]FELASA: Federation of European Laboratory Animal Science Associations

(Masterflex® L / S® Digital Standard drive)	
pH meter (inoLab® pH 7110)	WTW (Xylem Analytics), Weilheim
Pipettes	Eppendorf, Hamburg, Germany
SA3800 Spectral Analyzer	SONY Biotech, Tokyo, Japan
SH800 Sorter	SONY Biotech, Tokyo, Japan
Shaker Max Q400	Thermo Scientific, Waltham, USA
SP6800 Spectral Analyzer	SONY Biotech, Tokyo, Japan
Sterile workbench Safe 2020	Thermo Scientific, Waltham, USA
Thermostat	Julabo GmbH, Seelbach, Germany
XCelligence RTCA Multiplate	ACEA Biosciences, San Diego, USA

2.1.4 Consumables

Designation	Manufacturer
384 Microtiter plate for qPCR	Roche, Basel, Switzerland
96-well microtiter plate (V-shape)	Roth, Karlsruhe, Germany
Cannula	Braun, Melsungen, Germany
Cell sieve (40, 70, 100µm)	Corning, New York, USA
CellBIND Multiple Well Plate	Corning, New York, USA
Cover glasses (Menzel glasses)	Thermo Fisher Scientific, Waltham, USA
E-plate View 96	ACEA Biosciences, San Diego, USA
FACS tubes	Bio-Rad, Hercules, USA
Falcon tubes (15, 50ml)	Greiner Bio-One, Kremsmünster, Austria
MACS Separation column	Miltenyi Biotech, Bergisch Gladbach, Germany
Microtiter plates (6, 12, 24, 96 (F, U)	Sarstedt, Nümbrecht, Germany
PCR tubes	Thermo Fisher Scientific, Waltham, USA
Petri dish (100 mm)	Sarstedt, Nümbrecht, Germany
Pipette tips (10 - 1000 µl)	Sarstedt, Nümbrecht, Germany
Pipettes (2ml, 5ml, 10ml, 25ml)	Greiner Bio-One, Kremsmünster, Austria
Reaction vessels (1.5ml, 2ml)	Greiner Bio-One, Kremsmünster, Austria
Sterile reservoir	Corning, New York, USA
Syringes (2, 5, 20 ml)	Braun, Melsungen, Germany

Tissue culture flasks (25, 75, 150cm^2)	TPP, Trasadingen, Switzerland

2.1.5 Software

Software	Manufacturer
FlowJoTM	Becton-Dickinson, Franklin Lakes, USA
IDEAS® software	Luminex, Austin, USA
LightCycler® 480 software	Roche, Basel, Switzerland
MagellanTM (Tecan Reader)	Tecan GmbH, Crailsheim, Germany
Xcelligence® RTCA Software Pro	ACEA Biosciences, San Diego, USA

2.1.6 Reagents

Reagent	Manufacturer
Accutase® solution	Thermo Fisher Scientific, Waltham, USA
Adenosine triphosphate (ATP)	Sigma-Aldrich, St Louis, USA
Ammonium chloride (NH_4Cl)	Sigma-Aldrich, St Louis, USA
Anti-Biotin MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
Antimycin A	Sigma-Aldrich, St Louis, USA
Avidin HRP	Thermo Fisher Scientific, Waltham, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, St Louis, USA
Brefeldin A	Thermo Fisher Scientific, Waltham, USA
BSA	AppliChem, Darmstadt, Germany
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, St Louis, USA
Calcium Sensor Dye eFluor® 514	Thermo Fisher Scientific, Waltham, USA
CD11c Microbeads	Miltenyi Biotech, Bergisch Gladbach, Germany
CD146 Microbeads	Miltenyi Biotech, Bergisch Gladbach, Germany
CD19 Microbeads	Miltenyi Biotech, Bergisch Gladbach, Germany
CD8a Microbeads	Miltenyi Biotech, Bergisch Gladbach, Germany
Collagen R solution 0.2%	SERVA, Heidelberg, Germany
Collagenase	SERVA, Heidelberg, Germany

CpG oligodeoxynucleotides	InvivoGen, San Diego, CA
DMEM	Thermo Fisher Scientific, Waltham, USA
DMSO	Sigma-Aldrich, St Louis, USA
DNAse	Sigma-Aldrich, St Louis, USA
EDTA ($C_{10}H_{16}N_2O_8$)	AppliChem, Darmstadt, Germany
Ethanol (C_2H_6O)	Sigma-Aldrich, St Louis, USA
Fetal calf serum (FCS)	PAN-Biotech GmbH, Aidenbach, Germany
Gentamycin	Thermo Fisher Scientific, Waltham, USA
Gey's Balanced Salt Solution (GBSS)	Thermo Fisher Scientific, Waltham, USA
Glucose	AppliChem, Darmstadt, Germany
Glucose monohydrate	Sigma-Aldrich, St Louis, USA
Hank's balanced saline solution (HBSS)	Thermo Fisher Scientific, Waltham, USA
Heparin sodium (5000 IU / ml)	Braun, Melsungen, Germany
Horseradish peroxidase labeled streptavidin	Thermo Fisher Scientific, Waltham, USA
Hydrochloric acid (HCl)	Sigma-Aldrich, St Louis, USA
Hydrocortisone	Rotexmedica, Trittau, Germany
Insulin	Novo Nordisk, Copenhagen, Denmark
Ionomycin	Sigma-Aldrich, St Louis, USA
Isoflurane CP®	CP-Pharma, Burgdorf, Germany
Ketamine (10%)	Serumwerk Bernburg AG, Bernburg, Germany
L-glutamine 200 mM	PAN-Biotech GmbH, Aidenbach, Germany
Lipopolysaccharides from E. coli 055: B5	Sigma-Aldrich, St Louis, USA
Luciferin K salt	PJK, Kleinblittersdorf
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, St Louis, USA
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich, St Louis, USA
Methanol (CH_3OH)	Sigma-Aldrich, St Louis, USA
Monensin	Thermo Fisher Scientific, Waltham, USA
Nycodenz	Axis-Shield Diagnostics, Oslo, Norway
Ovalbumin	Sigma-Aldrich, St Louis, USA

Paraformaldehyde	Sigma-Aldrich, St Louis, USA
Penicillin / streptomycin 10,000 U / ml	Biochrome GmbH, Berlin, Germany
Percoll®	Thermo Fisher Scientific, Waltham, USA
PMA	Sigma-Aldrich, St Louis, USA
Potassium chloride (KCl)	Sigma-Aldrich, St Louis, USA
Potassium Hydrogen Phosphate (KH_2PO_4)	Sigma-Aldrich, St Louis, USA
Potassium hydrogen phosphate (NH_2PO_4)	Sigma-Aldrich, St Louis, USA
RPMI 1640	Thermo Fisher Scientific, Waltham, USA
Sodium azide NaN_3	Sigma-Aldrich, St Louis, USA
Sodium chloride	Sigma-Aldrich, St Louis, USA
Sodium hydrogen phosphate (NH_2PO_4)	Sigma-Aldrich, St Louis, USA
Sodium hydroxide (NaOH)	Sigma-Aldrich, St Louis, USA
Sulfuric acid (H_2SO_4)	Thermo Fisher Scientific, Waltham, USA
TMB Solution (1X)	Thermo Fisher Scientific, Waltham, USA
Trypan blue	Biochrome GmbH, Berlin, Germany
Tween 20	Sigma-Aldrich, St Louis, USA
Type II collagenase	Worthington Biochemical Cooperation, Lakewood, New York, USA
Type IV collagenase	Sigma-Aldrich, St Louis, USA
UltraComp eBeads	Thermo Fisher Scientific, Waltham, USA
William's Medium E	PAN-Biotech GmbH, Aidenbach, Germany
α-Galactosylceramide, α-Gal-Cer $C_{50}H_{99}NO_9$	Enzo Life Sciences GmbH, Loerrach, Germnay
β-Mercaptoethanol 50mM	Sigma-Aldrich, St Louis, USA

2.1.7 Antibodies

marker	Fluorochrome	clone	Dil./Conc.	Manufacturer
CCR2	BV421	SA203G11	1: 250	BioLegend
CCR5	Percp-Cy5.5, Percp-eF710	HM-CCR5 NP-6G4	1: 250	Thermo Fisher BioLegend
CD106	eF450, PE	429 (MVCAM.A)	1: 250	Thermo Fisher

				BioLegend
CD11b	PE-Dazzle594, BV605	M1/70	1: 250	BioLegend
CD11c	eF450, PE-Cy7, BV650, AF488	N418	1: 250	BioLegend
CD126 (IL-6R)	PE	D7715A7	1: 250	BioLegend
CD146	PE, AF488, Percp-vio700, FITC[2]	ME-9F1	1: 250	BioLegend
CD154 (CD40L)	PE	MR1	1: 250	BioLegend
CD178 (FasL)	PE	MFL3	1: 100	BioLegend
CD178.1 (FasL)	PE	Kay-10	1: 250	Biolegend
CD186 (CXCR6)	PE-Dazzle594, BV421, FITC, PE	SA051D1	1: 250	BioLegend
CD19	PE-eF610, FITC	1D3	1: 250	Thermo Fisher BioLegend
CD1d	PE, Percp-eF710	1B1	1: 250	Thermo Fisher
CD25	Percp-Cy5.5, BV650	PC61	1: 250	BioLegend
CD25	Percp-Cy5.5, BV650	PC61.5	1: 250	BioLegend
CD3ε	PE-Dazzle594, Percp-Cy5.5, BV605	17A2	1: 250	Thermo Fisher
CD31	BV421	390	1: 250	BioLegend
CD314 (NKG2D)	PE	CX5	1: 100	Thermo Fisher
CD335 (NKp46)	FITC, PE, AF647	29A1.4	1: 250	BioLegend
CD4	BV570, PerCP-Cy5.5, BV785	PM4-5	1: 250	BioLegend
CD40	Percp-Cy5.5, PE	3/23	1: 250	BioLegend
CD49b	FITC	DX5	1: 250	Biolegend
CD54 (ICAM)	PE, BV510	YN1 / 1.7.4	1: 250	Thermo Fisher
CD69	PE-Cy7, FITC, BV510, AF488	H1.2F3	1: 250	BioLegend

[2]FITC: Fluorescein isothiocyanate

CD80	BV650, PE-Cy7	16-10A1	1: 250	BioLegend
CD86	PE-Cy7, FITC	PO3 GL-1	1: 250	BioLegend
CD8a	PE-Cy7, PB, eF450, BV510, AF488, PE, BV711, FITC, BV421	53-6.7	1: 250	BioLegend
CD95 (Fas)	PE	SA367H8	1: 250	Biolegend
CD95 (Fas)	PE		1: 200	BioLegend
F4/80	BV421	BM8	1: 250	BioLegend
GM-CSF	Percp-Cy5.5	MP1-22E9	1: 250	BioLegend
GzmB	Percp-eF710, PE	GB11	1: 150	Thermo Fisher
IFN-γ	PE, BV711	XM61.2	1: 100	BioLegend
IL-10	Percp-Cy5.5	JES5-16E3	1: 250	BioLegend
IL-12R	PE	C15.6	1: 250	BioLegend
IL-17A	BV650	TC11-18H10 (R40)	1: 250	BD Bioscience
IL-2	BV510	JES6-5H4	1: 250	BioLegend
Ki-67	PE	SolA15	1: 100	BioLegend
MHC I	APC, FITC	AF6-88.5.5.3 34-1-25	1: 250	Thermo Fisher
MHC II	AF700, PE	M5/114.15.2	1: 250	BioLegend
NK1.1	BV421, BV570, PE, Percp	PK136	1: 250	BioLegend
PD-1	BV421	29F.1A12	1: 250	BioLegend
ROR- γ	PE	B2D	1: 250	Thermo Fisher
TNF	PE-Cy7, AF4800	MP6-XT22	1: 100	Thermo Fisher
Blocking				
anti-CCL2		4E2/MCP	10 µg / ml	BioLegend
Anti-CD178		MFL3	10 µg / ml	BioXcell
anti-CD1d		1B1	10 µg / ml	Thermo Fisher
anti-IFN-γ		H22	10 µg / ml	BioLegend
anti-IL-6		MP5-32C11	10 µg / ml	BioLegend

anti-LFA-1		M17 / 4	10 µg / ml	BioXcell
anti-P2RX7		W15180A	10 µg / ml	BioLegend
anti-TLR-2 (CD282)		QA16A01	10 µg / ml	BioLegend
anti-TNFR1		55R-593	10 µg / ml	BioLegend
anti-TNFR2		TR75-32.4	10 µg / ml	BioLegend
Activating				
anti-CD3		145-2C11	5 µg / ml	BioLegend
anti-CD28		37.51	5 µg / ml	Thermo Fisher

2.1.8 Cytokines

Surname	Species	Manufacturer
hIL-6	Mouse	R&D SYSTEMS, Minneapolis, USA
IFN-γ	Mouse	Miltenyi Biotech, Bergisch Gladbach, Germany
IL-12	Mouse	Miltenyi Biotech, Bergisch Gladbach, Germany
IL-15	Mouse	Peprotech, Hamburg, Germany
IL-15Rα/ IL-15	Mouse	Thermo Fisher Scientific, Waltham, USA
IL-1β	Human, Mouse	Peprotech, Hamburg, Germany
IL-2	Human, Mouse	Novartis, Basel, Switzerland
IL-4	Mouse	Peprotech, Hamburg, Germany
IL-6	Mouse	Miltenyi Biotech, Bergisch Gladbach, Germany
SAA[3]	Human	Peprotech, Hamburg, Germany
TGF-β	Human, Mouse	Peprotech, Hamburg, Germany
TNF	Mouse	Thermo Fisher Scientific, Waltham, USA

2.1.9 Primers

Gene	Species	Sequence (5´-> 3´)	Manufacturer
CCL2	mouse	fw: CCC AAT GAG TAG GCT GGA GA rev: AAA ATG GAT CCA CAC CTT GC	Eurofins

[3]SAA: Serum amyloid A1 protein

CXCL16	mouse	fw: AAA GAG TGT GGA ACT GGT CAT G rev: AGC TGG TGT GCT AGC TCC AG	Eurofins
IL-12p35	mouse	fw: AAA TGA AGC TCT GCA TCC TGC rev: TCA CCC TGT TGA TGG TCA CG	Eurofins
IL-15	mouse	fw: CAT TTT GGG CTG TGT CAG TGT rev: ACT GGG ATG AAA GTC ACT GTC AGT C	Eurofins
IL-1b	mouse	fw: GCA ACT GTT CCT GAA CTC AAC T rev: ACT TTT TGG GGT CCG TCA ACT	Eurofins
IL-21	mouse	fw: CGC CTC CTG ATT AGA CTT CG rev: TGG GTG TCC TTT TCT CAT ACG	Eurofins
SAA	mouse	fw: GCT TTG ATG GAC ACA CTA GCC rev: CTC AGG CCA GGA GTA GTT TCA A	Eurofins
TNF	mouse	fw: CGA TGG GTT GTA CCT TGT C rev: CGG ACT CCG CAA AGT CTA AG	Eurofins

2.1.10 Buffer

Surname	Content	Final conc.
ACK lysis buffer	H_2O	100%
	NH_4Cl	150 mM
	$KHCO_3$	10 mM
	Na_2EDTA	0.1 mM
FACS[4]-Buffer	PBS	100%
	FCS	1 %
	EDTA	20 mM
	NaN_3	0.01%
MACS[5]-Buffer	PBS	100%
	FCS	1 %
	EDTA	20 mM
PBS	H_2O	100%
	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	10 mM
	KH_2PO_4	1.8 mM
Hepatocyte Isolation		

[4]FACS: Flow cytometry staining
[5]MACS: Magnetic activated cell sorting

Buffer A (EGTA buffer)	Glucose solution (9.9 g / L)	5.7 g / L
	KH buffer	10% (v / v)
	HEPES buffer (pH 8.5)	10% (v / v)
	Amino acid solution	15% (v / v)
	L-glutamine (200 mM)	0.5 mm
	EGTA (125 mM)	0.5 mM
Buffer B (collagenase buffer)	Glucose solution (9.9 g / L)	5.7 g / L
	KH buffer	10% (v / v)
	HEPES buffer (pH 8.5)	10% (v / v)
	Amino acid solution	12% (v / v)
	$CaCl_2$ x 2 H_2O (130 mM)	2 mM
	$MgCl_2$ x 6 H_2O (130 mM)	3.5 mM
	L-glutamine (200 mM)	0.5 mM
	Collagenase NB 4G	0.46 mg / ml
Buffer C (suspension buffer)	Glucose solution (9.9 g / L)	5.7 g / L
	KH buffer	10% (v / v)
	HEPES buffer (pH 7.6)	10% (v / v)
	Amino acid solution	12% (v / v)
	$CaCl_2$ x 2 H_2O (130 mM)	1.1 mM
	$MgSO_4$ x 7 H_2O (100 mM)	0.41 mM
	BSA	0.17% (w / v)

2.1.11 Cell-culture media

Surname	Content	Final conc.
Hepatocyte medium	William's Medium E (without Glc, Gln)	100%
	L-glutamine	2 mM
	Glucose solution	0.5 g / L
	HEPES buffer (pH 7.4)	20 mM
	Penicillin / streptomycin (10,000 U / ml)	50 U / ml
	Gentamycin	89 µg / ml
	Hydrocortisone	5.6 µg / ml

	Insulin	23 IU / L
	DMSO	1.6%
LSECs[6] medium	DMEM	100 %
	Glucose solution	4.5 g / L
	FCS	10% (v / v)
	L-glutamine	2 mM (2%)
	Penicillin / streptomycin (10,000 U / ml)	100 U / ml (2%)
T-cell medium	RPMI 1640	100%
	FCS	10% (v / v)
	Penicillin / streptomycin (10,000 U / ml)	100 U / ml
	L-glutamine	2 mM
	β-Mercaptoethanol	0.1 mM

2.1.12 Kits

designation	purpose	Manufacturer
Bio-Plex Pro Mouse Cytokine 23-plex	Cytokine immunoassay	Bio-Rad GmbH, Munich, Germnay
Foxp3 / Transcription Factor Staining Buffer Set	Staining of nuclear proteins	Thermo Fisher Scientific, Waltham, USA
NucleoSpin® RNA XS	RNA isolation	Macherey-Nagel, Düren, Germany
SensiFAST® cDNA[7] Synthesis kit	cDNA synthesis	Bioline,
StayBrite® Highly Stable ATP Bioluminescence Assay Kit	ATP concentration	BioVision, Milpitas, USA

[6]LSECs: Liver sinusoidal endothelial cells
[7]cDNA: complementary DNA

2.2 Methods

2.2.1 Isolation of murine primary cells

Sterility of space, instruments and personnel was applied during harvesting of the animal tissues. For each experiment, mice of the same sex and 6 to 12 weeks age were sacrificed for isolation of different primary cells. Cervical dislocation was used for sacrificing the mice after isoflurane anesthesia. After horizontally cutting through the abdominal skin, animal body was soaked again with 70% Alcohol and repositioned to expose the targeted organs to be harvested via cutting through the abdominal muscles and releasing the organ from all other attachments.

2.2.1.1 Isolation of primary cells by magnetic separation (MACS)

Density gradient centrifugation protocols were used to prepare different cell suspensions from murine tissues. Primary cells were then resuspended in a defined volume of MACS buffer ($1x10^7$ cells in 40 µl[8] MACS buffer) and magnetically labelled with the recommended concentration of paramagnetic MACS particles (10 µl of Microbeads per 10^7 total cells), which bound with the related cell-surface antibody:
CD8a > For T-cell.
NKp46 > For NK cell.
CD19 > For B cell.
CD11c > For Dendritic cell.
CD146 > For LSECs.
Cell suspension was mixed and incubated for 15 minutes in the refrigerator (2-8°c). Unbound microbeads were removed by washing the cell suspension in MACS buffer (10 mins, 480g) and then filtered through a nylon sieve to get rid of possible aggregates. Cell suspension in 500 µl buffer/ 10^8 cells was finally prepared and the recommended Auto-MACS program (Possel, Posseld2, Deplete or Deplete05) were used to positively/negatively collect a single-cell suspension.

2.2.1.2 Isolation of primary cells by FACS[9]-based sorting

The FACS-based isolation of primary cells was carried out with the SH800 (Sony). A cell suspension was prepared and stained with fluorescein-conjugated antibodies for 15 minutes in dark in the refrigerator (2-8°c). The unbound antibodies were then

[8]µl: Microliters
[9]FACS: Fluorescence activated cell sorting

removed, and the cells of interest were purely isolated using gating strategies to exclusively collect the cells of interest.

2.2.1.3 Isolation of single cells suspension from the spleen

Murine spleen was isolated and placed in a tube with 5 ml ice-cold PBS[10]. To generate a cell suspension, the organ was mashed and passed through a metal sieve of 100 µm pore-size using the plunger of 5 ml syringe. The flow through was brought up to a final volume of 40ml using PBS and centrifuged (480g for 10 minutes). The cell-pellet was treated with ACK lysis for 2 minutes to remove erythrocytes, again brought up to a volume of 40 ml using PBS and passed through a nylon filter of 100 µm pore-size to discard cell aggregates. The flow through was then centrifuged using the same condition and the final cell pellet was resuspended in 10 ml of MACS buffer, cell numbers were determined and immunomagnetic separation was performed as recommended by the manufacturer (Miltenyi).

2.2.1.4 Isolation of single cells from mouse liver

A pump with a flow rate of 4 ml / min was used for perfusion by inserting the cannula into the portal vein while cutting the vena cava at the same time. Perfusion was performed until complete the blood was flushed out and the liver turned light brown in color. After removing of the gallbladder and transferring the liver into 5 ml ice-cold PBS, the organ was mashed and passed through a metal sieve of 100 µM pore-size using the plunger of 5 ml syringe. The flow through was made up to 40ml using PBS and spined down at 480g for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in 8 ml of a GBSS[11] + Collagenase IV (1:400) solution while shaking for 15 minutes at 37°c (240 rpm[12]) for digestion. Cell suspension was then made up to 40 ml with PBS and spined down at 480g for 10 minutes. A density gradient centrifugation was used to separate the lymphocytes from other cell components from the liver. For this, the cell pellet was resuspended in 3 ml of a 40% Percoll solution and carefully layered on 3 ml of an 80% Percoll solution in a 15 ml Falcon tube. The centrifugation took place at 1440 g (acceleration 7, brake 1) for 20 minutes at room temperature (RT). The layer with the lymphocytes between the two phases was removed, washed again with PBS and then the final cell pellet was

[10]PBS: Phosphate buffered saline
[11]GBSS: Gey's balanced salt solution
[12]rpm: rounds per minute

resuspended in 10 ml of MACS buffer to determine the cell-number before continuing with the positive selection isolation (MACS separation).

2.2.1.5 Isolation of pure NKT cells from mouse liver

We developed an optimized protocol including two steps of depletion and positive selection to isolate pure NKT cells from mouse liver.

Depletion (Step 1): For that, we prepared cell suspension of liver lymphocytes (3.1.1.4) that were centrifuged at 480g for 5 minutes and then resuspended in a defined volume of MACS buffer ($1x10^7$ cells in 40 µl MACS buffer) with 0.25 µg / 10^8 total cells of Biotin-labelled antibodies (anti-CD11c, anti-CD11b, anti-CD19, anti-NKp46, anti-F4/80). Cell suspension was mixed and incubated for 15 minutes at 2-8°C. Unbound antibodies were removed by washing the cell suspension with MACS buffer (5 mins, 480g). Cell pellet was again resuspended in a defined volume of MACS buffer ($1x10^7$ cells in 40 µl MACS buffer), magnetically labelled with the recommended concentration of anti-Biotin microbeads (10 µl of Microbeads per 10^7 total cells), mixed and then incubated for 15 minutes in the refrigerator (2-8°c). Unbound microbeads were removed by washing the cell suspension in MACS buffer (5 mins, 480g) and then filtered through a nylon sieve to get rid of possible aggregates. Cell suspension in 500 µl buffer/ 10^8 cells was finally prepared and the recommended Auto-MACS program (Deplete05) was used to deplete the undesired cells.

Positive Selection (Step 2): For that, the negative fraction was collected from step 1, brought up to a volume of 10 ml with MACS buffer and centrifuged down at 480g for 5 mins. Supernatant was removed and cells were then resuspended in a defined volume of MACS buffer ($1x10^7$ cells in 40 µl MACS buffer) with 0.25 µg / 10^8 total cells of Biotin-labelled antibody (anti-NK1.1). Cell suspension was well mixed and incubated for 15 minutes in the refrigerator (2-8°c). Unbound antibodies were removed by washing the cell suspension in MACS buffer (5 mins, 480g). Cell pellet was again resuspended in a defined volume of MACS buffer ($1x10^7$ cells in 40 µl MACS buffer), magnetically labelled with the recommended concentration of anti-Biotin microbeads (10 µl of Microbeads per 10^7 total cells), mixed and then incubated for 15 minutes at 2-8°C. Unbound microbeads were removed by washing the cell suspension in MACS buffer (5 mins, 480g) and then filtered through a nylon sieve to get rid of possible aggregates. Cell suspension in 500 µl buffer/ 10^8 cells was finally

prepared and the recommended Auto-MACS program (Posseld2, Miltenyi) was used to collect the positive fraction of pure-NKT cells.

2.2.1.6 Isolation of primary liver sinusoidal endothelial cells (LSECs)

A warm 1:80 (0.6 mg/ml) perfusion solution of collagenase type II was prepared in Ca2+ deprived buffer for a pump with a flow rate of 4 ml / min. After mouse sacrifice and preparation, liver perfusion was performed by inserting the pump-cannula in the portal vein while cutting the vena cava at the same time, allowing 4ml / liver of perfusion solution to digest the liver tissues. After removing of the gallbladder and transferring the liver into 5 ml of warm GBSS, the organ was placed in 100 mm Petri dish and the Glisson's capsule of the liver was gently scratched to release the LSECs until compete disruption of the whole liver. Cell suspension was then transferred using the second digestion solution (10ml GBSS + Collagenase II (1:100) to a 50 ml falcon tube and incubated for 15 minutes at 37°c with shaking (240 rpm) for digestion. Cell suspension was then passed through a metal sieve of 100 µM pore-size using the plunger of 5 ml syringe and finally made up to 40 ml using GBSS. After 10 mins centrifugation at 480g, cell pellet was resuspended into gradient solution (GBSS in 1:1.23 with 30% Nycodenz). Cell suspension was then transferred to a 15 ml falcon tube and centrifuged at 1400g (acceleration 7, brake 1) for 20 mins at room temperature (RT). The layer of LSECs was collected from the top of the gradient solution and washed for 2x using 40 ml of PBS at 480g for 10 mins. Cell pellet was then resuspended in a defined volume of MACS buffer ($1x10^7$ cells in 40 µl MACS buffer) and magnetically labelled with the recommended concentration of paramagnetic Microbeads bound to anti-CD146 antibody (10 µl of Microbeads per 10^7 total cells). Cell suspension was mixed and incubated for 15 minutes in the refrigerator (2-8°c). Unbound microbeads were removed by washing the cell suspension in MACS buffer (10 mins, 480g) and then filtered through a nylon sieve to get rid of possible aggregates. Cell suspension in 500 µl buffer/ 10^8 cells was finally prepared and the recommended Auto-MACS program (Possel) was used to collect the positive fraction of LSECs.

Isolated cells were seeded in Corning Cellbind plates in concentration of ($1,6 \times 10^5$/ 96 well, $0,8 \times 10^6$/24 well, $1,6 \times 10^6$ / 12 well) using LSECs media.

2.2.1.7 Isolation of primary mouse hepatocytes

To avoid coagulation, 150 µl of heparin (10000 U / 5 ml dissolved in a saline solution) were ip13 injected 15 minutes before the mouse was sacrificed. Next, mouse was anesthetized via ip injection of 150 µl ketamine (5% of the initial concentration) - Xylazine (20 mg / ml initial concentration) solution (ratio 3:1). After absence of the mouse reflexes, horizontal cuts through the abdominal skin /and muscle were applied and the liver was pumped with buffer A via the portal vein for 8 minutes together with cutting through the vena cava. Liver was then further perfused using 40 ml of buffer B using the same speed. The liver was then gently detached from the mouse together with removing the gallbladder and then placed in a petri dish contained 2 ml of buffer B. Hepatocytes were released by cutting through the Glisson's capsule using a curved scissor, the cell suspension was then passed through a metal sieve of 100 µM pore-size. Cell suspension was then made up to 50 ml with buffer C and spined down for 2 minutes at 50g. To get rid of the dead cells, density gradient was used by resuspending the cell pellet into 5 ml of Percoll/ PBS solution (1:10), overlayed on 5.5 ml of 80% Percoll solution. Gradient tube was centrifuged at 600g for 20 minutes (acceleration: 7, brake: 1) at RT. The viable hepatocytes were collected from the middle layer, made up to 40 ml with buffer C and centrifuged down for 2 minutes at 50g. The final cell pellet was then carefully resuspended in 5 ml of prewarmed hepatocyte medium to determine the cell number. Isolated cells were seeded at $1x10^4$ cells/well in 96 well-plates, which were pre-coated with a collagen solution (CollagenR® solution with H2O, 1:10) for 30 minutes.

2.2.1.8 Cell counting

The cell count was determined using the Neubauer counting chamber. For this, the cell suspension was mixed with a trypan blue solution in a ratio of 1:10 and 10 µl of it was pipetted into the counting chamber. Live cells that were not stained blue were counted in all four large corner squares. The number of cells resulted from the following formula:

Concentration = Number of Cells x 10,000 / Number of squares counted x dilution

[13]Ip: Intraperitoneally

2.2.2 In vitro culture

2.2.2.1 Cell culture

All cells were kept under the following conditions: 37°c., relative humidity 90%, CO_2 content 5%.

2.2.2.2 Cocultures of NKT cells with LSECs, DCs and Hepatocytes

For all the experiments studied different conditions of NKT cells response to immune-agonists, and their further immune crosstalk with other immune cells, experimental protocols were developed. As APCs[14], LSECs, DCs and hepatocytes were isolated according to the protocols mentioned before (3.1.1.6, 3.1.1.3, 3.1.1.7), respectively. APCs were then seeded in either Cell-bind or E-plates using the respective cell number and medium mentioned in the previous protocols. During 4 hours of NKT isolation process from liver-isolated lymphocytes (3.1.1.6), APCs were treated with different concentrations of agonists, specifically NKT specific agonist (α-GC). The isolated NKT cells were then loaded to respective culture using $4x10^4$ cells/well in the same medium used for every APC studied. Cocultures were kept under cell-culture conditions (3.1.2.1)

2.2.2.3 CD8+ T and NK cells culture in NKT-APCs primed in vitro culture

To investigate the further contribution of activated NKT cells in liver immunity, NK and CD8+ T-cells were isolated as previously mentioned (3.1.1.1, 3.1.1.3, 3.1.1.4). After 8 hours of NKT cells in vitro crosstalk with APCs (3.1.2.2), Isolated NK and CD8 T-cells were loaded separately as $4x10^4$ cells/well to the previous culture using the relative APCs culture-medium. Final cocultures were then kept under cell culture-conditions (3.1.2.1).

2.2.3 Immunological methods

2.2.3.1 Flow cytometry (FACS[15])

Flow cytometry was used as an essential method to characterize and monitor the different phenotypes, reactions and functions of the cells. By hydrodynamic mean, cells were passed through laser beams to allow sufficient excitation of fluoresceine

[14]APCs: Antigen presenting cells
[15]FACS: fluorescence activated cell sorting

bound to surface or intracellular markers. Emission from such fluorochromes was passed through different filters and finally collected via different detectors.

2.2.3.1.1 Surface staining

To determine the expression levels of antigens expressed at the cell surface, cells were transferred to 96-well microtiter plates with a V-bottom and spined down at (600 g, 2 min, 4°c.). After discarding the supernatant, cell pellet was resuspended in 150 µl of FACS buffer and spined down using the same condition. This washing step was repeated for 2 times before the cells were then resuspended in 50 µl of the staining solution (FACS buffer with recommended concentration of each fluorochrome-conjugated antibody). Cell suspension was mixed and kept in dark for 30 minutes at 2-8°C. The unbound antibodies were then washed for two times using 150 µl of FACS buffer. After last centrifugation, cell pellet was either resuspended in 150 µl of FACS buffer for direct analysis by flow cytometer or fixed by fixation buffer for intracellular protein staining.

2.2.3.1.2 Intracellular staining

For the intracellular staining of cytokines, protein transport between endoplasmic reticulum and Golgi apparatus was blocked by 1:1000 of Brefeldin (1000x) + Monensin (1000x) for 6 hours before collection of cells from the cells-culture for surface staining. After the surface staining, cells were fixed with 100 µl of the "IC Fixation Buffer" (Thermo Fisher) for 1 hour in dark in the refrigerator (2-8°c). For the staining of nuclear components, cell suspension was centrifuged using the same condition before and then resuspended into 100 µl of fixation/permeabilization solution from the "Foxp3[16]/ transcriptions factor staining kit "(Thermo Fisher) in dark in the refrigerator (2-8°c). After centrifugation, cells were resuspended into 50 µl of the intracellular staining solution (Fixation/permeabilization buffer from Foxp3 kit + the recommended concentrations of each fluorochrome-conjugated antibody). Cell suspension was mixed and kept in dark for at least 1 hours or overnight in the refrigerator (2-8°c). The cells were washed for 2 times with 150 µl of FACS buffer to discard the unbound antibodies and the cell pellet was finally resuspended into 50 µl of FACS buffer to be analyzed by the flow cytometer.

[16]Foxp3: forkhead box protein P3

2.2.3.2 Enzyme-linked Immunosorbent Assay (ELISA)

For the detection of cytokines and chemokines in cell culture supernatants of LSECs /or Hepatocyte /or Dendritic cells with NKT cells in presence of NKT cell-glycolipid agonist (α-GC), antibodies for the respective antigen were dissolved in PBS (1 µg /ml). 100 µl of this solution were distributed into each well of an uncoated 96-well microtiter plate as a coating antibody solution and incubated overnight in the refrigerator (2-8°c). The next day, unbound antibodies were removed by washing the plate three times with a device that washes 96-well microtiter plates (BioPlex Pro II) automatically. Unspecific binding was blocked by pipetting 100 µl/well of the blocking solution (1% BSA[17] in PBS) and then incubated for one hour at RT. A standard solution of the different cytokines or chemokines to be investigated was prepared using 100 ng /ml (0.05% Tween-20, 0.1% BSA in PBS) as the highest concentration and diluted at constant intervals. After three times of plate washing, 100 µl /well of the standard and the samples were incubated overnight in the refrigerator (2-8°c). After incubation, the plate was washed for three times like before and then 100 µl/well of biotinylated antibodies (0.5 µg /ml), which are directed against the corresponding antigen to be detected, were pipetted into each well and incubated for two hours in the refrigerator (2-8°c). Unbound biotinylated antibodies were then removed by washing for 3 times and 100 µl/well of an avidin-HRP[18] Load (horseradish peroxidase) conjugate were added and stored for 1 hour in the refrigerator (2-8°c). After the plate was washed three times, 100 µl/well of a substrate solution (TMB[19]) were added and incubated at room temperature until a clear color change to blue became apparent. Finally, the enzymatic reaction was stopped with a sulfuric acid solution (1.8%) and the absorbance was detected at 450 nm wavelength.

2.2.3.3 Bio-Plex Pro™ cytokine, chemokine and growth factor assay

As a big advantage over the other immune-assays, Bio-Plex was used to measure multiple cytokines, chemokines and growth factors in tissue culture supernatants. For that, Bio-Plex Pro Mouse Cytokine 23 kits was used, and the tissue culture supernatants were collected and kept at -20°c according to the guidelines of Bio-Rad GmbH, Germany. All reagents were warmed up to room temperature before starting

[17]BSA: Bovine serum albumin
[18]HRP: Horse-radish peroxidase
[19]TMB: 3,3 ', 5,5'-tetramethylbenzidine

the assay and at the same time the washing buffer of 1x dilution was prepared in dist. H_2O. The standards of the different analytes were reconstituted using the calibrating pipets provided and then agitated for at least for 15 minutes without vortex. Microparticles were also prepared by spinning the vials for 30 seconds at 1000g followed by gentle vortex to redisperse the particles. Using the calibrated diluent, the microparticles and standard cocktails were diluted for 1x dilution, while the biotin-antibody and streptavidin-PE were diluted to 1x using washing buffer. Cell culture supernatants were then centrifuged at 16000g for 4 minutes to get rid of possible cell-debris. Loading of the assay plate was started by adding 50 µl/well of each sample, standards dilutions and blank and then 50 µl/well of pre-vortexed microparticles were added. The plate was securely covered using a foil plate-sealer and incubated for 2 hours on a shaker at room temperature. After incubation, the plate was kept on a magnetic device for one minute to collect the microparticles before the plate wash washed for 3 times using 100 µl/well of washing buffer. 50 µl/well of Biotin antibody cocktail were then added and the sealed plate was incubated on a shaker for one hour at room temperature. After 3 times washing by washing buffer, 50 µl/well of streptavidin-PE/well were added and the plate was again incubated 30 minutes on a shaker at room temperature. After the incubation, the plate was washed by 100 µl/well of washing buffer for 3 times and the microparticles were then resuspended in 100 µl/well of washing buffer, incubated for 2 minutes on a shaker and finally measured using Bio-RAD Bio-Plex 200 system.

2.2.3.4 Molecular biological methods

2.2.3.4.1 RNA isolation and quantitative real-time PCR (qPCR)

The RNA from different cell-types including LSECs, hepatocytes, DCs and NKT cells were isolated using the NucleoSpin RNA XS Kit (Macherey Nagel) according to the manufacturer's protocol. The concentration and purity of the RNA was determined using the nanophotometer (Implen). Transcription of RNA into cDNA was carried out with using SensiFASTTM cDNA synthesis kit (Bioline) according to the manufacturer's protocol. For the qPCR[20], cDNA samples were loaded on a 384-well microtiter plate together with 2x Takyon Mix SYBR Assay (Eurogentec) and the corresponding primers (Eurofins) and measured with the LightCycler 480 (Roche).

[20]qPCR: quantitative polymerase chain reaction

2.2.3.5 Metabolic Methods

2.2.3.5.1 Analysis of ATP[21] release by LSECs and DCs using Bio-luminescence assay

To assess the immune response of LSECs and DCs to immune challenging agonist, released ATP was quantitatively measured in the supernatant of cell cultures based on the Luciferin-Luciferase test. For that, standard reaction solution, ATP-standard curve and supernatant from the cell cultures were prepared and kept in dark according to ATP Determination Kit, Invitrogen, USA guidelines. In 96 well plate, 90 µl/well of the standard reaction solution were added and 10 µl/well of standard curve dilution and sample supernatant were. Finally, luminescence was read using Infinite M1 1000 PRO reader.

2.2.3.5.2 Analysis of Ca^{2+} influx in activated T-cells using calcium sensing dye

To determine the increase or decrease in intracellular free calcium concentrations in the activated CD8+ T-cells, pre-cultured T-cells with LSECs were collected and transferred to V bottom 96 well-plate. Using the same protocol for surface staining (3.1.2.1.1), eFlour™ 514 Calcium Sensor Dye was added to the staining solution and kept in dark in the refrigerator (2-8°c) for 30 minutes. After incubation, unbound antibodies and free calcium dye were washed, while esterases cleaved the dye take up by Ca^{2+} rich-cells, yielding a membrane-impermeable dye. The fluoresceine was finally detected at 520nm using SA3800 flow cytometer (SONY).

2.2.3.6 Functional Analysis

2.2.3.6.1 Cytotoxicity Assay

2.2.3.6.1.1 Live Cell Analysis System xCELLigence (Real-Time Cell Analyzer)

In this assay, the biological processes were assessed using electronic sensor to measure the changes in the electronic impedance. Presence, absence or change in the properties of attached cells was measured by the passage of the electrons and ions on sensor surfaces. Live cells which were efficiently adhering to the electronic plate were acting as a resistance to the electrons passage on the surface, while de-attachment of the adherent cells in response to biological changes/death recapitulate the normal flow of the electrons through the plate. A longitudinal dynamic measurement of the adherence was recorded as impedance factor which was then

[21]ATP: Adenosine triphosphate

translated to reflect a percentage of cell-viability. To determine the viability of LSECs / hepatocytes co-cultured with NKT cells in presence of a cognate NKT cell agonist (α-GC), LSECs / Hepatocytes real-time viability was monitored using the RTCA SP instrument. For that, E-96 well plate was coated by collagen solution (Collagen R® solution with H_2O, 1:10) for 30 minutes. Before seeding of the cells, 100 µl of corresponding medium was added for each well and the impedance background was measured and calculated by the software. After that, isolated LSECs / Hepatocytes were seeded as 1,6 x 10^5 or 1 x 10^4 /well, respectively into the E-plate. A real-time detection of the cell-viability was measured and the influence of activated NKT cells on the viability of LSECs / Hepatocytes was detected every 15 minutes for 96 hours at 37°c.

2.2.3.7 Statistical Analysis

Statistics were calculated using the GraphPad Prism software (GraphPad Inc., CA). Statistical differences were analyzed using One-way ANOVA, Two-ways ANOVA with Tukey's multiple comparison correction, Kruskal Wallis test with Dunn's multiple comparison correction, Mann Whitney test and unpaired or paired t test.

3. RESULTS

3.1 Expression of CD1 by different cell populations in the liver

At the beginning, we tried to find out how likely the lipid antigens are to be presented by hepatic cells via CD1d molecule. For that, murine liver sinusoidal endothelial cells (LSECs), liver resident dendritic cells (DCs) and hepatocytes were isolated using gradient centrifugation and immunomagnetic cell isolation protocols. By using flow cytometry, different gating strategies and cell-specific markers, we analysed LSECs, DCs and Hepatocytes (Figures 7a-7c).

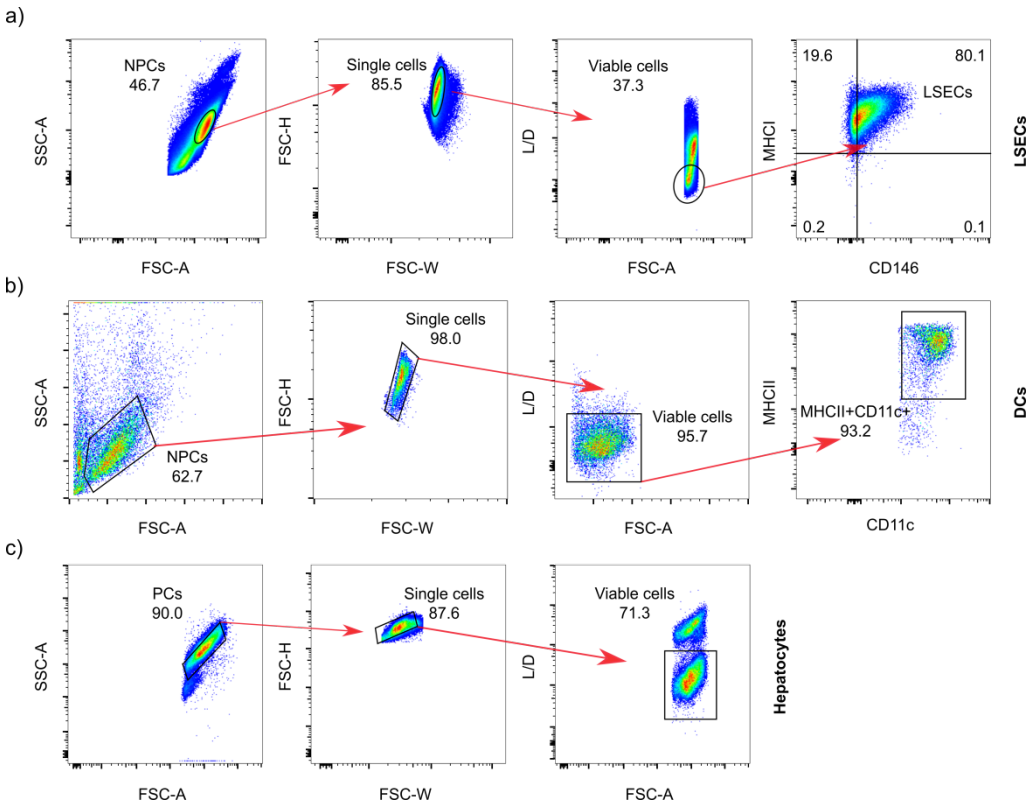

Figure 7. Gating strategies used by flow cytometry to identify LSECs, DCs and Hepatocytes
(a-c) After isolation, cells were stained with fluorescence-labelled monoclonal antibodies and representative flow cytometry plots are shown with the gating strategies for identification of (a) LSECs, (b) DCs and (c) hepatocytes. Each panel is representative of at least three independent experiments.

Later, the percentages of CD1d expression cells and the mean fluorescence intensity (MFI) were determined. We observed high expression levels of CD1d, with a more than 85% expression on all three cell-populations investigated (Figures 8a and 8b).

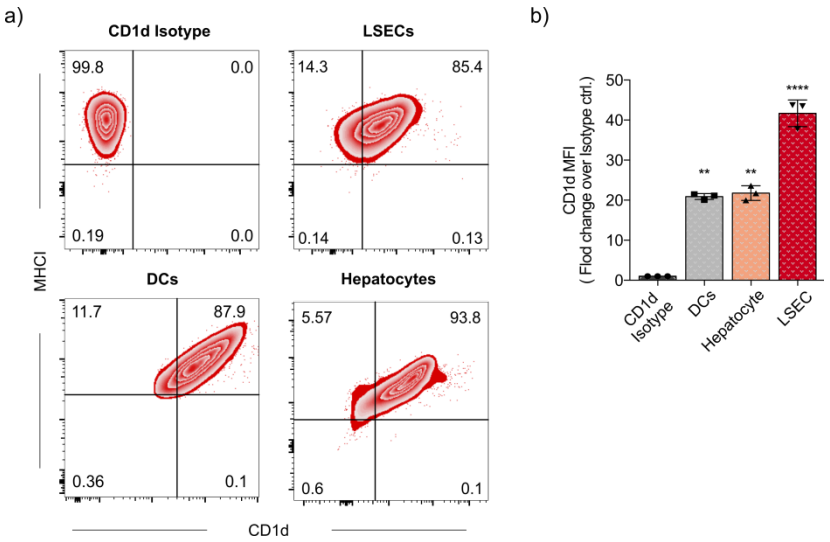

Figure 8. Expression levels of MHC-I (H-2K^b) and CD1d on different liver cell populations
*(a) Representative images from flow cytometry analysis showing dot plots demonstrating CD1d and H-2K^b expression by LSECs, DCs and hepatocytes compared to staining with an isotype-specific control antibody. (b) Mean fluorescence intensity (MFI) values quantified using Flow Jo software, reflecting fold changes in CD1d expression over the isotype control. Error bars represent mean ± SD of triplicate samples. **p < 0.003, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three independent experiments.*

These results suggested that all liver cell populations investigated here had the capacity to interact in a cognate fashion with natural killer T-cells (NKT cells) that recognize their antigens in the context of CD1d.

3.2 Proportion and vitality of NKT cell in liver circulating immune cells

NKT are particularly abundant in the liver and are localized within the liver sinusoids where they can interact with LSECs and DCs as well as with hepatocytes. We first established a protocol to isolate NKT cells from the liver. Using the gating strategy introduced in (Figure 9a), for analysing liver associated lymphocytes obtained by density gradient centrifugation, we detected a population of NKT cells accounting for 11% of total liver lymphocytes. This population could be further separated into three distinct subsets of NKT cells being CD4+, CD8+ or double negative (DN) (Figure 9b). We used immunomagnetic cell sorting to deplete NK cells, B cells and monocytes from liver-associated lymphocytes, which was then followed by a positive selection of NKT cells. Using this protocol, we were able to isolate hepatic NKT cells with more than 91% purity (Figure 9c).

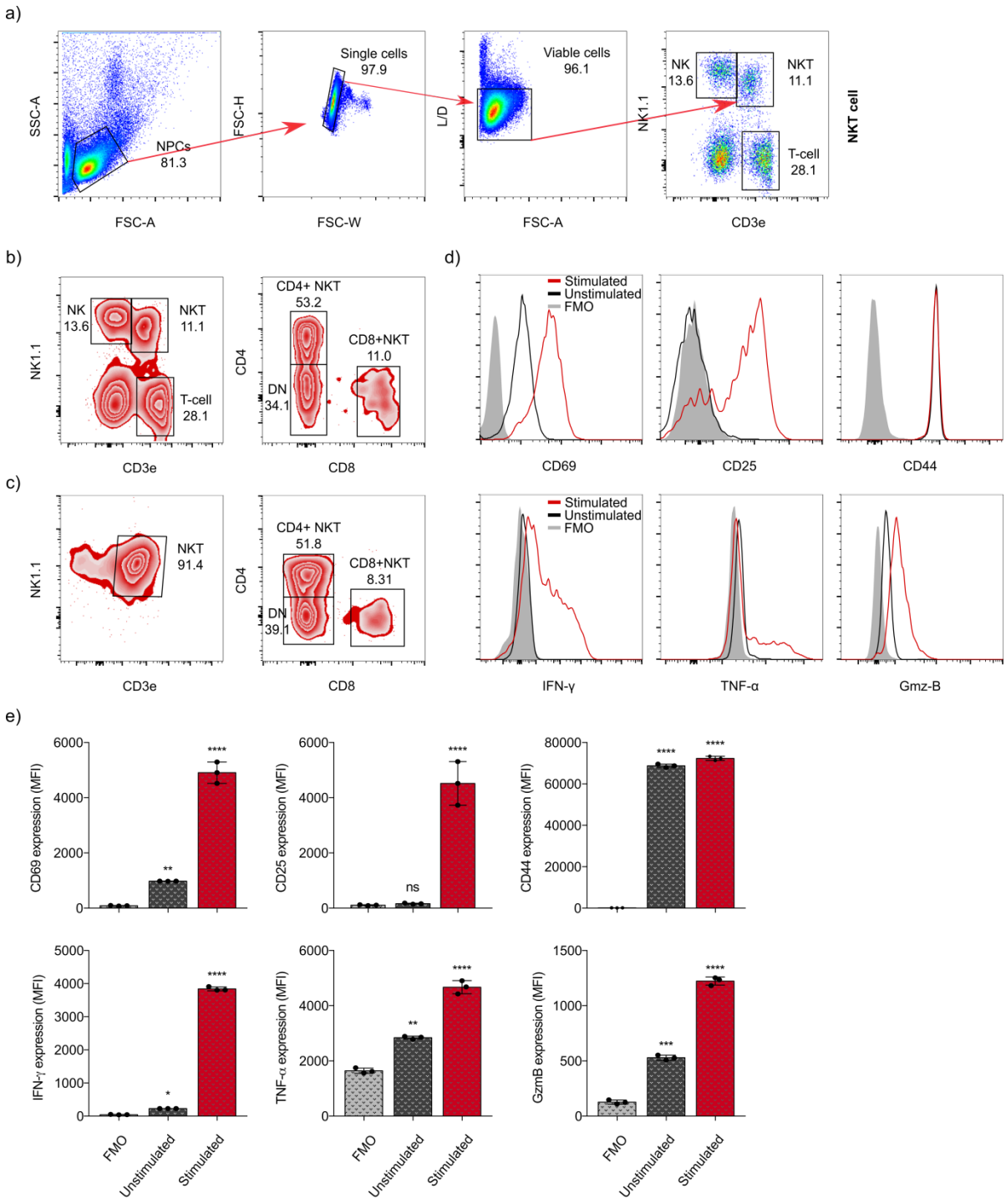

Figure 9. Isolation of NKT cells from the liver and characterization of their function after stimulation
*(a) Representative flow cytometry plots demonstrating the gating strategy for identification of NKT cell in the liver. (b) Liver-associated lymphocytes were isolated by density gradient centrifugation, were analysed by flow cytometry for expression of CD4+, CD8+ and double negative (DN). (c) Liver NKT cells were isolated using in-house developed protocol, phenotype and purity were identified using the demonstrated flow cytometry plots. (d and e) NKT cells were stimulated with CD3/CD28 micro-beads for 10 hours in the absence or presence of Brefeldin A. Surface activation markers and intracellular cytokines expression were analysed by flow cytometry, demonstrated as (d) Histogram and (e) MFI. Error bars represent mean ± SD of triplicate samples. ns < 0.98, *p < 0.029, **p < 0.0051, ***p < 0.0010, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

To investigate the response to stimulation, 40.000 of CD4, CD8 and DN NKT cells were incubated for 5 hours in a 96 well-plate coated with anti-CD3/CD28 antibodies. Using flow cytometry, we detected a significant up-regulation of the expression of the activation markers CD69 and CD25. Furthermore, we found a significant increase in IFN-γ, TNF-α and GzmB expression after stimulation (Figures 9d and 9e). These results demonstrated that NKT cells can be isolated to a high purity from the liver and that the cells remained responsive to stimulation.

3.3 NKT cell response to lipid antigens presented by LSECs and DCs

Building on the finding of constitutive expression of CD1d by LSECs, we compared the efficacy of LSECs and DCs in presenting α-Galactosyl Ceramide (α-GC) as a known CD1d-restricted lipid antigen to NKT cells, that are known to have a restricted TCR repertoire and respond to α-GC stimulation. For that, mouse LSECs were seeded in a cell-bind 96 well-plate for 48 hours, where the confluence and purity of LSECs were detected using light microscopy (Figure 10a) and flow cytometry (Figure 10b). LSECs and DCs were pulsed with 30 ng/ml of α-GC, washed to remove free α-GC and 40.000 NKT cells/well were added for 10 hours co-culture period. Using ELISA, we detected significantly higher expression and secretion of IFN-γ by NKT cells after incubation with α-GC-exposed LSECs compared to α-GC exposed DCs (Figure 10c). Moreover, flow cytometric analysis of NKT cell activation markers showed significantly higher expression of CD69, CD25 and CD44 after interaction of liver NKT cells with α-GC-exposed LSECs compared to α-GC-exposed DCs (Figures 10c and 10d).

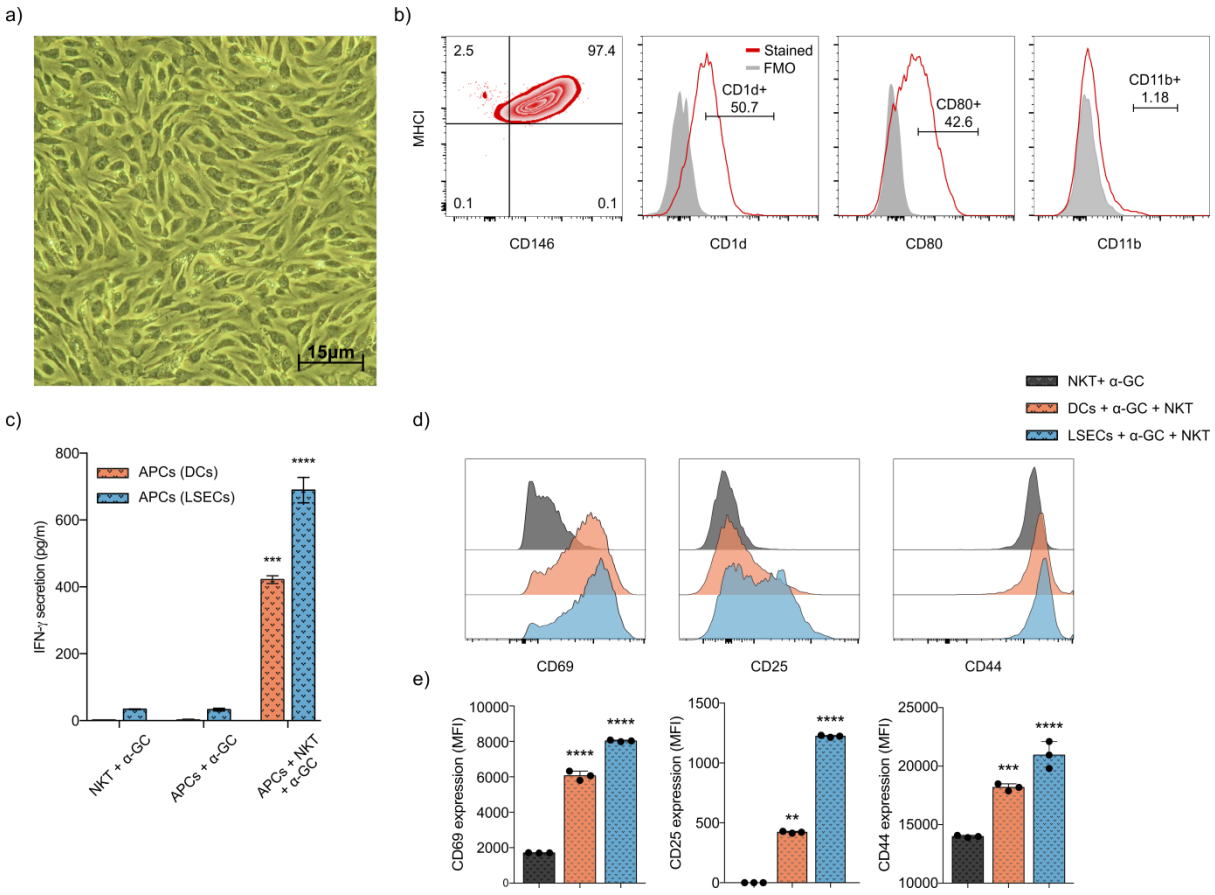

Figure 10. Stronger response of liver NKT cells to lipid antigens presented by LSECs compared to DCs

*(a) 10x microscopic images of mouse LSECs seeded in a cell-bind 96-well plate for 48 hours. (b) Seeded LSECs were detached with accutase treatment for 10 minutes and the phenotype of cells was determined by labeling with fluorescently labeled antibodies and detected by flow cytometry. (c and d) LSECs and DCs were co-cultured for 10 hours with 30 ng/ml of α-GC in presence or absence of NKT cells. (c) IFN-γ release from activated NKT cells was quantified by ELISA. (d, e) CD69, CD25 and CD44 surface expression by NKT cell was determined by flow cytometry and are shown by dot plots and mean fluorescence intensity (MFI) values. Error bars represent mean ± SD of triplicate samples. **p < 0.0029, ***p < 0.0006, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

To identify how efficient LSECs or DCs can present α-GC in the context of CD1d to NKT cells, LSECs and DCs were pulsed with a dose-kinetic of 0.03 to 2.7 µg/ml α-GC, washed and co-incubated for 10 hours with 40.000 NKT cells. By using ELISA, we detected a concentration-dependent increase of IFN-γ secretion by NKT cell upon exposure to increasing doses of α-GC. Importantly, the magnitude of NKT cell activation by LSECs presenting α-GC on CD1d was significantly higher compared to DCs (Figure 11).

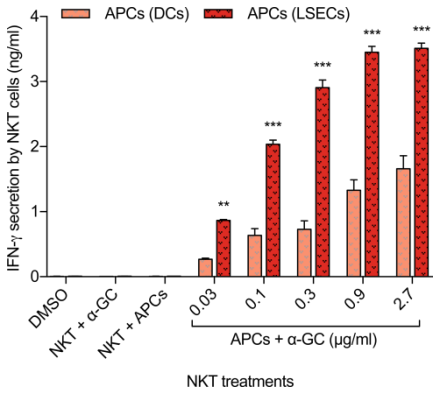

Figure 11. α-GC titration reveals higher antigen-presentation capacity of LSECs compared to DCS for NKT cells

*LSECs and DCs were cultured for 10 hours with the indicated doses of α-GC with or without NKT cells. IFN-γ release was quantified by ELISA reflecting the activation-magnitude of NKT cells to α-GC. Error bars represent mean ± SD of triplicate samples. **p < 0.003, ***p < 0.0008, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

3.4 LSEC collaborate with NKT cells to boost adaptive and innate immune response against infections

Having established the ability of LSEC to antigen-specifically activate NKT cells via lipid antigens presented in the context of CD1d, we were interested to explore how such interaction might influence CD8+ T-cell and NK cell activation as part of local adaptive and innate immune responses in the liver. For that, we used our established system to co-culture 40.000 NKT cells with LSECs and DCs, in presence of α-GC, and then added 40.000 CD8+ T-cells or NK cells (see illustrations 1a and 1b).

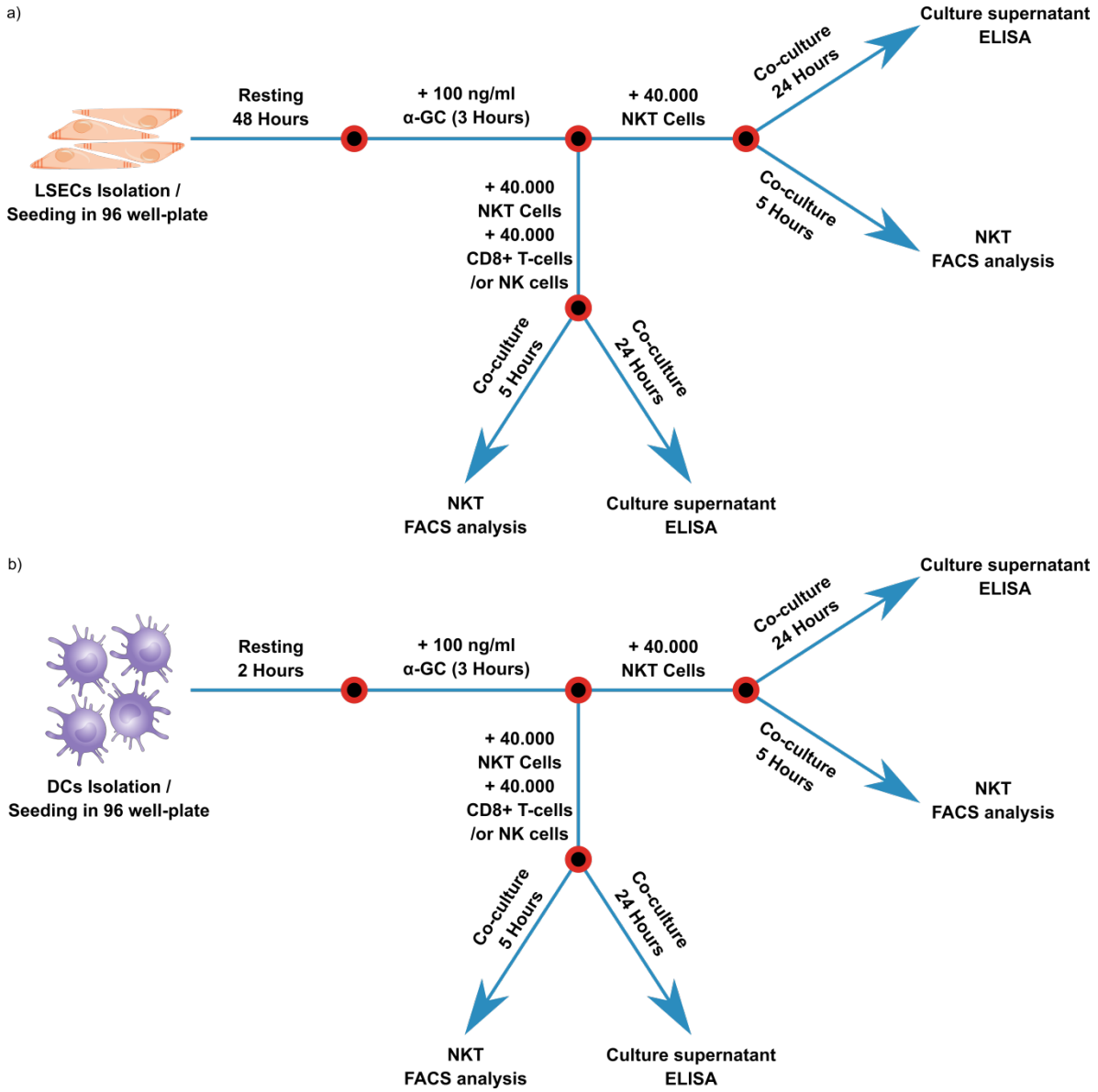

Illustration 1. Schematic representation of the experimental outline to study LSEC-NKT cognate crosstalk and its effect on adaptive and innate immune responses
(a) LSECs and (b) DCs were isolated according to the mentioned protocols. The experimental timeline, samples collection and data analysis were done according to the representative illustration.

We used ELISA to quantify IFN-γ concentrations in cell culture supernatants, while flow cytometry was used to identify the phenotypic changes of NKT cells (Figure 12).

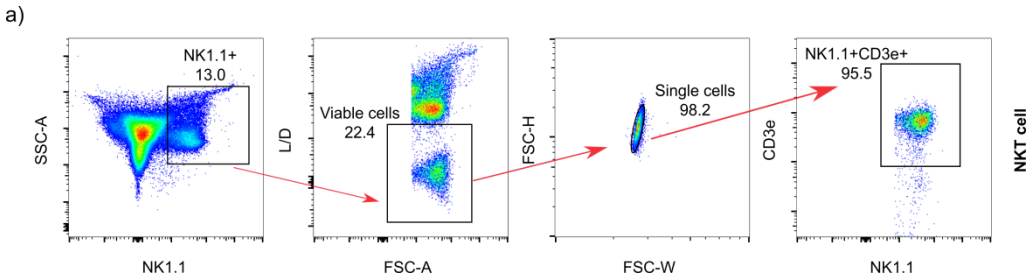

Figure 12. Gating strategies used by flow cytometer to identify NKT cells

NKT cells cultured in vitro in presence or absence of CD8 T-cell or NK cell were collected after 10 hours of co-culture and the representative flow cytometry plots demonstrating the gating strategy for identification of NKT.

We observed a significant increase in IFN-γ secretion within LSECs-NKT co-culture in presence of CD8+ T-cells (Figure 13a). In contrast, there was no significant change in IFN-γ concentration in presence or absence of CD8+ T-cells within DCs-NKT co-culture (Figure 13b).

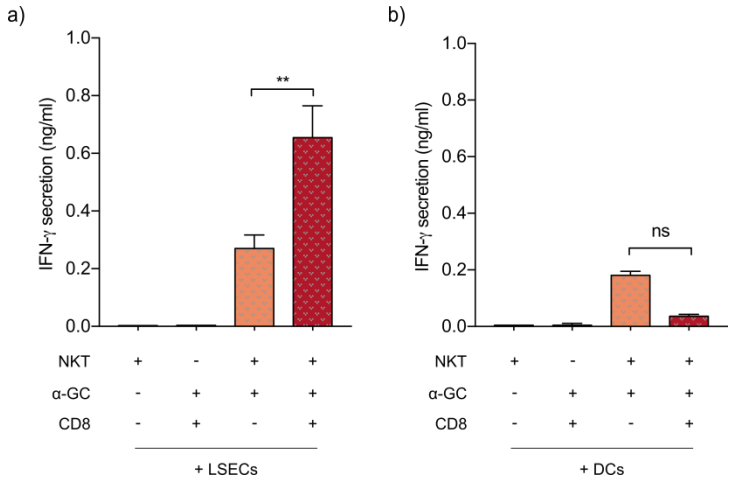

Figure 13. LSEC-induced NKT activation triggers increase in IFN-γ production in presence of CD8 T-cell

*(a and b) α-GC pulsed-LSEC or DCs, were co-cultured with NKT cells for 10 hours before addition of CD8+ T-cells for 24 hours. IFN-γ release into the cell culture supernatant was quantified by ELISA. Error bars represent mean ± SD of triplicate samples. ns < 0.043, **p < 0.0011, as determined by one-way ANOVA. Each panel is representative of at least three independent experiments.*

Furthermore, flow cytometry analysis of NKT cells after activation with α-GC pulsed-LSEC did not show an increase in CD69, IFN-γ, TNF-α and GzmB expression after co-culture with CD8 T cells (Figures 14a and 14b). These findings suggested that the activation of NKT cells through LSECs supported a polyclonal activation of CD8+ T-cells.

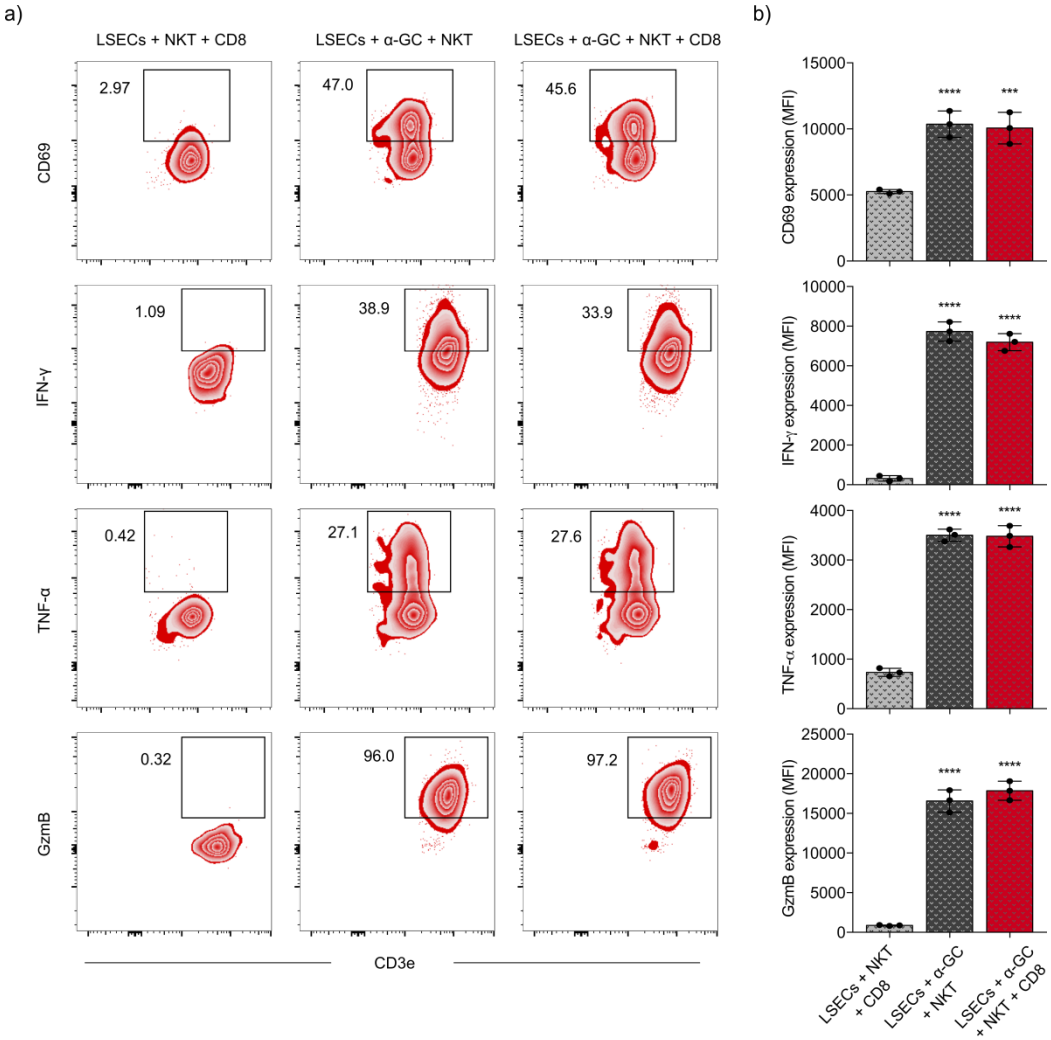

Figure 14. LSEC-induced NKT activation leads to polyclonal activation of CD8+ T-cells
*(a and b) Phenotype of activated NKT cell in presence or absence of CD8 T-cell was quantified by detection of activation markers and intracellular analysis of NKT activation and showed as FACS plots (a) and MFI values (b). Error bars represent mean ± SD of triplicate samples. ***p < 0.0008, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three independent experiments.*

Similarly, we used ELISA and flow cytometry to identify whether LSEC-induced NKT cell activation similarly increased effector cytokine production in the presence of NK cells. Again, we co-cultured α-GC-pulsed LSEC and NKT cells for 10 hrs before adding NK cells. We detected a significant increase in IFN-γ secretion after addition of NK cells (Figure 15a). Strikingly, when α-GC-pulsed DCs were co-cultured with NKT cells, addition of NK cells did not further increase IFN-γ expression (Figure 15b).

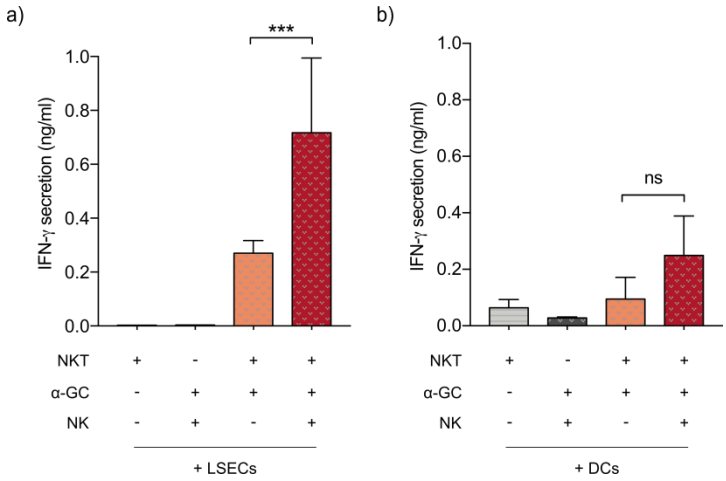

Figure 15. LSEC-induced NKT activation increases IFN-γ production in presence of NK cell
*(a and b) α-GC primed-LSEC and DCs, were co-cultured with NKT cells for 10 hours before addition of NK cells for 24 hours. IFN-γ release was quantified by ELISA. Error bars represent mean ± SD of triplicate samples. ns < 0.1795, ***p < 0.0002, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

Furthermore, we analysed the activation potential of NKT cells in presence or absence of NK cells using flow cytometry. We found no insignificant increase of CD69, IFN-γ, TNF-α and GzmB expression in NKT cells (Figures 16a and 16b), suggesting a polyclonal activation of NK cells after NKT cells were activated by α-GC-pulsed LSECs.

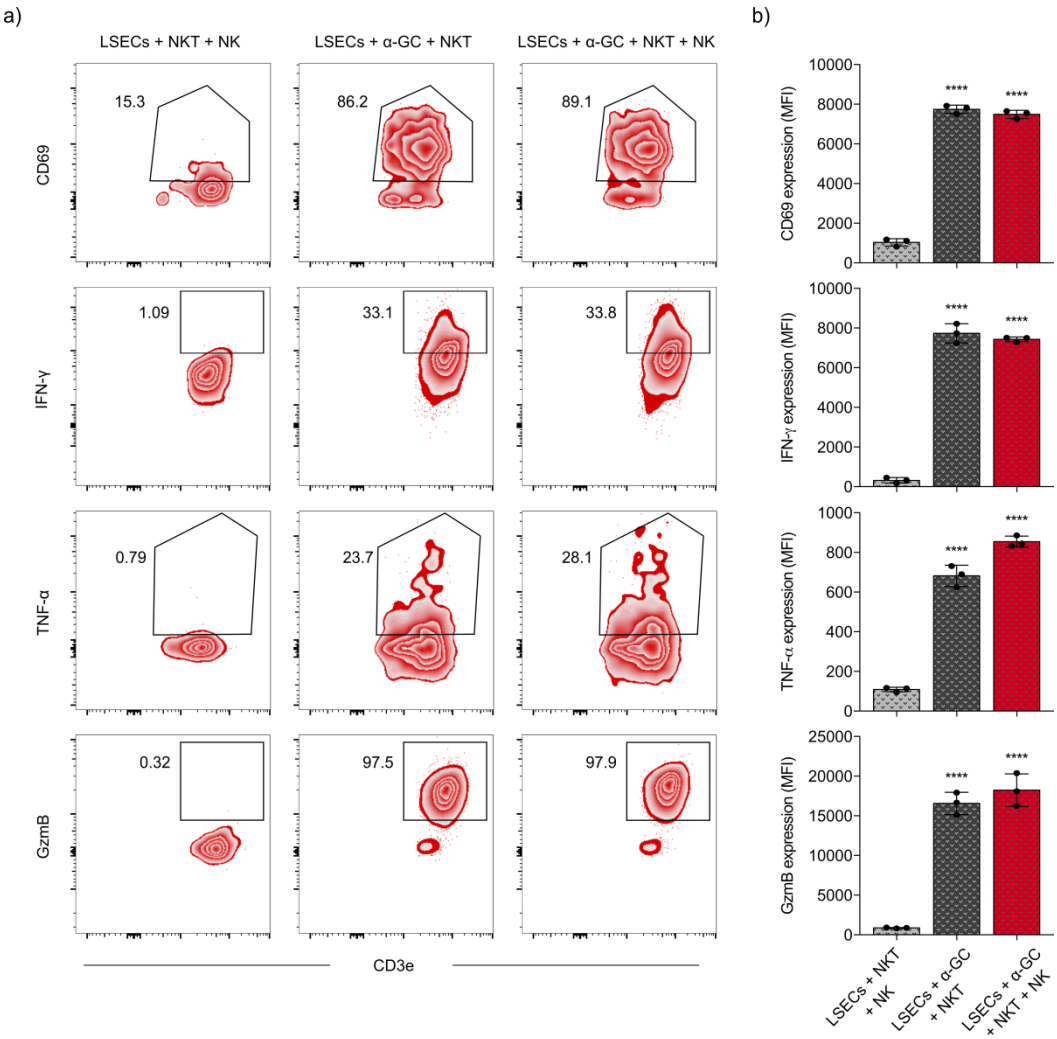

Figure 16. LSEC-mediated CD1d-restricted NKT cell activation evokes subsequent NK cell activation
*(a and b) Phenotype of activated NKT cell in presence or absence of NK cells was determined by measuring the expression of activation markers and intracellular cytokines. Error bars represent mean ± SD of triplicate samples. ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

From these results, we inferred that recognition of lipid antigens presented by LSECs on CD1d molecules led to NKT cell activation, and in turn caused a polyclonal activation of CD8 T cells as well as NK cells.

3.5 Characteristics of LSECs-induced NKT cell activation and concomitant CD8 T cell or NK cell activation

Next, we aimed to investigate in more detail if CD8+ T-cells and NK cells were activated following LSEC-induced NKT cell activation. For that, we used our established system to co-culture 40.000 NKT cells with LSECs or DCs after pulsing with a defined concentration (100 ng/ml) of α-GC, and then followed by addition of 40.000 CD8+ T-cells or NK cells. Using flow cytometry, the activation of NKT cells,

CD8+ T-cells and NK cells was determined by quantifying activation markers on the different cell populations in a time kinetic fashion over a period of 20 hours (see illustrations 2a and 2b).

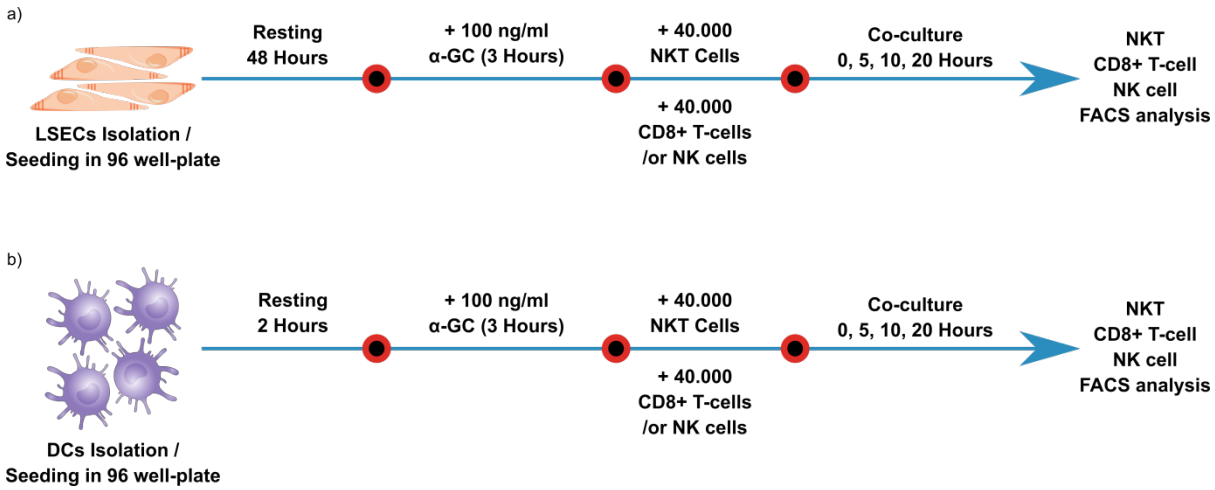

Illustration 2. Schematic representation of the study addressed the concatenation and strength of NKT, CD8+ T-cell and NK cell activation is response for lipid antigen presentation by LSECs
(a) LSECs and (b) DCs were isolated according to the mentioned protocols. The experimental timeline, samples collection and data analysis were done according to the representative illustration.

For the flow cytometry analysis, previously showed gating strategy was used for NKT cell, while the gating strategy in (Figure 17) was used to identify the co-cultured CD8+ T-cells.

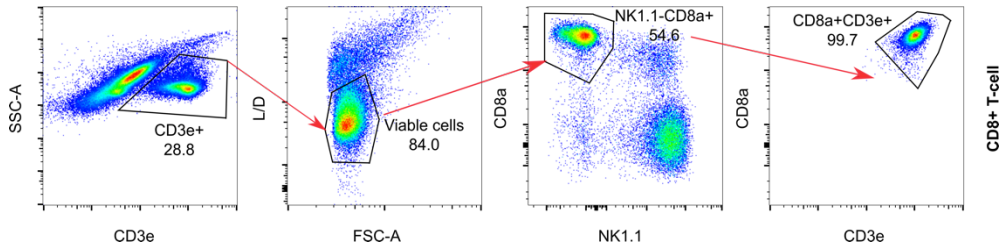

Figure 17. Gating strategies used by flow cytometer to selectively identify CD8+ T-cells
Cultured cells were collected after 10 hours of co-culture and the representative flow cytometry plots demonstrating the gating strategy for identification of CD8+ T-cells.

After 5 hours co-culture, NKT cell already showed an increase in intracellular expression of INF-γ and increased GzmB levels, while CD8+ T-cells only started to express INF-γ and increased GzmB levels at 10 hrs (Figures 18a and 18b).

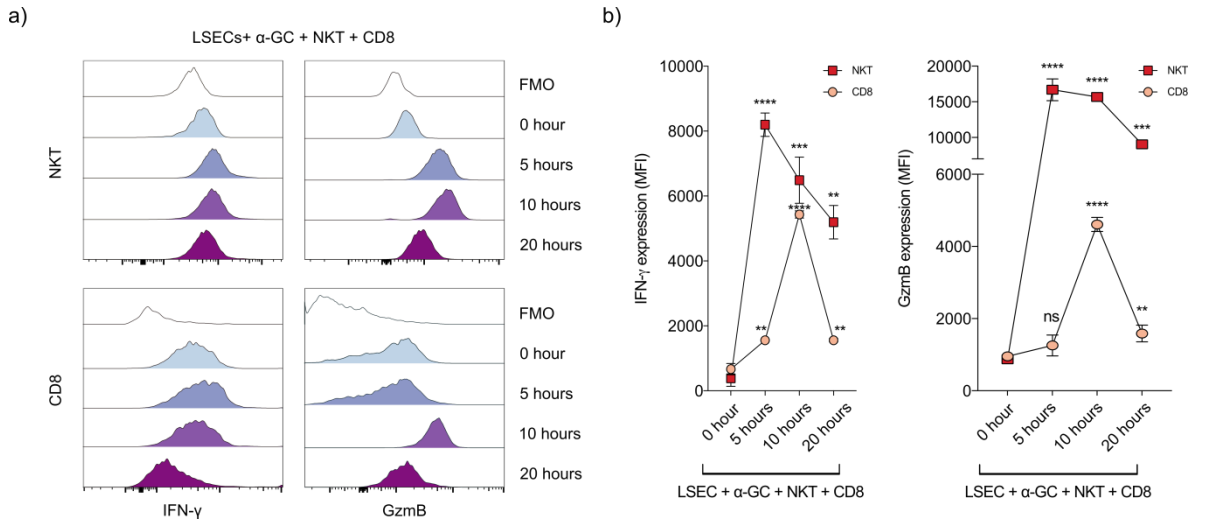

Figure 18. Distinct time kinetics of NKT cell and CD8 T cell activation in co-culture with α-GC pulsed LSECs

*(a and b) α-GC primed-LSECs were cultured with NKT cells in presence of CD8+ T-cells for the indicated time course to monitor cell activation. (a) Flow cytometry histogram plots and (b) MFI values at the different time points for intracellular INF-γ expression or GzmB expression in NKT and CD8+ T-cells. Error bars represent mean ± SD of triplicate samples. ns < 0.32, **p < 0.0033, ***p < 0.0002, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

Therefore, we characterized the phenotype of CD8 T cells by flow cytometry at 10 hrs after co-culture in more detail. We observed a significant increase in CD69 and TNF-α in addition to IFN-γ and GzmB in CD8+ T-cells co-cultured with α-GC-pulsed LSECs and NKT cells (Figures 19a and 19b), whereas in consistence with our previous results, we did not detect any activation of CD8+ T-cells after co-culture with α-GC-pulsed DCs and NKT cells (Figures 19c and 19d). These results suggested that upon first CD1d-restricted activation of NKT cells by LSECs there was a second phase of CD8 T cell activation, which occurred only in the presence of LSECs but not DCs.

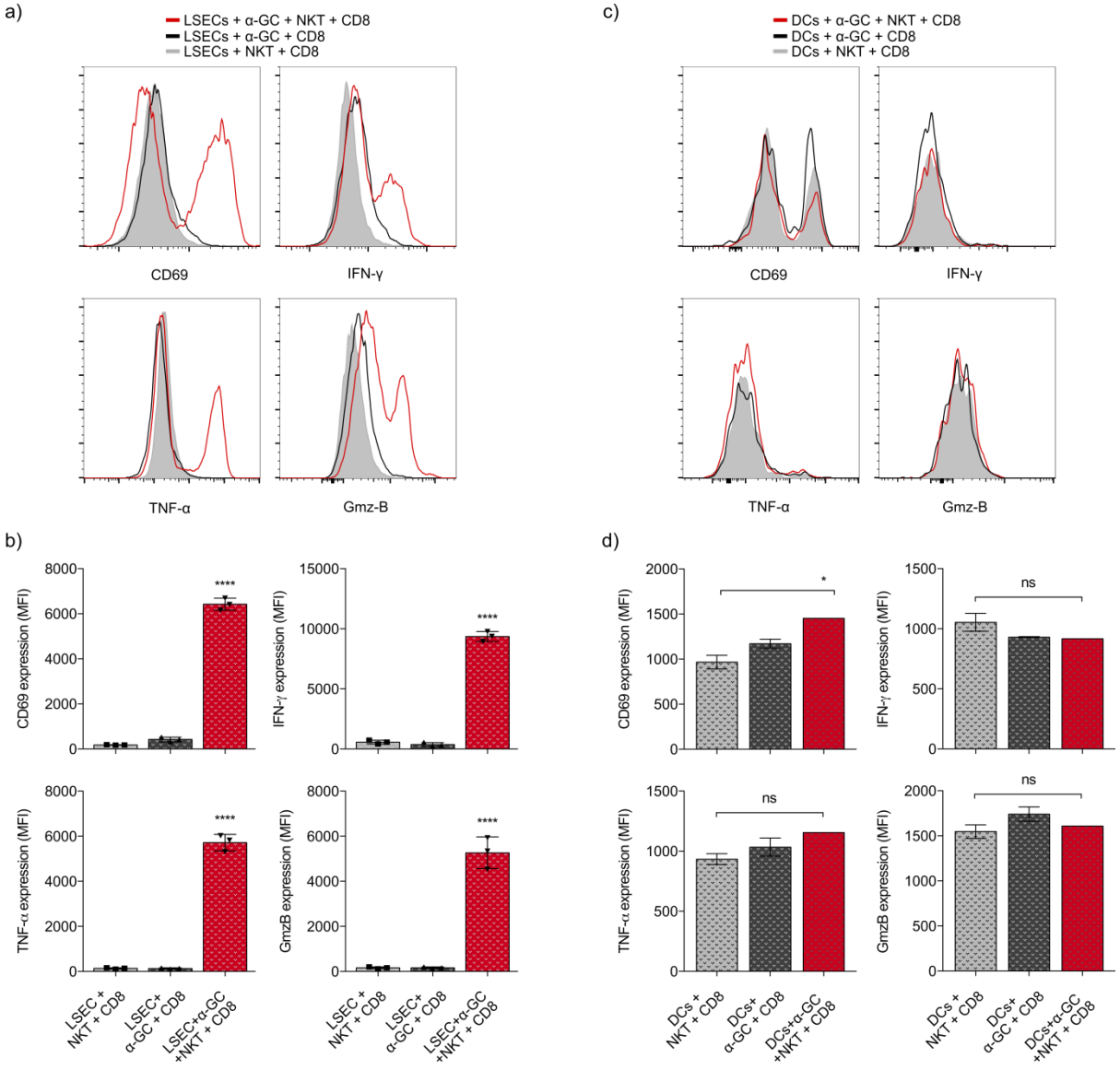

Figure 19. Activation of CD8 T cell in response to LSEC-mediated NKT cell activation
*(a-d) Phenotypic characterization of CD8+ T-cells after 10 hours co-culture with α-GC primed-LSECs or DCs in presence of NKT cells shown as dot plots and MFI values. CD8 T-cell expression of CD69, INF-γ, TNF-α and GzmB was measured in presence LSECs (a, b) compared to DCs (c, d). Error bars represent mean ± SD of triplicate samples. ns < 0.32, *p < 0.027, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

Likewise, to study the time kinetics of the activation sequence of NKT and NK cells after contact with α-GC-pulsed LSECs, we used the gating strategy below (Figure 20) to characterize NK cells after 0, 5, 10 and 20 hours of co-culture.

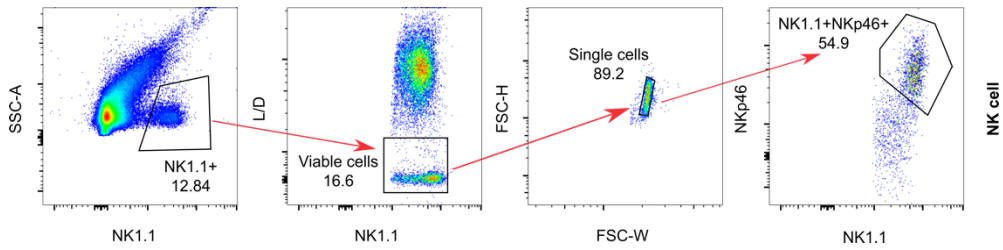

Figure 20. Gating strategies used by flow cytometer to selectively identify NK cells
Cultured cells were collected after 10 hours of co-culture and the representative flow cytometry plots demonstrating the gating strategy for identification of NK cells.

The results showed an early activation of NKT cells after 5 hours of co-culture as compared to 10 hours needed for NK cell to reach its highest expression of intracellular INF-γ and GzmB (Figures 21a and 21b), suggesting a later activation of NK cells to NKT cell activation.

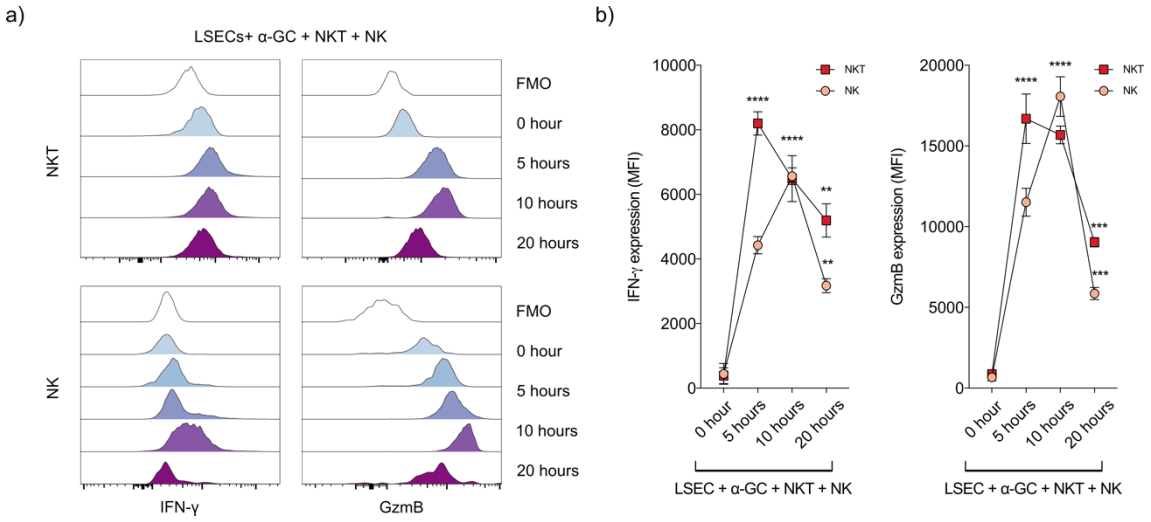

Figure 21. Time kinetics of NKT cell vs NK cell activation in co-culture with α-GC pulsed LSECs
*(a and b) α-GC primed-LSECs were cultured with NKT cells in presence of CD8+ T-cells for the indicated time course to monitor cell activation. (a) Flow cytometry histogram plots and (b) MFI values at the different time points for intracellular INF-γ expression or GzmB expression in NKT and CD8+ T-cells. Error bars represent mean ± SD of triplicate samples. **p < 0.0087, ***p < 0.0006, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

Using the identified time-point of 10 hours, we recorded a worthy polyclonal activation of NK cells shown by increased CD69, IFN-γ, TNF-α and GzmB expressing NK cells after 10 hours within LSECs-NKT co-culture (Figures 22a and 22b), but we did not observe a considerable activation of NK cells within DCs-NKT co-culture (Figures 22c and 22d).

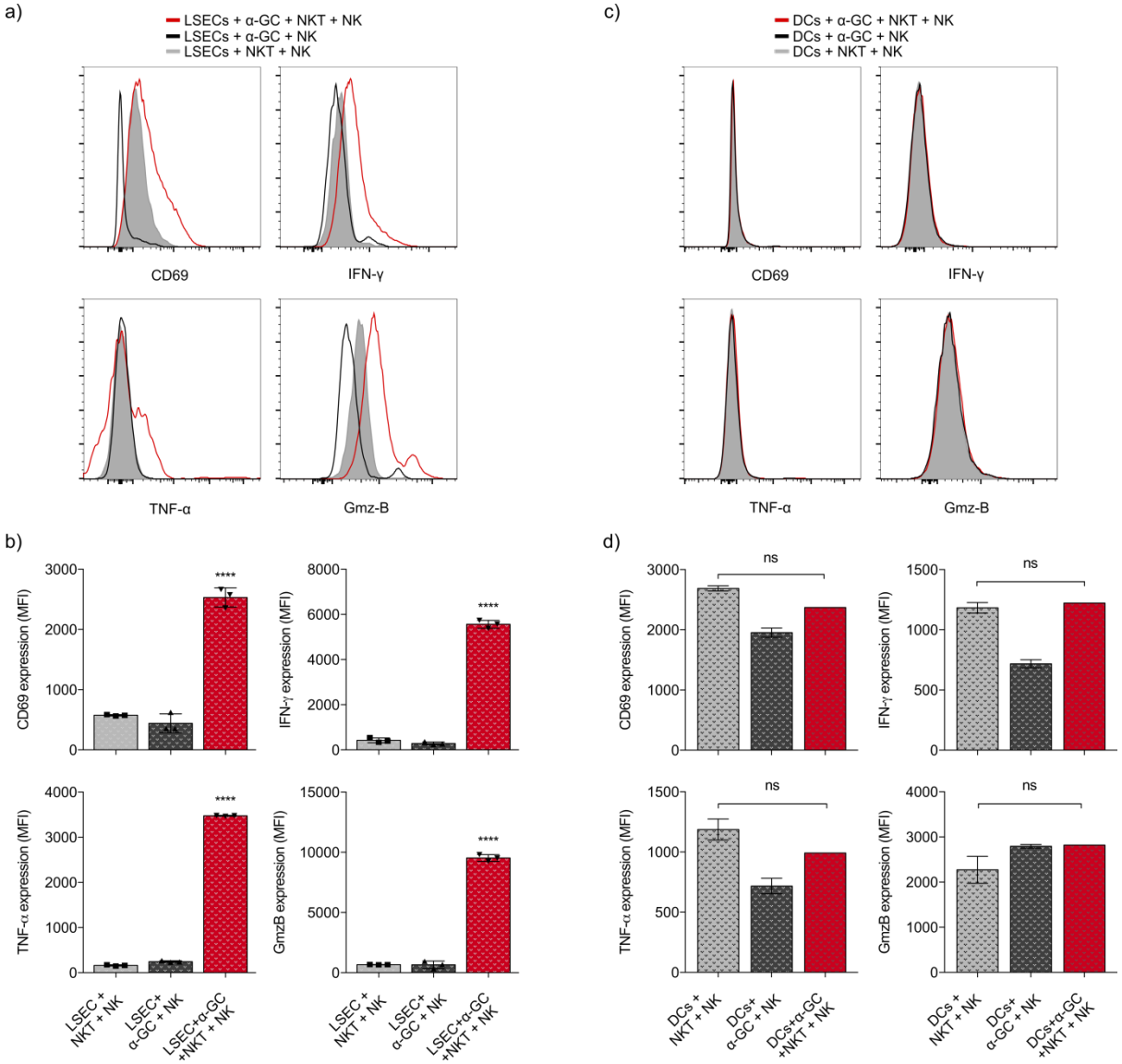

Figure 22. NK cells acquire cytotoxic-phenotype in response to LSEC-mediated NKT cell activation
*(a-d) Phenotypic characterization of NK cells after 10 hours co-culture with α-GC primed-LSECs or DCs in presence of NKT cells shown as dot plots and MFI values. NK cells expression of CD69, INF-γ, TNF-α and GzmB was measured in presence LSECs (a, b) compared to DCs (c, d). Error bars represent mean ± SD of triplicate samples. ns < 0.29, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

These results supported the notion that the LSEC-induced activation of NKT cells led to subsequent activation of CD8 T cells as well as NK cells.

3.6 Coherence of LSEC-platform during the polyclonal activation of immune responses

Along with the early activation potential of NKT cell we previously determined due to its interaction with LSECs, we investigated here the real-time viability of LSECs during such interaction to estimate their individual or synergetic contribution in the polyclonal activation of CD8+ T-cell and NK cell. For that, we used the XCELLigence

technique, in which the measured electron-impedance is directly proportional to the LSEC-monolayer seeded on a 96-well e-plate. However, in order to use adherent hepatocytes as a control, we had to investigate the ability of hepatocytes to crosstalk with 40.000 NKT cells in presence of a 100 ng/ml of α-GC. After 24 hours of NKT cells co-culture with 10.000 hepatocytes seeded on 96 well-plate (Figure 23a), we observed a significant increase in soluble IFN-γ concentration using ELISA (Figure 23b), demonstrating the capacity of hepatocytes to present lipid antigens to NKT cells.

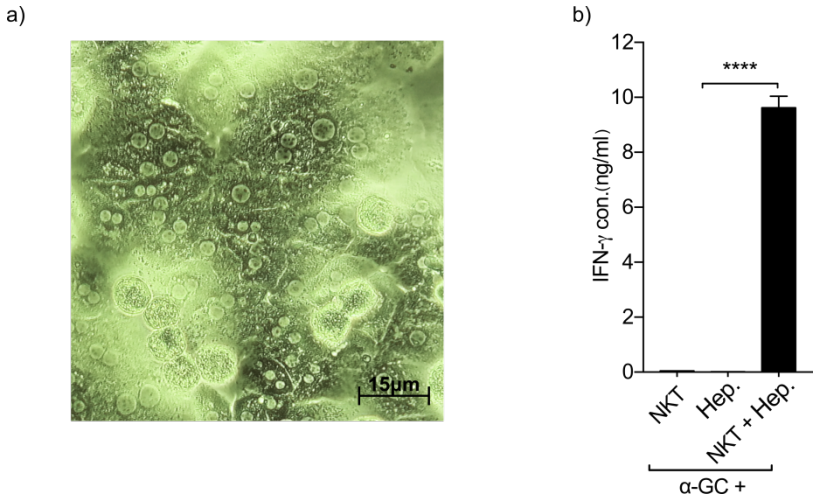

Figure 23. Hepatocytes mediate a CD1d-restricted activation of NKT cells in presence of α-GC
*(a) 10x microscopic imaging of Hepatocytes seeded in a cell-bind 96-well plate for 24 hours. (b) ELISA measurements of the soluble IFN-γ secreted by NKT cells in response for the crosstalk with α-GC-pulsed Hepatocytes. Error bars represent mean ± SD of triplicate samples. ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

We wondered whether CD1d-mediated activation of NKT led to death of the antigen-presenting cells. To investigate this for liver cell populations that are adherent and therefore amenable for a time-resolved cytotoxicity assay based on electrical impedance generated by cells adherent to a special analysis plate, we directly compared the fate of LSECs and hepatocytes when serving as antigen-presenting cells for NKT cells. To this end, we seed hepatocytes or LSECs into e-plates, pulsed the cells with α-GC and then added 40.000 NKT cells (Figure 24a). We observed a rapid decline of the cell index of hepatocyte cultures that were pulsed with α-GC and where NKT cells were added (Figure 24b). Of note, addition of NKT cells to hepatocytes that were not pulsed with α-GC did not cause any change in cell index, indicating normal viability of hepatocytes that were in direct contact with NKT cells. Together, these data indicated that NKT cells killed hepatocytes after recognizing α-GC presented in the context of CD1d molecules.

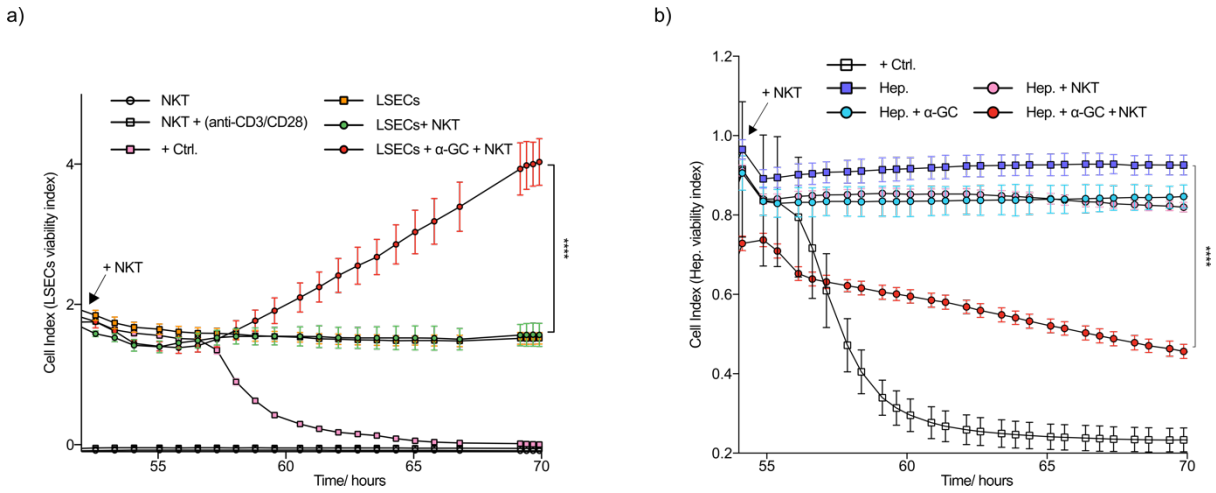

Figure 24. LSECs remain viable after activating NKT cells
*(a and b) LSECs or Hepatocytes were seeded on E-plates and pulsed with α-GC in presence of NKT cells. impedance measurements were performed every 15 minutes and were calculated as cell index for LSECs (a) and Hepatocytes (b). Reagents and cells were added in the time sequence outlined in the figures above. Error bars represent mean ± SD of triplicate samples. ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

In contrast, the cell index of LSECs did not decrease after addition of NKT cells irrespective of whether LSECs had been pulsed with α-GC before. Surprisingly, we even observed increased cell index values that indicate higher electrical impedance when LSECs served as antigen-presenting cells and activated NKT cells. Since this change in electrical impedance occurred so quickly, it is most likely explained by increased intercellular adhesion that increases impedance rather than proliferation of adherent LSECs. These results suggested that LSECs served as antigen-presenting cells for NKT cells but did resisted elimination through activated NKT cells, which support the notion that LSECs may serve as a sustainable sinusoidal platform for NKT cell activation.

Next, we addressed the question whether polyclonal activation of CD8 T cells or NK cells resulting from previous LSEC-mediated activation of NKT cells would damage the LSECs. To this end, we added CD8 T cells or NK cells to the co-cultures of α-GC-pulsed LSECs and NKT cells. Clearly, the addition of CD8 T cells and NK cells – which led to their activation as measured by cytokine expression, upregulation of activation markers and effector molecules – did not affect the viability of LSECs (Figure 25). These results further supported the notion that LSECs served as an activation platform for sinusoidal cell populations like NKT cells, CD8 T cells and NK cells without becoming target of effector functions that would lead to loss of sinusoidal integrity.

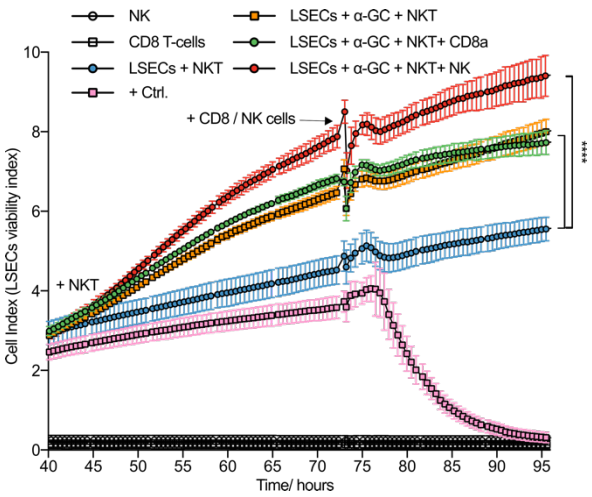

Figure 25. Polyclonal activation of CD8+ T-cell and NK cell does not affect the integrity of LSEC-NKT platform

*LSECs were seeded on E-plate and primed with α-GC in presence of NKT cells. Primed-LSECs viability was measured during polyclonal activation of CD8+ T-cells by real-time measurements of the LSECs cell index. Error bars represent mean ± SD of triplicate samples. ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

3.7 The principle of CD8+ T-cell and NK cell polyclonal activation in response for LSECs-NKT cognate interaction

In the previous experiments, we showed that LSEC-NKT cognate interaction was responsible for the induced polyclonal activation of CD8+ T-cell and NK cells. Therefore, we were interested to characterize whether this activation resulted from physical cell-cell contact or a paracrine signalling. For that, we incubated CD8+ T-cells and NK cells with the cell culture supernatants of co-cultures of α-GC pulsed LSECs and NKT cells for 10hrs. CD8+ T-cell and NK cell from the different culture-conditions were then analysed by flow cytometer after 10 hours to determine their activation (see illustration 3).

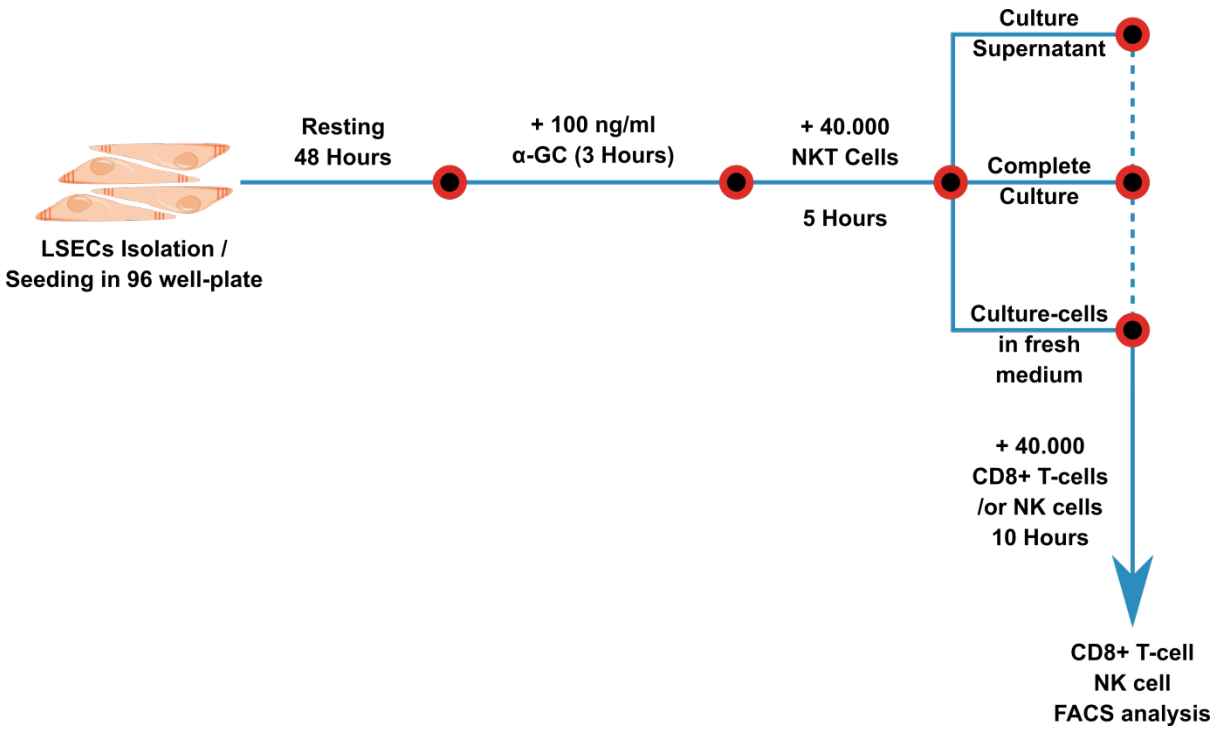

Illustration 3. Schematic representation of the study addressed the principle of CD8+ T-cell and NK cell polyclonal activation in response for LSEC-NKT cognate interaction
LSEC was isolated according to the mentioned protocols. Culture of CD8+ T-cells and NK cells with the three different culture-conditions, the experimental timeline, samples collection and data analysis were done according to the representative illustration.

CD8 T cells being in direct co-culture with LSECs/NKT cells showed increased expression of activation markers, expression of cytokines and increase in GzmB levels. However, we did not detect increased expression of activation markers, no expression of cytokines and no increase in GzmB levels when CD8 T cells were only exposed to LSEC/NKT cell culture supernatants (Figures 26a and 26b). This demonstrated that a physical contact was required for CD8 T cell activation.

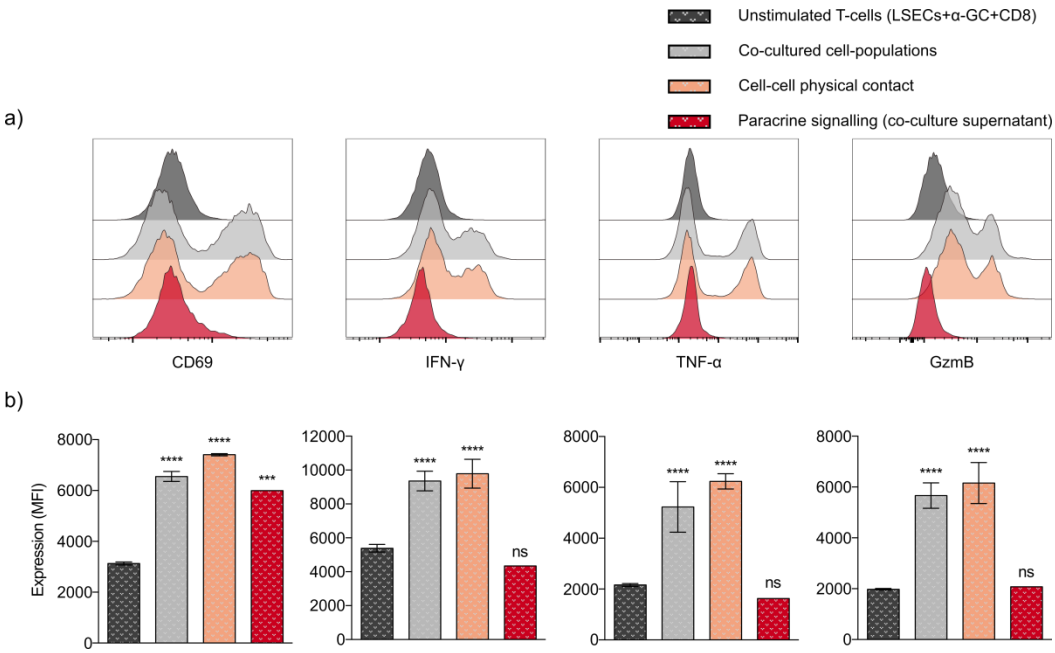

Figure 26. Cell-cell contact is required for CD8 T cell activation following LSEC-mediated NKT cell activation

*(a and b) Mode of CD8+ T-cell polyclonal activation through LSECs-NKT crosstalk was determined by culturing CD8+ T-cells in LSECs-NKT co-culture, LSECs-NKT cell-cell contact (without supernatant) and in LSECs-NKT supernatant only. (a) Histogram and (b) MFI analysis for both surface and intracellular activation signals of CD8+ T-cells was done after 10 hours incubation in the indicated culture-condition. Error bars represent mean ± SD of triplicate samples. ns < 0.10, ***p < 0.0004, ****p < 0.0001, as determined by two-way ANOVA. Each panel is representative of at least three different experiments.*

Similarly, we observed a significant increase in CD69 and intracellular cytokine (IFN-γ, TNF-α and GzmB) expressing NK cells within the LSEC-NKT cell-cell contact and LSEC-NKT co-culture, whereas there was a complete lack activation of NK cells in LSEC-NKT culture-supernatant (paracrine-signalling) (Figures 27a and 27b).

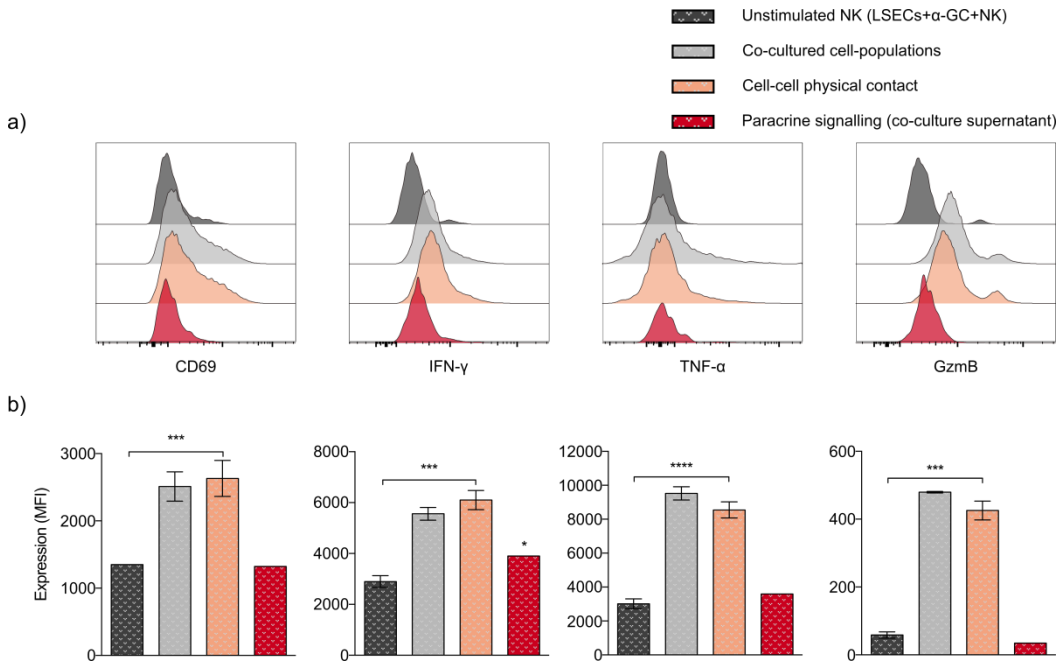

Figure 27. Cell-cell physical contact with LSECs is mandatory for NK cell activation following LSEC-mediated NKT cell activation

*(a and b) Mode of NK cell polyclonal activation through LSECs-NKT crosstalk was determined by culturing NK cells in LSECs-NKT co-culture, LSECs-NKT cell-cell contact (without supernatant) and in LSECs-NKT supernatant only. (a) Histogram and (b) MFI analysis for both surface and intracellular activation signals of NK cells was done after 10 hours incubation in the indicated culture-condition. Error bars represent mean ± SD of triplicate samples. *p < 0.0265, ***p < 0.0003, ****p < 0.0001, as determined by two-way ANOVA. Each panel is representative of at least three different experiments.*

Given dependence of cell-cell physical contact-driven CD8 T cell and NK cell activation we searched for increased expression of chemokine receptors such as CCR2 and CCR5. Using flow cytometry, we detected increased levels of CCR2 and CCR5 expression on CD8+ T-cells at 10 hours after co-culture with α-GC pulsed LSECs and NKT cells (Figures 28a and 28b). Similarly, NK cell activation by LSEC-NKT was similarly accompanied by increased surface-expression levels of CCR2 and CCR5 (Figures 28c and 28d).

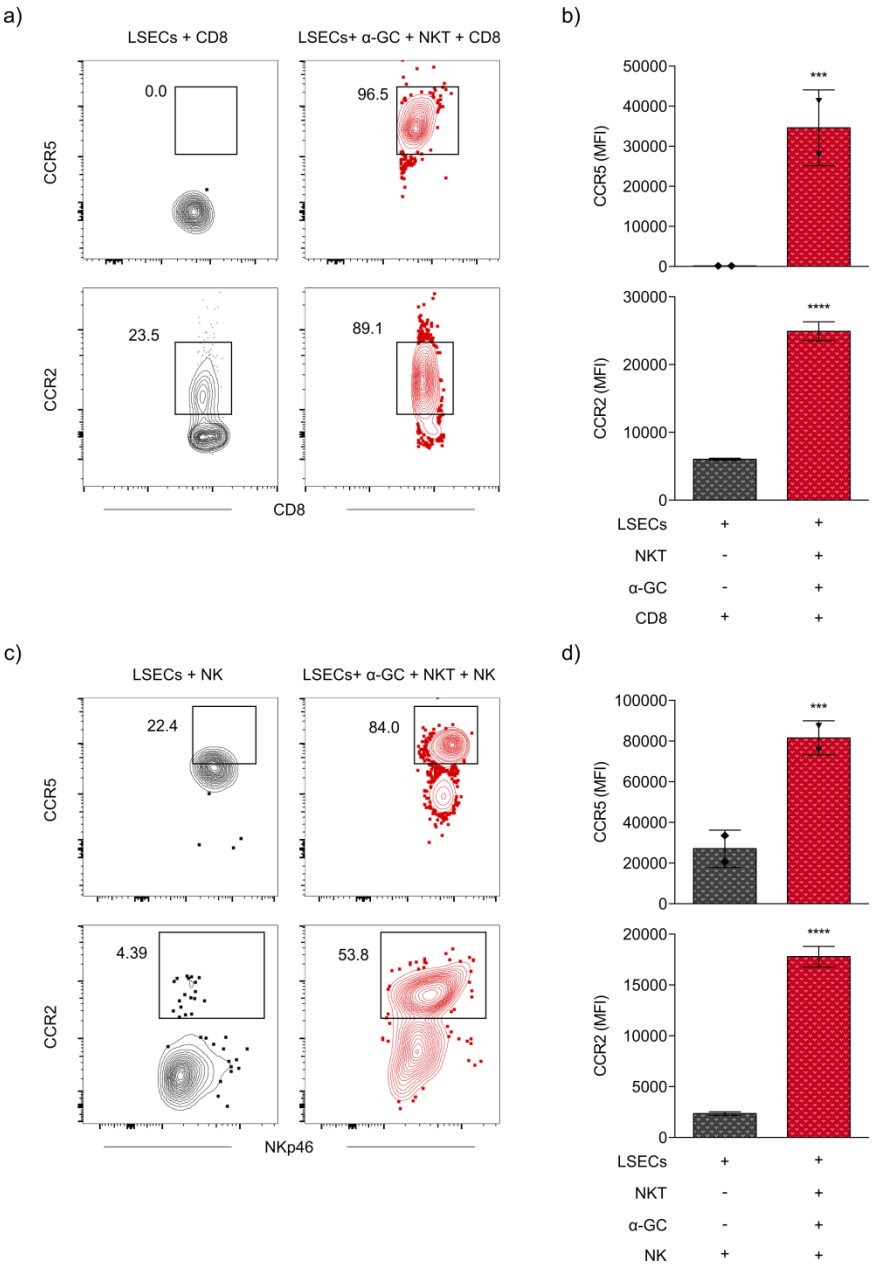

Figure 28. The CD8 T cell and NK cell activation during LSEC/NKT cell activation is accompanied by increased CCR2 and CCR5 expression

*(a-d) Flow cytometric analysis of expression of chemokine receptors in CD8 T cells and NK cells after 10 hours of co-culture with LSEC/NKT cells. Flow cytometry plots and MFI analysis of CCR5 and CCR2 expression (a, b) CD8+ T-cells and (c, d) NK cells. Error bars represent mean ± SD of triplicate samples. ***p < 0.0004, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

Since expression of CCR5 is a marker of immune cell activation (Dyer et al., 2019; Lefebvre et al., 2016), this provides further evidence for the polyclonal activation of CD8 T cells and NK cells following contact with NKT cells activated through antigen presenting LSECs.

3.8 Identification of the inflammatory mediators produced by LSEC-NKT cognate interaction

Although we had excluded a role of paracrine signalling in CD8 T cell or NK cell activation in coculture with LSEC-activated NKT cells, we wondered which cytokines were induced during NKT cell activation as well as during further CD8 T cell and NK cell activation. The release of cytokines and chemokines into the supernatant of cell co-cultures was measured using a Bio-Plex bead array assay.

Compared with DCs and hepatocytes, CD1d-restricted antigen-presentation by LSECs and activation of NKT cells led to a markedly higher secretion of a wide range of cytokines and chemokines especially IL-1, IL-6, IFN-γ, TNF-α and MCP-1. Of note, antigen presentation by DCs led to induction of IL-2, which was not observed during NKT cell activation by other antigen-presenting cells. Importantly, after addition of CD8 T cells or NK cells, there was a substantial increase in production of cytokines and chemokines (Figure 29), indicating that NKT cell activation through LSECs in the direct vicinity to CD8 T cells and NK cells leads to a vast induction of pro-inflammatory mediators.

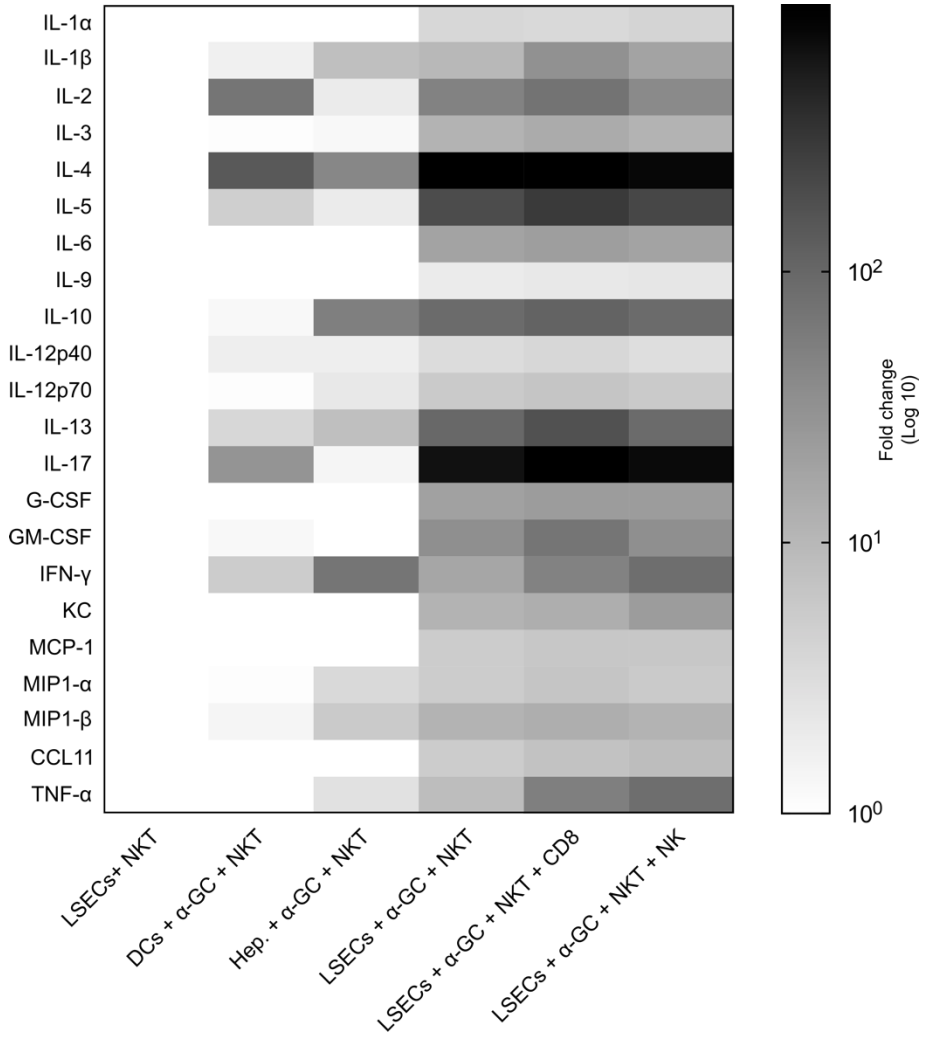

Figure 29. Expression of mediators (cytokines and chemokines) after activation of NKT cells through antigen presenting LSECs or DCs or hepatocytes in presence of absence of further CD8 T cells or NK cells.

DCs, hepatocytes and LSECs were pulsed with α-GC and cocultured with NKT cells, CD8 T cells and NK cells for 10 hours. Heat map visualization of cytokine and chemokine release in culture-supernatant measured by a multiplex cytokine bead array assay. Colors represent log10 fold change of concentration compared to untreated samples. Each panel is representative of at least three different experiments.

3.9 Role of IFN-γ and IL-6 in the polyclonal activation of immune response via LSEC-NKT platform

Following up on the broad induction of cytokine/chemokine expression after LSEC-mediated NKT cells activation, we first validated the results from the bead array. Clearly, IFN-γ and IL-6 concentrations were increased in cell culture supernatants from α-GC pulsed LSECs co-cultured with NKT cells in response for LSEC-NKT cognate interaction as determined by conventional ELISA (Figures 30a and 30b). Importantly, neither IFN-γ nor 60 ng/ml of recombinant IL-6 or hIL-6 were capable of inducing CD8 T cell activation measured by cytokine expression (Figures 30c and 30d).

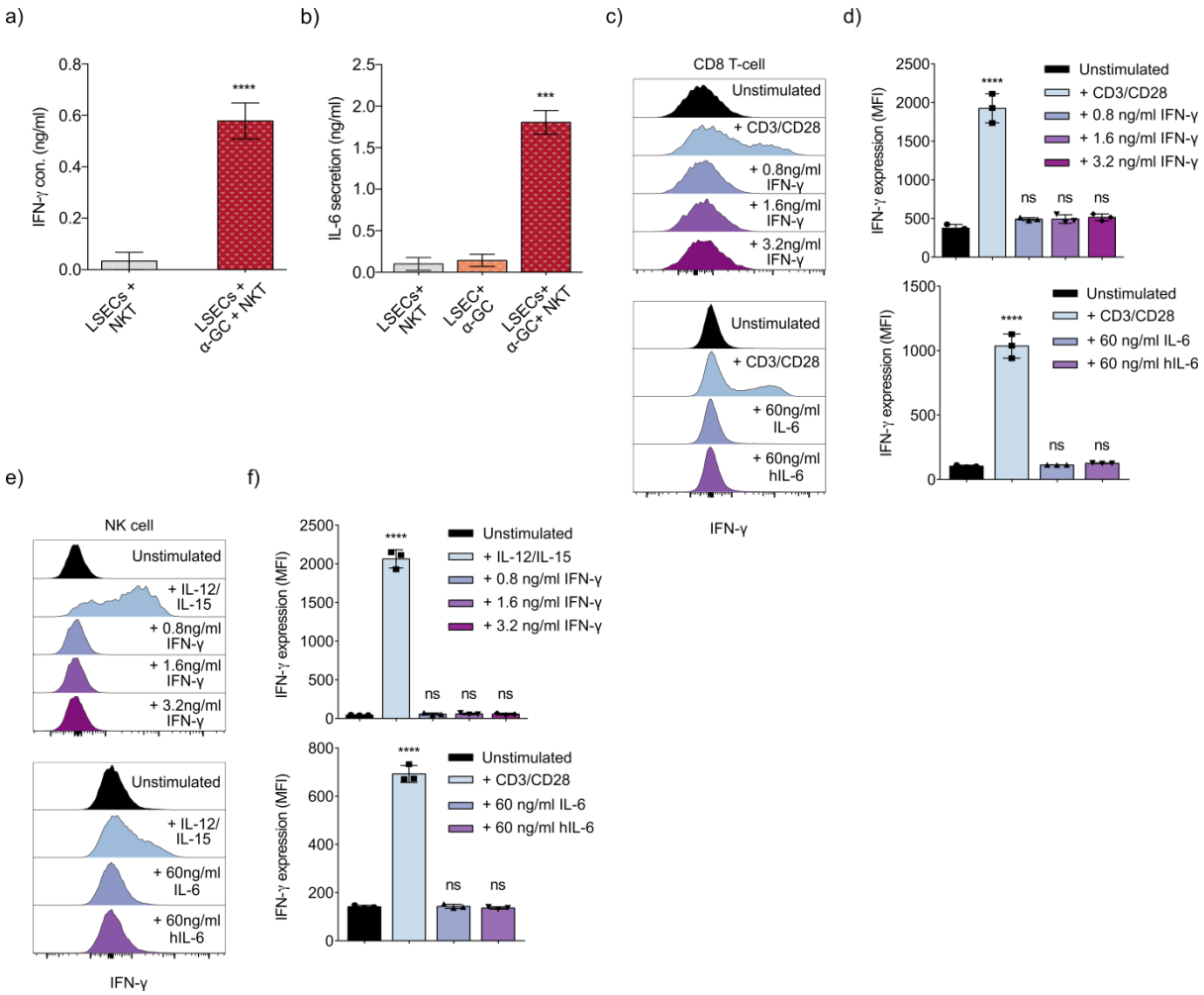

Figure 30. IFN-γ and IL-6 do not cause polyclonal activation of CD8+ T-cell and NK cell
*(a and b) α-GC primed-LSECs were cultured with NKT cells for 10 hours. ELISA measurements of soluble (a) IFN-γ and (b) IL-6 (b) in culture-supernatant after 10 hours of LSEC-NKT cell co-culture. (c) FACS plots and (d) MFI values, showing intracellular IFN-γ expression by CD8+ T-cells following 10 hours culture with the indicated doses of IFN-γ, IL-6 and hIL-6. (e) FACS plots and (f) MFI values, represents the intracellular IFN-γ expression by NK cells following 10 hours culture with the indicated doses of IFN-γ, IL-6 and hIL-6. ns < 0.9148, ***p < 0.0005, ****p < 0.0001, as determined by two-way ANOVA. Each panel is representative of at least three different experiments.*

Similarly, NK cells after exposure to recombinant IFN-γ, IL-6 or hIL-6 did not show increased IFN-γ expression (Figures 30e and 30f). These results indicated that neither IFN-γ nor IL-6 played a key role in CD8 T cell activation or NK cell activation following LSEC-mediated NKT cell activation.

3.10 Role of TNF in the polyclonal activation of CD8 T cells or NK cells in presence of LSECs

This led us to investigate the role of TNF. First, we determined whether TNF was expressed by NKT cells following stimulation with α-GC-pulsed LSECs. We detected a more than 5-fold increase in TNF gene expression in NKT cells after activation through antigen-presenting LSECs (Figure 31a), which led us to characterize the relevance of TNF released from activated NKT cells for subsequent CD8 T cell or NK cell activation. CD8+ T-cells were therefore exposed to 10 ng/ml of recombinant TNF in the presence or absence of LSECs. Activation of CD8+ T-cells was then determined after 10 hours by detection of increased expression of CD69, CD25, IFN-γ, TNF-α and GzmB.

Clearly, CD8+ T-cells cultured with LSEC and exposed to recombinant TNF but not CD8 T cells exposed to TNF alone showed increased CD69, CD25, IFN-γ, TNF and GzmB expression (Figures 31b and 31c). Thus, TNF sufficed to initiate polyclonal activation of CD8 T cells provided these cells were cultured in the presence of LSECs.

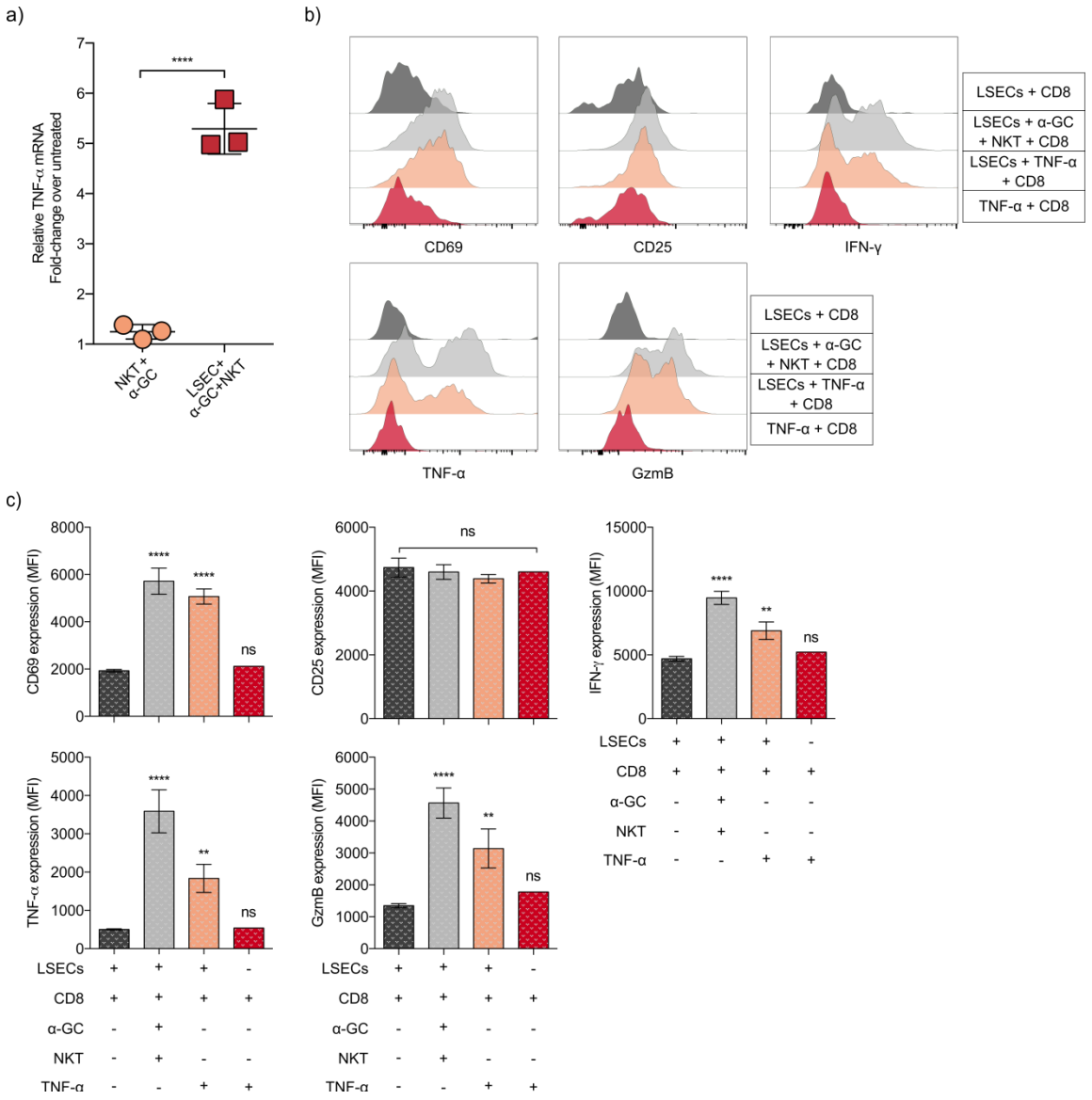

Figure 31. TNF secreted during LSEC-mediated NKT cell activation supports CD8 T cell activation
*(a) TNF mRNA expression by NKT cells stimulated by α-GC pulsed LSECs was analysed by real-time PCR and is shown as fold increased (compared to 18s as ICR). (b and c) Detection of CD8+ T-cell polyclonal activation following 10 hours culture using the indicated conditions, in presence or absence of Brefeldin A. (b) FACS plots and (c) MFI values represent the surface markers and intracellular cytokine expression by CD8+ T-cells. Error bars represent mean ± SD of triplicate samples. ns < 0.19, **p < 0.0011, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

Likewise, we performed similar experiments with NK cells. Also, here TNF induced activation of NK cells if they were cultured in the presence of LSECs, whereas NK cells stimulated with TNF in the absence of LSECs did not show any evidence for activation (Figures 32a and 32b).

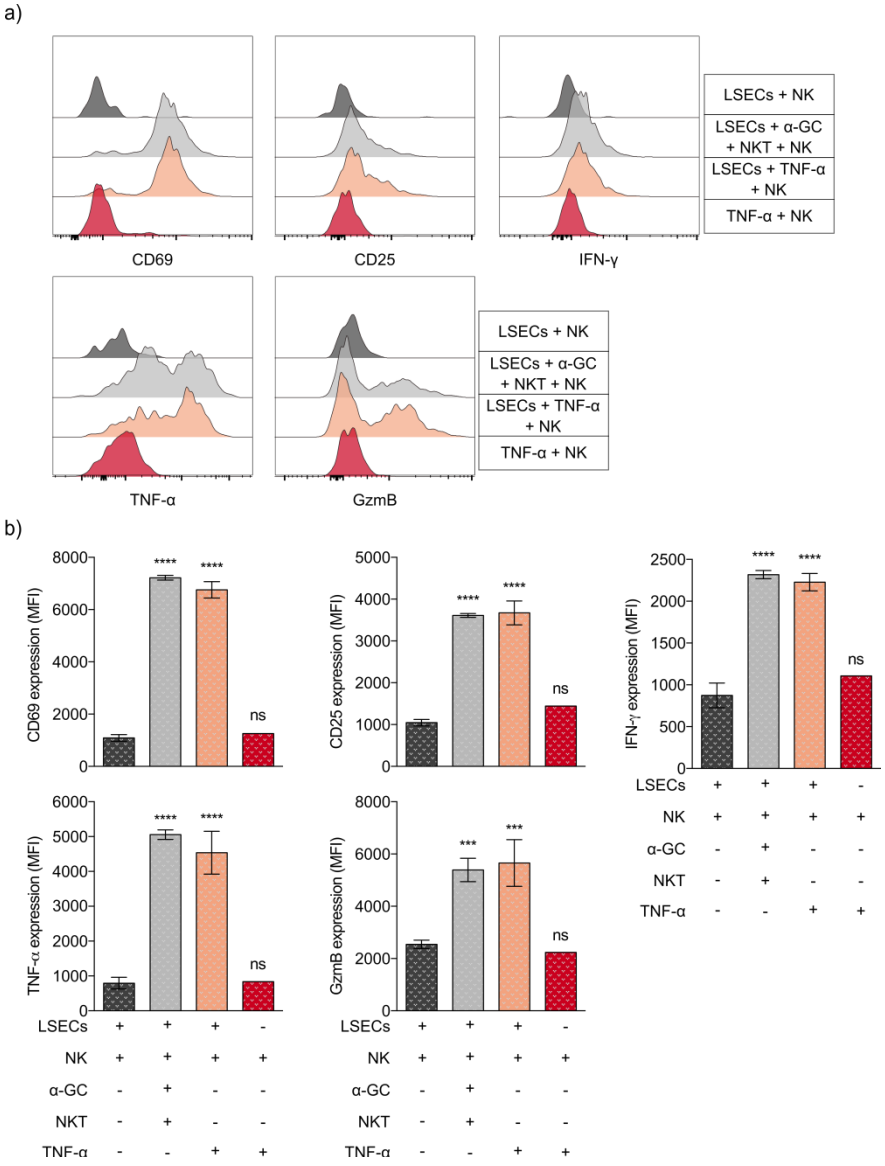

Figure 32. TNF-α secreted by LSEC-NKT cognate-interaction indirectly induces a polyclonal activation of NK cell through LSEC-platform

*(a and b) Detection of Liver NK cell polyclonal activation following 10 hours culture using the indicated conditions, in presence or absence of Brefeldin A. (a) FACS plots and (b) MFI values represent the surface markers and intracellular cytokine expression by NK cells. Error bars represent mean ± SD of triplicate samples. ns < 0.5539, ***p < 0.0004, ****p < 0.0001, as determined by one-way ANOVA. Each panel is representative of at least three different experiments.*

These findings further corroborated the notion that LSECs served as a platform to stimulate NKT cells in a CD1d-dependent fashion and at the same time further promoted activation of other sinusoidal cell populations, such as CD8 T cells and NK cells.

3.11 Role of LSEC-metabolites in polyclonal activation of the immune response

We proved in the previous experiments that a cell-cell physical contact was needed for the activation of CD8+ T-cell and NK cell during LSEC-NKT activation. Meanwhile, in response to LSEC-meditated activation, our data showed increased expression of TNF-α, which is known to increase the sensitization to extracellular ATP via Nlrp3 inflammasome activation. Accordingly, we further investigated the possible transfer of adenosine triphosphate (ATP) through LSEC-platform. For that, ATP secretion by LSECs was measured in the culture-supernatant using a bio-luminescence assay. We observed a significant secretion of ATP by LSECs compared to DCs (Figure 33a). However, CD8+ T-cells did not show increase in the intracellular Calcium (Ca^{2+}) concentration in response for this increase in LSEC ATP-secretion (Figure 33b). Meanwhile, loss of function experiments were done using a blocking antibody to neutralize the ATP binding receptor (P2RX7) and we analysed the polyclonal activation of CD8+ T-cell using flow cytometry. By measuring the surface markers (CD69 and CD25) and intracellular cytokines (IFN-γ, TNF-α and GzmB), we did not find a significant decrease in CD8+ T-cell polyclonal activation in presence of P2RX7 inhibitor compared with the control (Figures 33c and 33d).

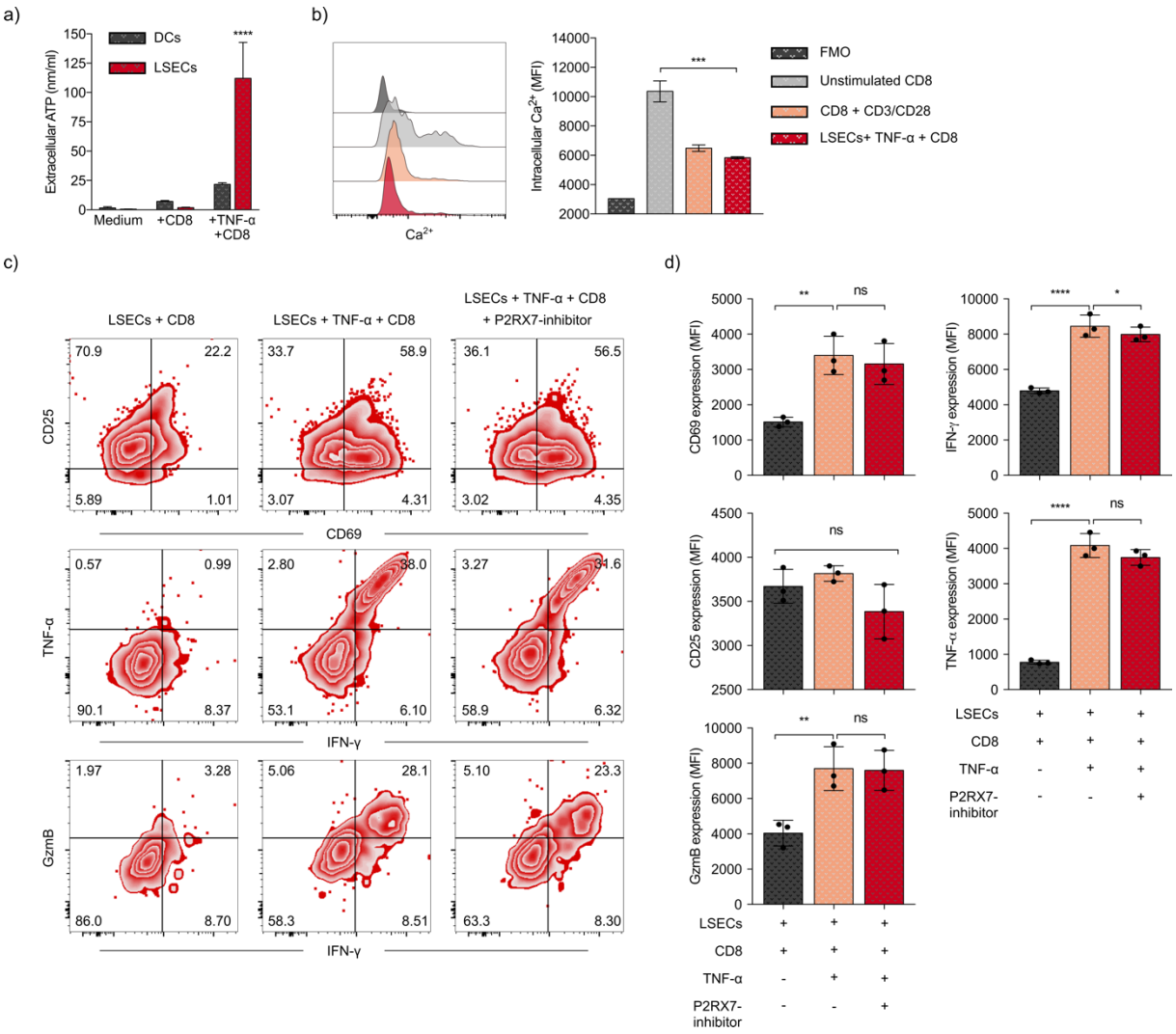

Figure 33. LSEC-soluble ATP is dispensable for CD8+ T-cell polyclonal activation through LSECs in presence of TNF

*(a) LSECs or DCs were cultured with CD8+ T-cells in presence or absence of TNF-α for 10 hours and the released ATP was measured using bioluminescence assay. (b) For Intracellular Calcium level-measurement, CD8+ T-cells were cultured with primed LSECs in presence of 100 ng/ml recombinant murine TNF-α and intracellular Calcium (Ca2+) concentration of CD8+ T-cells was measured using Ca2+ sensing dye and represented in FACS plot and MFI value. (c and d) CD8+ T-cells were cultured with TNF-α-primed LSECs using the indicated conditions for 10 hours in presence of 10 μg/ml anti-P2RX7 antibody. Polyclonal activation of CD8+ T-cells was analysed by surface and intracellular staining of the activation-mediators represented by (c) FACS plots and (d) MFI values. Error bars represent mean ± SD of triplicate samples. ns < 0.19, *p < 0.05, **p < 0.004, ***p < 0.0003, ****p < 0.0001, as determined by one/two-way ANOVA. Each panel is representative of at least three different experiments.*

We concluded from these results that compared to DCs, LSECs secret a high concentration of ATP in response for the inflammatory signals. However, this soluble ATP did not cause an increased Ca^{2+} influx or polyclonal activation of CD8+ T-cells.

3.12 Liver CD8 T cells retain their CXCR6 expression during physical contact with LSECs

Given the necessity for LSECs being in physical contact with CD8 T cells to respond with activation to TNF exposure, we characterized the expression of a chemokine-receptor pair that is known to be operative in the liver, i.e., CXCL16 and CXCR6. LSECs showed increased expressed of the chemokine CXCL16 during CD1d-restricted antigen presentation to NKT cells (Figure 34a). This is also consistent with an activation of LSECs themselves following CD1d-restricted activation of NKT cells. Moreover, expression of the receptor of CXCL16, i.e., CXCR6, on liver CD8 T cells was stabilized after isolation in vitro, when hepatic CD8 T cells were kept in co-culture with LSECs (Figures 34b and 34c). In the absence of LSECs, previously CXCR6+CD8 T cells were not detected any more at 24hrs after isolation and in vitro culture, suggesting an active process in maintaining CXCR6 expression on CD8 T cells. CXCR6 is found on liver resident CD8 T cell and NK cell populations (Fernandez-Ruiz et al., 2016; Stegmann et al., 2016), and the stabilized expression levels of CXCR6 by hepatic CD8 T cells in the presence of LSECs suggests crosstalk between LSECs and CD8 T cells.

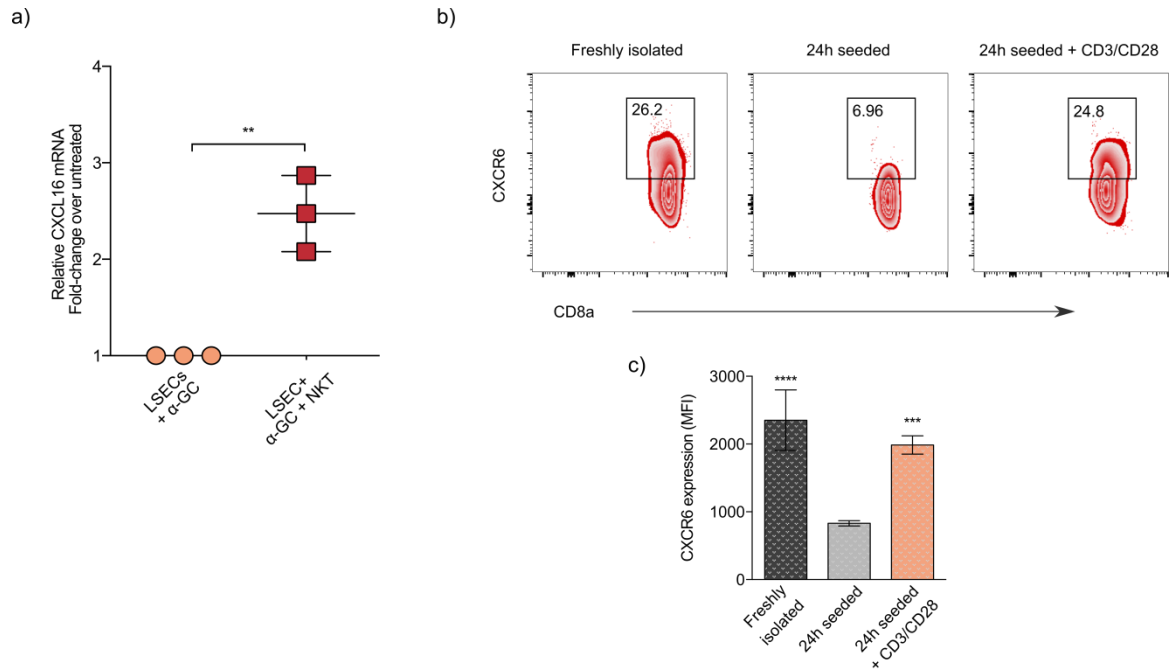

Figure 34. Increased CXCL16 expression in LSECs after CD1d-restricted antigen-presentation to NKT cells and maintenance of CXCR6 expression on CD8 T cells in physical contact with LSECs.
*(a) α-GC primed-LSECs were cultured with NKT cells for 10 hours. Increase in CXCL16 mRNA expression by LSECs was analysed by real-time PCR. (b and c) Flow cytometric detection of CXCR6 expression by liver CD8 T cells. **p < 0.0017, ***p < 0.0005, ****p < 0.0001, as determined by two-way*

ANOVA. Error bars represent mean ± SD of triplicate samples. Each panel is representative of at least three different experiments.

Similarly, stabilization of CXCR6 expression on hepatic NK cells after isolation in vitro was determined by activation in presence of stimulatory molecules or LSECs (Figures 35a and 35b). However, in absence of LSECs, previously CXCR6+NK cell were undetectable anymore 24hrs after isolation and in vitro culture, suggesting the importance of CXCL16-CXCR6 axis in NK cell activation.

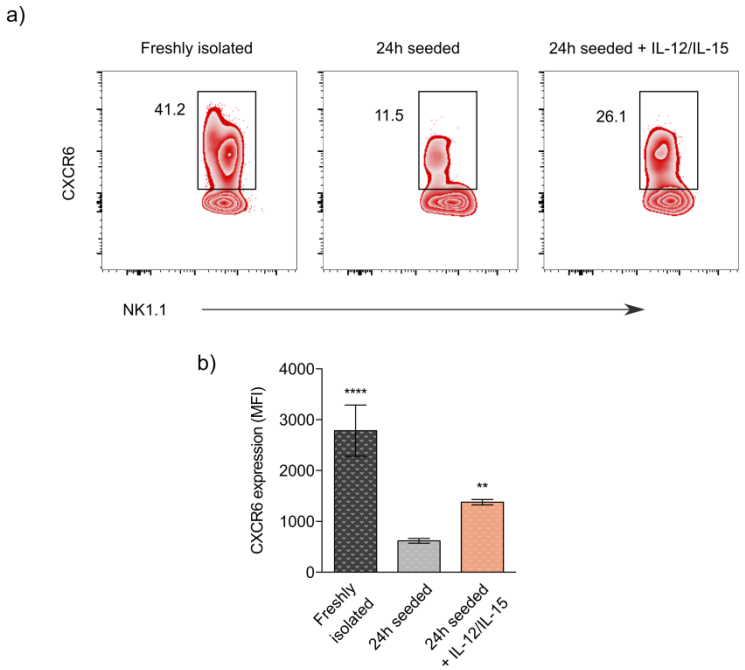

Figure 35. Activated liver NK cells maintain a significant expression of CXCL16 receptor (CXCR6)
*(a) FACS plots and (b) MFI values of CXCL16 receptor (CXCR6) surface-regulation on liver NK cells within the indicated conditions. **p < 0.0030, ****p < 0.0001, as determined by two-way ANOVA. Error bars represent mean ± SD of triplicate samples. Each panel is representative of at least three different experiments.*

These results indicated that LSECs maintained liver CD8 T cells as well as liver NK cells in a differentiated form, where they expressed the chemokine receptor CXCR6 that is a hallmark of liver resident immune cells.

3.13 CXCR6-expressing CD8 T cells and NK cells are activated by TNF

We therefore next addressed whether CXCR6-expressing lymphocytes showed unique responsiveness to stimulation with TNF. CD8 T cells were cultured in presence or absence of LSECs / DCs and subjected to exposure with recombinant TNF. Consistent with our data that LSEC was needed to stabilize the expression of CXCR6 on CD8 T-cells, our flow cytometry showed a preserved expression (30.2%) of CXCR6 on CD8 T-cell after 10hrs in vitro co-culture with LSECs compared to DCs (Figures 36a and 36b). Additionally, a strong CD8 T cell activation shown by increase in CD69, CD25, IFN-γ, TNF-α and GzmB expression was detected only on CXCR6+ CD8+ T-cells in presence of LSECs (Figure 36c) but not DCs (Figure 36d). In contrast, a complete absence of CD8 T cell activation was observed in the CXCR6- CD8 T cells in both LSECs and DCs co-culture (Figures 36c and 36d).

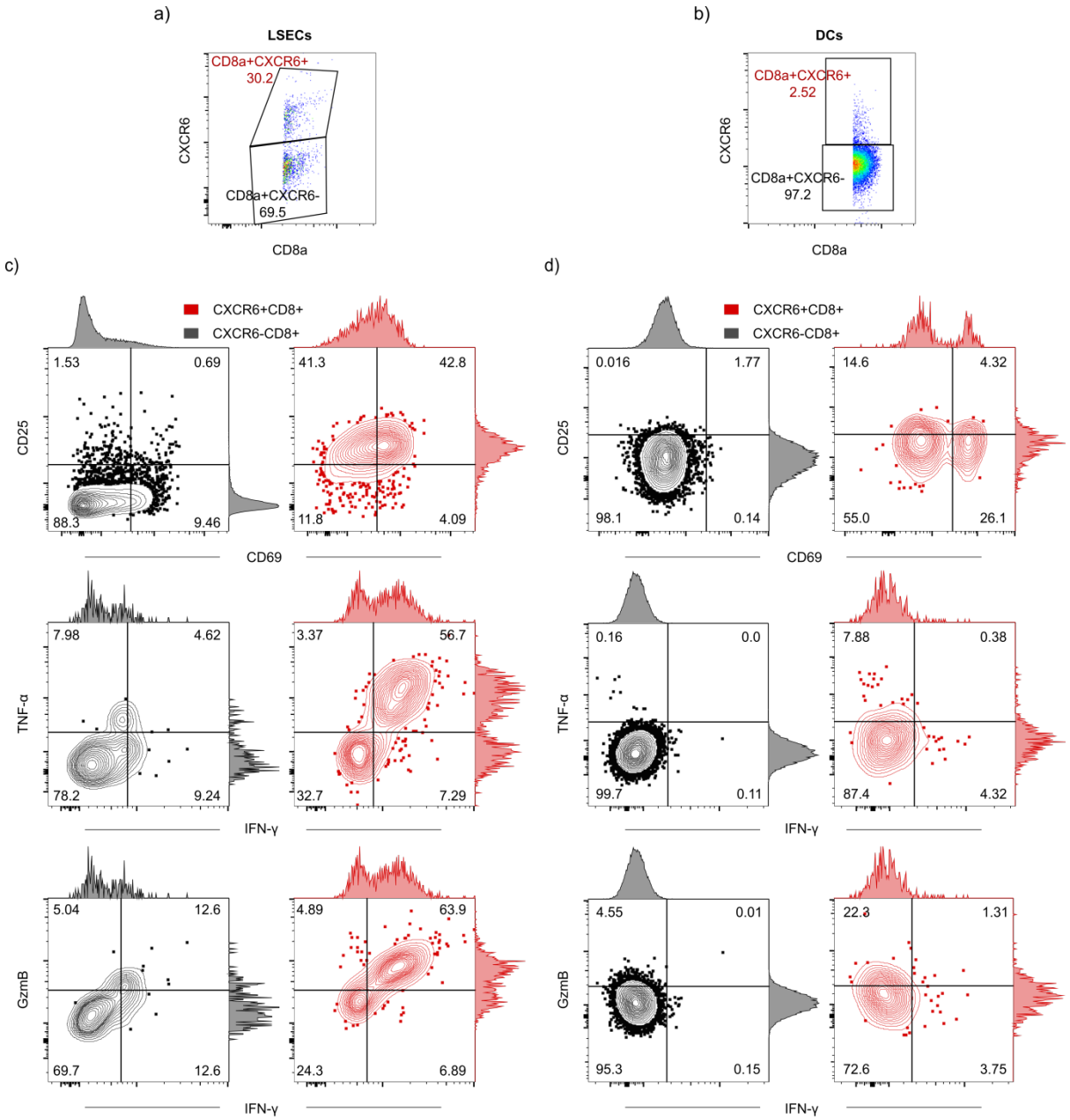

Figure 36. TNF-α activates CXCR6+ CD8 T-cells in the presence of LSECs

(a-d) Liver CD8+ T-cells were co-cultured for 10 hours with LSECs or DCs and exposed to 100 ng/ml of recombinant TNF, in presence or absence of Brefeldin A. (a and b) FACS plots represent CXCR6 expression on CD8 T-cell after 10hrs in vitro culture with LSECs (a) and DCs (b). (c and d) FACS plots compare the polyclonal response of CXCR6- and CXCR6+ fractions of CD8+ T-cell in response to TNF-exposed LSECs (c) and DCs (d). Each panel is representative of at least three different experiments.

Additionally, a strong CD8 T cell activation shown by increase in CD69, CD25, IFN-γ, TNF-α and GzmB expression was detected only on CXCR6+ CD8+ T-cells in presence of LSECs (Figure 36c) but not DCs (Figure 36d). In contrast, a complete absence of CD8 T cell activation was observed in the CXCR6- CD8 T cells in both LSECs and DCs co-culture (Figures 36c and 36d).

Similarly, we looked at NK cell response to TNF stimulation through their in vitro culture in presence or absence of LSECs / DCs and subjected to exposure with recombinant TNF. After 10hrs in vitro culture with LSECs, NK cells showed more stabilized expression of CXCR6 (29.4%) compared to its culture with DCs (Figures 37a and 37b). Furthermore, we observed a significant polyclonal activation and increased expression of both extracellular markers (CD69 and CD25) and intracellular cytokines (IFN-γ, TNF-α and GzmB) by the CXCR6+NK cells cultured with LSECs not with DCs (Figures 37c and 37d). Yet, the cultured CXCR6- NK cells with both LSECs and DCs were completely irresponsive to TNF-α signalling (Figures 37c and 37d), suggesting a conserved activation of CXCR+ CD8 T-cell and NK cell in response to TNF in presence of CXCL16 expressing LSECs.

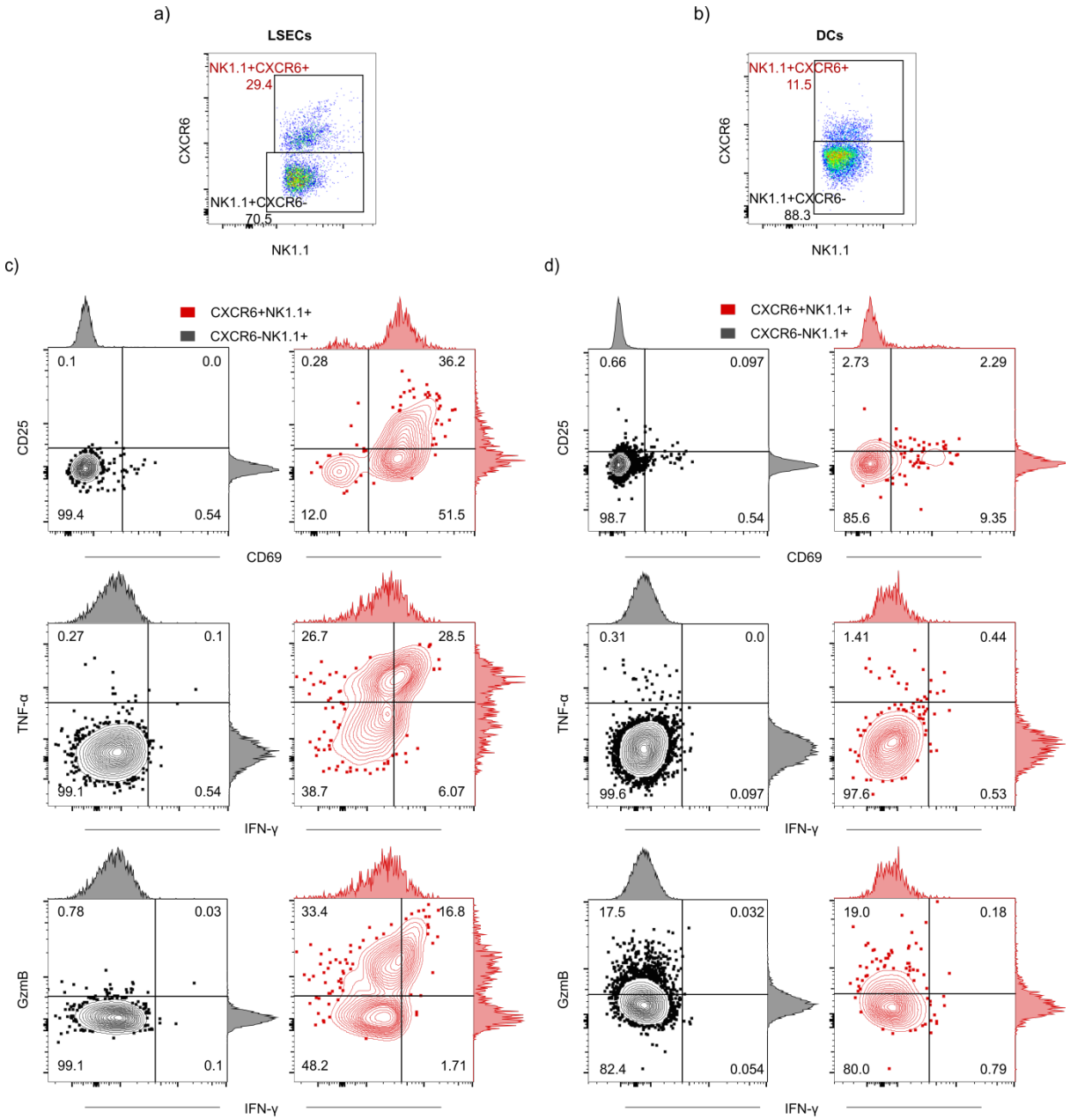

Figure 37. TNF-α activates CXCR6+ NK cells in the presence of LSECs

(a-d) Liver NK cells were co-cultured for 10 hours with LSECs or DCs and exposed to 100 ng/ml of recombinant TNF, in presence or absence of Brefeldin A. (a and b) FACS plots represent CXCR6 expression on NK cell after 10hrs in vitro culture with LSECs (a) and DCs (b). (c and d) FACS plots compare the polyclonal response of CXCR6- and CXCR6+ fractions of NK cell in response to TNF-exposed LSECs (c) and DCs (d). Each panel is representative of at least three different experiments.

From these findings we conclude that CD1d meditated antigen presentation by LSECs was capable of inducing NKT cell activation. This cognate interaction was followed by expression of inflammatory mediators, which indirectly induced polyclonal activation of CD8 and NK cells provided these cells were in direct contact with LSECs. This dual function of LSECs in activating NKT cells in an antigen-specific form and promoting antigen-independent activation of NK cells and CD8 T cells with a liver-resident phenotype was unique. LSECs may therefore serve as a platform to amplify signals among immune cells adherent to the sinusoids in the liver. This intercellular crosstalk could be further exploited to determine its importance to curtail the threshold of immune response to liver infections.

4. DISCUSSION

iNKT cells represent a major part of unconventional T-cell populations including γδ T-cells and mucosal-associated invariant T (MAIT) cells, with an invariant TCR of Vα14-Jα18-Vβ11 in mouse and Vα24-Jα18-Vβ11 in human. These cells are transcriptionally divided into three distinct subsets: NKT1, NKT2 and NKT17. Previous studies demonstrated that NKT cells are mainly distributed between liver, lungs and intestine, with residency characteristics especially in the liver, accounting for a 40% of the total hepatic lymphocyte in mice and 3-5% in human and suggesting an in-negligible contribution in hepatic immunology. Accumulating evidence demonstrated that NKT cells recognize lipid antigens in the liver presented on the MHC-I like molecule CD1d by both professional and unprofessional antigens presenting cells (APCs) in the hepatic milieu (Geissmann et al., 2005; Zeissig et al., 2017). However, whether these APCs were separately able to provide NKT cells with sufficient activation signals, the strength and outcome of NKT cell cognate-crosstalk with each APCs in the liver remain to be elucidated.

Here we showed using an *ex vivo* system that liver NKT cells reacted to the lipid-antigens presented by different populations of APCs in the liver including LSECs, hepatocytes and DCs but with more robust and dynamic extent toward LSECs-presented lipid-antigens. Furthermore, we found that LSEC-induced NKT cell activation mounted a concomitant polyclonal activation of CD8 T-cells and NK cells in a cell-cell contact-dependent fashion. More importantly, we noticed that NKT cell cognate crosstalk with LSECs induces a panoply production of interleukins, cytokines and chemokines, which efficiently targeted CXCR6-expressing liver CD8 T-cells and NK cells, inducing their activation in an antigen-independent fashion. Our findings are in line with previous studies showing that NKT-induced liver injury in autoimmune disease or in response for α-galactosylceramide (α-GalCer) injection is associated with a rapid recruitment of neutrophils and T_h1 T-cells to the liver vasculature (Bennstein, 2018; Georgiev et al., 2016; Santodomingo-Garzon and Swain, 2011). Therefore, our findings provide important insight for further development of iNKT cell-based immunotherapy targeting its cognate crosstalk with liver endothelium.

4.1 Hepatic capsules harbor a high expression of CD1d molecule by several APCs together with three distinct subsets of NKT cell

CD1d is a MHC-I like antigen-binding molecule belonging to the CD1 family of CD1a, CD1b, CD1c and CD1d (Zeng et al., 1997). Previous work showed that the CD1 family comprises the MHC-I like fold associated with deep pockets and hydrophobic antigen-binding clefts, reflecting their conserved specificity to bind lipid antigens (Godfrey et al., 2004; Koch et al., 2005). Interestingly, recognition of CD1-antigen complex has been shown to be restricted to NKT cells, which express an invariant Vα14-Jα18-Vβ11 TCR. For instance, binding of glycolipid antigen such as α-GalCer to pocket A´ of CD1d was found to induce binding of Vα14-Jα18-Vβ11 chain of NKT to pocket F´ and CD3R loop of CD1d, leading to activation of NKT cells (Cohen et al., 2009; Pellicci et al., 2009). Previous studies showed a bright staining of CD1d molecule in liver tissues, suggesting a broadly distributed expression (Geissmann et al., 2005). In this study, we show that LSECs, DCs and hepatocytes have high expression levels of CD1d giving NKT cells an easy access to CD1d-presented lipid antigens in the liver. Whether CD1d molecules expressed by hepatic cells were also instrumental in presenting other lipid-antigens need to be further investigated.

NKT cells have been classified into three different subsets: NKT1, NKT2 and NKT17 depending on their transcriptional regulation (Watarai et al., 2012). In liver, NKT cells have been shown to represent 40% of the total hepatic lymphocytes in mice and 3-5% in human (Syn et al., 2010). Due to the lack of a selective group of markers to identify the different subsets of NKT cells, previous work used CD1d-lipid complexes known as CD1d tetramers for isolation and studying of NKT cells (Benlagha et al., 2000; Matsuda et al., 2000). However, the limitation of this protocol is that it induces TCR activation of NKT cells, perhaps affecting the integrity of the isolated cells (Zeissig and Blumberg, 2019). Here, we exploited the surface-expression of innate and adaptive markers by NKT cells to isolate pure populations of NKT cells without triggering TCR activation. In concordance with previous studies, we show that the liver comprises CD4+, CD4- and a small proportion of CD8+ NKT cells (Gumperz et al., 2002; Lee et al., 2002).

Previous studies showed that the activation via TCR or inflammatory cytokines such as IL-12 up-regulates CD69 and CD25 expression by NKT cells and induces cytokine production, eliciting an immune response for instance against pathogens invading the

liver (Albarran et al., 2005; Engel et al., 2016). To mimic the *in vivo* activation of NKT cells, we used in this study micromagnetic beads coated with antibodies against CD3/CD28 surface molecules and recombinant IL-12 (Huang et al., 2013). Here, we show that the isolated NKT cells up-regulated the surface activation markers and showed increased cytokine production in response to the activation, with no impact of our isolation-protocol. We postulate that our optimized protocol could be used to study the distinct phenotypes of NKT cells in the future. Further enhancement of the protocol could be applied for more detailed study of hepatic NKT cell subsets by sorting CD4+, CD4- and CD8+ NKT cells separately.

4.2 NKT cells respond to lipid antigens presented by LSECs and DCs on CD1d

α-GalCer is a glycolipid derived from marine sponge *Agelas mauritianus* and its synthetic form has been used in many studies to synchronize the lipid-antigen presentation in human and mouse (Kawano et al., 1997). Previous work showed that α-GalCer develops a highly stable complex with the lipid-presenting molecule CD1d through its buried acyl chain in A´ pocket of CD1d molecule, suggesting its exclusive recognition by NKT cells (Burdin et al., 2000; Burdin et al., 1998; Salio et al., 2010). Subsequently, several studies targeting cancer or other infections used DCs- α-GalCer complexes to activate NKT cell and induce production of IFN-γ and IL-12 (King et al., 2018; Yamashita et al., 2018). Meanwhile, microscopic imaging showed that intravenous injection of α-GalCer triggered arrest of liver-patrolling NKT cells on the sinusoid-lining endothelium, suggesting a crosstalk between NKT cells and LSECs (Geissmann et al., 2005). Here, we demonstrated that a confluent layer of α-GalCer-pulsed LSECs was able to *ex vivo* elicit NKT cell activation in a robust manner, inducing high production of IFN-γ. Furthermore, our data showed that IFN-γ production by NKT cells occurred in concentration dependent fashion after increasing doses of α-GalCer and shows a much higher magnitude when α-GalCer was presented by LSECs compared to DCs. In addition to previous studies highlighting the role of MHC-I antigen-presentation by LSECs in the function of naïve and effector CD8 T-cells (Knolle and Wohlleber, 2016), our findings shed light on the immune functions of LSECs derived by lipid-antigen presentation via CD1 molecule.

4.3 LSECs induce rapid activation of NKT cells concomitant with sustained production of IFN-γ and polyclonal activation of CD8 T-cell and NK cell

IFN-γ belongs to the canonical Th-1 cytokine and represents the type II IFNs family. Studies showed that the primary sources of IFN-γ are NKT cell, NK cell, CD8 and Th-1 CD4 T-cells (Schroder et al., 2004a). Of note, previous work showed that NKT and NK cells constitutively express IFN-γ mRNA, allowing a rapid production and secretion of IFN-γ on infections (Kronenberg, 2005; Stetson et al., 2003). Contrastingly, naïve CD8 T-cells secret a substantial amount of IFN-γ immediately after TCR or pro-inflammatory activation, followed with a copious production of IFN-γ, which triggers more MHC-I expression by the infected cells (Glimcher et al., 2004). In this study, we show that LSEC induces a rapid activation of NKT cells associated with the highest magnitude of IFN-γ secretion after 5 hours of the activation. More importantly, addition of CD8 cells or NK cells to LSEC-activated NKT cells increased IFN-γ release within 10 hours of their co-culture, suggesting additional cell activation. Strikingly, we did not find this increase in IFN-γ release after culture of CD8 T-cells or NK cells with DCs-activated NKT cells. Consistent with this data, previous work showed increased levels of serum IFN-γ after four hours of intraperitoneal (IP) injection of α-GalCer (Cao et al., 2009; Hasegawa et al., 2016; Uchida et al., 2018). In conclusion, lipid-antigen presentation by LSECs to NKT cells might initiate further intercellular crosstalk with other immune effector cells like CD8 T cells and NK cells.

Of note, NKT cell has been shown to self-regulate its responsiveness to inflammatory challenges using intrinsic and extrinsic mechanisms. This regulation induces a hypo-responsiveness, which is later circumvented by the increased expression of antigens or secretion of IL-12 (Chiba et al., 2008; Choi et al., 2008). In line with these findings, our data show that altered IFN-γ release was not originating from NKT cells, suggesting a concomitant activation of CD8 T-cells and NK cells.

Previous studies of autoimmune diseases and α-GalCer administration showed that activation of NKT cells induces recruitment and activation of Th1 T-cells and NK cells (Santodomingo-Garzon and Swain, 2011; Smyth et al., 2002). This influence is attributed to a copious secretion of cytokines such as IFN-γ, TNF-α and IL-4 (Biburger and Tiegs, 2005; Wu and Van Kaer, 2011). Moreover, LSECs were shown to mount effector Th1 immune response via IL-6 secretion, and up-regulation of MHC-I and adhesion molecules (Böttcher et al., 2014; Schurich et al., 2010). Here we

show that the increased IFN-γ production originated from activation of CD8 T-cells and NK cells in response to LSEC but not DCs-induced NKT cell activation. In addition, we also observed their potential expression of other pro-inflammatory cytokines including TNF-α and the death-inducing molecule granzyme B (GzmB). From these findings, we postulate an amplifying role of LSEC-NKT activation in further activation of other immune cell populations within the liver that may help to mount a rapid defense against infection.

Liver injury is a stage of liver diseases associated with severe liver failure and elevated levels of serum alanine aminotransferase (ALT). Several studies showed that NKT cell-induced liver injury contributes in the development of different forms of liver diseases such as alcoholic liver diseases (ALD), non-alcoholic steatohepatitis (NASH) and non-alcoholic fatty liver disease (NAFLD) (Bandyopadhyay et al., 2016; Schrumpf et al., 2015; Takeda et al., 2000). Although measurements of serum ALT level and immunohistochemistry staining are widely used to assess liver failure, whether elevated ALT originates from dying hepatocytes or dysfunctional LSECs remains to be elucidated (Giannini et al., 2005; Kim et al., 2008). In this study, we used the xCELLigence technology, which measures the real-time cellular adhesion to high-density gold electrode printed on ex vivo culture-plate, to unveil the NKT cell-induced cytotoxicity against hepatocytes and LSECs. The index of cellular adhesion to the plate is directly proportional to cell viability and coherence of the cells (Kho et al., 2015).

Indeed, activated NKT cells produce various cytokines such IFN-γ, TNF-α, IL-4 and IL-17, which can support the recruitment and activation of Th-1 lymphocytes and neutrophils (Wang et al., 2013). Previous studies showed that accumulated-neutrophils can trigger oxidative killing of hepatocytes, while NKT cells induce direct killing of hepatocytes via Fas-FasL axis and TNF-α (Takeda et al., 2000). In line with this, we show here that NKT cells killed hepatocytes after recognizing α-GalCer presented in the context of CD1d molecules. In contrast, we did not find any decrease in the cell index of LSECs even after α-GalCer presentation to NKT cells and concomitant activation of CD8 T-cell or NK cell. These results supported the notion that LSECs served as an activation-platform for NKT cells, CD8 T cells and NK cells without becoming target of effector functions that would lead to loss of

sinusoidal integrity. Transcriptional and metabolic fingerprint of cross-primed LSECs need to be further investigated.

4.4 LSEC-mediated NKT cell activation induces CD8 T-cell and NK cell activation in a cell-cell contact dependent fashion

LSECs are considered as the gatekeeper of the liver due to their lining architecture of the hepatic vasculature. As non-professional antigen presenting cells, previous studies by others and us showed that LSECs present antigens to CD4 and CD8 T-lymphocytes via both MHC-II and MHC-I molecules (Burgdorf et al., 2007; Knolle et al., 1999; Limmer et al., 2000). Besides, LSECs show a copious expression of IL-6 and C-type lectins in response for antigen sensing, inducing adhesion and activation of patrolling leukocytes (Bashirova et al., 2001; Böttcher et al., 2014; Liu et al., 2004). For instance, stabilin-1, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion protein-1 (VAP1) were shown to regulate the T-cell migration across LSECs (Bertolino et al., 2005; Shetty et al., 2011). Moreover, chemokines such CXCL9, CXCL10 and CXCL16 represent also a selective route of leukocytes-arrest by LSECs through binding of G-protein coupled receptors (GPCRs) (Heydtmann and Adams, 2009; Heydtmann et al., 2005; Hokeness et al., 2007). Interestingly, we found here that co-culture of CD8 T-cells or NK cell with α-Galcer pulsed-LSECs in presence of NKT cells elicited their robust activation in a cell-cell contact dependent fashion. Consistently, paracrine signaling via LSEC/NKT cell culture supernatants failed to trigger any CD8 T-cell or NK cell activation. These results suggested a so far un-described role of LSECs in the activation of CD8 T-cells and NK cell after having induced NKT cell activation.

More recent studies showed that the chemokine receptors 2 and 5 (CCR2 and CCR5) are widely expressed on immune cells including NK cells, T-lymphocytes and monocytes, with an important role in hepatic inflammation (Dyer et al., 2019; Lefebvre et al., 2016). Additional studies used CCR2/CCR5 antagonists to treat hepatic impairment by inhibiting the lymphocyte adhesion to the endothelium (Silva-Vilches et al., 2018). These studies are in line with our results showing increased surface expression of CCR2/CCR5 on CD8 T-cell and NK cell upon their physical contact in LSEC-NKT co-culture. These findings open more questions regarding the underlying mechanism harbored by LSEC, and orchestrate the adhesion, activation or even regulation of immune cells in hepatic milieu.

4.5 LSEC/NKT cell activation produces panoply of pro-inflammatory mediators supporting physical contact-dependent CD8 T-cell and NK cell activation

Several studies showed that activation of NKT cell by α-GalCer induces the production of Th1-type and Th2 cytokines (Coquet et al., 2008; Gumperz et al., 2002; Lee et al., 2002). Other studies showed that NKT-induced liver injury was associated with increased expression of other pro-inflammatory cytokines such IL-1β, IL-6 and chemokines (Cua and Tato, 2010; Santodomingo-Garzon and Swain, 2011). Here, our comparison between the cytokine profile found in co-cultures of α-GalCer-pulsed LSEC, Hepatocytes and DCs with NKT cells confirmed this secretion of inflammatory cytokines. Interestingly, we noticed that the magnitude of cytokine production during LSEC/NKT cell activation was higher compared to DC/NKT cell activation. Furthermore, CD8 T-cell and NK cell activation in response for the LSEC/NKT cell activation significantly altered the cytokines secretion, perhaps highlighting the indispensable role of LSECs in NKT-induced hepatitis. Indeed, previous in vivo work showed that LSECs and chemokine production were critical to induce NKT cell accumulation and hepatic inflammation. Meanwhile, neutralization of chemokine-mediated LSEC function prohibited the NKT cell-mediated hepatitis (Wehr et al., 2013). Whether the activation of CD8 T-cell and NK cell is taking place in response for cytokine signaling or transcriptional regulation remains to be further addressed.

IL-6 is one of the main cytokines produced by LSECs upon antigen cross-presentation to T-lymphocytes. Although IL-6 signaling requires expression of two molecules; gp130 and gp80 resemble the IL-6 receptor, studies showed that IL-6 is able to form a receptor-ligand complex and induce a trans-signaling to neighboring cells lacking the gp80 expression such as CD8 T-cells (Knolle and Wohlleber, 2016; Rose-John, 2012). Along this line, studies showed that LSEC-derived antigen presentation to antigen-specific CD8 T-cells induced a rapid expression of GzmB in T-cells, which was attributed to Il-6 trans-signaling. However, these cells were not able to produce cytokines such as IFN-γ, IL-2 and TNF-α despite their cytotoxic capacity, suggesting that the rapid acquisition of cytotoxicity via IL-6 might not be enough to mount cytokine production (Böttcher et al., 2014). Here we showed that α-GalCer-pulsed LSECs produce high concentration of IL-6, whereas culturing of LSECs with CD8 T-cell or NK cell and in presence of external IL6 or hyper IL-6 (hIL-

6) failed to induce cytokine production. These results supported the notion that LSEC reacted to antigen cross-presentation by secretion of cytokines including IL-6 that might work synergistically to elicit a further immune response.

Another cytokine known to induce transcriptional activities and significantly produced during NKT-induced activation is IFN-γ. IFN-γ was shown to strongly mediate activity of various proteins, transcription factors, surface molecules and inflammatory cytokines (Hu and Ivashkiv, 2009; Schroder et al., 2004b). Here, we also show a rapid and exponential secretion of IFN-γ by LSEC-activated NKT cells leading to subsequent CD8 T-cell and NK cell activation. However, external IFN-γ did not elicit CD8 T-cell or NK cell activation upon a co-culture with LSECs. Consistently, in vivo studies reported that neutralization of IFN-γ failed to block α-GalCer-mediated hepatic injury. On the other hand, neutralizing antibodies against TNF-α were shown to reduce the hepatic injury in α-GalCer treated mice (Biburger and Tiegs, 2005; Inui et al., 2002). These findings propose the importance of IFN-γ to recruit neutrophils and lymphocytes to α-GalCer-induced heaptic injury and raise the question about TNF mediated immune response (Wang et al., 2013).

TNF-α is a pleiotropic cytokine that is secreted by different immune cells and induces various responses such as proliferation, transcriptional activations, pro-inflammatory cytokines production and cell death (Schwabe and Brenner, 2006). It binds to two known sets of TNF receptors (TNFR); TNFR1 and TNFR2, which are distinctly expressed on many cells and direct TNF function either for cell proliferation or cell death (Zhao et al., 2020). Of note, studies showed that TNF-α exists in two forms either membrane bound (mTNF) or soluble (sTNF) and both of them are capable to bind with TNFRs and induce intracellular processes (Sedger and McDermott, 2014). Meanwhile, several reports showed the increased hepatic-expression of TNF-α during α-GalCer or Concanavalin A (Con-A) mediated hepatitis (Biburger and Tiegs, 2005; Wu and Van Kaer, 2011). Here, our transcriptional flow cytometry and ELISA-based results showed that NKT cells produced high concentrations of TNF-α in response to LSECs presenting α-GalCer. Meanwhile, LSEC/NKT cell activation mediated further increase of TNF-α through a physical cell-contact with CD8 T-cell and NK cells. Furthermore, our data show that the replacement of NKT-derived TNF-α with a recombinant TNF-α could mount the activation of CD8 T-cell and NK cell. Consistent with our previous data, we also show that recombinant TNF-α elicit CD8

T-cell and NK cell activation upon their physical contact with LSECs, whereas this activation was completely abolished when cultured in the absence of LSECs. Our data suggested that TNF exerted its stimulatory function during α-GalCer mediated hepatitis indirectly through LSECs. Whether loss of function can be achieved by neutralizing TNF in vivo and the underlying molecular mechanisms for inducing CD8 T-cell or NK cell response via TNF-primed LSECs remain to be further investigated.

4.6 Extracellular ATP does not control CD8 T-cell and NK cell activation in response for LSEC/NKT cell activation

Previous studies showed that TNF-α can mediate an inflammatory response via metabolite sensitization independent of antigen recognition by MHC molecules and TLRs (Franchi et al., 2009; Han et al., 2020). In this pathway, TNF-α triggers Nod-like receptor p3 (Nlrp3) inflammasome activation through NF-κB transcriptional activation and increased expression of P2X7 receptors. Activated Nlrp3 was found to induce IL-1β secretion via the activation of caspase-1 in response to extracellular adenosine triphosphate (ATP) (Eigenbrod and Núñez, 2009). ATP is widely known as the primary energy-store within cells. More recently, extracellular ATP release by membrane channels (gap junctions, pannexin channels) was reported to play a pro-inflammatory function (Shyer et al., 2020). ATP can enter the cell through engagement with P2X7 receptor, which internalizes ATP and opens pores for cations such as sodium (Na^+), calcium (Ca^{2+}) and potassium (K^+) influx (Fantuzzi et al., 2019; Zhao et al., 2019). This ion influx induces a series of intracellular transcriptional-events including the activation of Nlrp3 inflammasome, which in turn triggers the production of various pro-inflammatory cytokines such IL-1β, IL-6, CXCL8 and CCL2 (Bachelerie et al., 2013; Martins et al., 2018). In this study we show that α-GalCer-dependent LSEC/NKT cell activation leads to a high secretion of ATP compared to a substantial secretion during DC/NKT cell activation, suggesting CD8 T-cell activation via LSEC-derived ATP. However, using monoclonal antibodies against P2RX7 to block ATP detection was unable to hamper the TNF-induced activation of CD8 T-cells. Consistent with our data, the activated CD8 T-cells did not show increase in the intracellular Ca^{2+} levels, suggesting that stimulation with TNF and the concomitantly secreted IL-1β might be sufficient to maintain caspase-1 activation even in presence of ATP inhibitors.

Additionally, recent studies demonstrated that ATP requires acetate to mount T-cell activation and cytokine secretion (Balmer et al., 2016; Degauque et al., 2018). Determination of TNF extent to induce Nlrp3 activation independent of ATP and analysis of acetate secretion by LSEC/NKT cell activation in response to α-GalCer antigen presentation remain to be studied.

4.7 CXCL16-CXCR6 axis and TNF-induced activation of CD8 T-cell and NK cell in contact with LSECs

LSECs were demonstrated to express high concentration of chemokines especially CXCL16 in response for hepatic infections. These molecules were shown to play inevitable role in the recruitment and regulation of immune cells within the liver (Bettelli et al., 2008). CXCR6 is the conserved receptor for CXCL16 and is highly expressed on NKT cell, NK cells and T-lymphocytes (Fernandez-Ruiz et al., 2016; Stegmann et al., 2016). Studies showed that LSECs up-regulate the level of CXCL16 molecules in hepatic viral infection or α-GalCer / Con-A induced hepatitis, inducing recruitment and retention of CXCR6+ lymphocytes on the endothelium (Heydtmann and Adams, 2009; Heydtmann et al., 2005). Here, we show that LSECs had increased mRNA expression of CXCL16 after CD1d-mediated antigen presentation to NKT cells. Several studies showed that liver resident NKT cells, NK and CD8 T-cells are characterized by high CXCR6 expression and low circulating capacity (Fernandez-Ruiz et al., 2016; Stegmann et al., 2016). In this study, we noticed that CXCR6 expression was stabilized on liver CD8 T-cells and NK cells after isolation in vitro, as they were kept in co-culture with LSECs. However, in absence of LSECs, previously CXCR6+ T-cells and NK cells lost their CXCR6 expression, becoming undetected any more at 24 hours after isolation and in vitro culture, suggesting an active process in maintaining CXCR6 expression, which is promoted by LSECs.

More recent studies reported that TNF contributes in endometriosis progression by activating the CXCL16-CXCR6 axis through the regulation of ERK1/2 signaling (Peng et al., 2019). Meanwhile, other studies showed the activation of CXCL16-CXCR6 axis within diverse models of hepatic injury (Darash-Yahana et al., 2009; Ma et al., 2018; Wehr et al., 2013). In line with these reports, our data show that CXCR6+ CD8 T-cells and NK cells co-cultured on LSECs were uniquely responsive to TNF-α induced activation. In contrast, we found a complete absence of CXCR6- CD8 T-cell and NK cell activation similar to that in absence of LSEC. This validated that CXCR6+ liver-

resident T cells have a potent and indirect response to the LSEC CD1d-mediated antigen presentation to NKT cells.

In sum, we have identified an important so far unknown role of liver sinusoidal endothelial cells to present lipid-antigens on CD1d molecules to NKT cells, which leads to an indirect activation of CXCR6+ CD8 memory T-cells and NK cells. We believe that our findings raise the prospect of harnessing the interplay between endothelial cells and NKT cells as well as immune effector cells locally in liver sinusoids and may help to design future immune therapies to increase intrahepatic immunity against infection or cancer.

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6. PUBLICATION AND POSTER PRESENTATION (Pre-publication)

6.1 Publication

- Four expected manuscripts are under writing process.

6.2 Poster presentation (Pre-publication)

- **Abdallah A. Yassin**, Bastian B. Hoechst, Percy A. Knolle (2020) “NKT cells crosstalk with liver sinusoidal endothelial cells and polyclonal induction of local-immune response”- Keystone Symposia on Molecular and Cellular Biology-Tissue Immunity (A6) Poster Number: 2044
- **Abdallah A. Yassin**, Bastian B. Hoechst, Percy A. Knolle (2019) “Crosstalk between liver NKT cells and liver sinusoidal endothelial cells”- EASL ILC 2019 poster number: SAT-382

7. LIST OF ILLUSTRATIONS AND FIGURES

7.1 ILLUSTRATIONS

7.2 FIGURES

8. LIST OF ABBREVIATIONS

ACK	Ammonium-Chloride-Potassium
Ag	Antigen
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
anti-	Antibodies
APCs	Antigen presenting cells
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
avidin-HRP	Avidin horseradish peroxidase
Bcl-2	B-cell lymphoma 2
Bim	Bcl-2-interacting mediator of cell death
BSA	Bovine serum albumin
BSA	Bovine serum albumin
BSL	Biosafety level
C57BL/ 6	Genetically modified mice for use as models of human disease
CAR-	Chimeric antigen receptor (CAR)-
CAR.GD2	GD2 ganglioside
Caspase	Cysteinyl aspartic acid protease
CCR	C-C chemokine receptor family
CD	Cluster of differentiation
cDNA	Complementary DNA
Con-A	Concanavalin A
CpG ODN	Cytosine triphosphate deoxynucleotide (C) followed by a guanine triphosphate deoxynucleotide (G) oligodeoxynucleotides
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand family
CXCR	C-X-C chemokine receptor family
DCs	Dendritic cells
DMEM	Dublecco´s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide

LIST OF ABBREVIATIONS

DN	Double negative
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme linked immune absorbent spot
EOMES	Eomesodermin (T-box brain protein)
FACS	Fluorescence-activated cell sorting
FAS	Fas-Membrane protein of the death receptor family
FASL	CD95L or CD178 - Type-II transmembrane protein of tumor necrosis factor (TNF) family
FCS	Fetal calf serum
FELASA	Federation of European Laboratory Animal Science Associations
Fig	Figure
FMO	Fluorescence minus one control
g	Gravitational force / Gram
G-CSF	Granulocyte colony-stimulating factor
GATA3	Transcription factor GATA3
GBSS	Gey's Balanced Salt Solution
GM-CSF	Granulocyte-macrophage colony stimulating factor.
GPCRs	G-protein coupled receptors
GzmB	Granzyme B
HBSS	Hank's Balanced Salt Solution
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
hIL-6	Hyper Interleukin-6
HLA	Human leukocyte antigen
HSCs	Hepatic stellate cells
i.v.	Intravenous
ICR	Internal control RNA
IDO	Indoleamine 2,3-dioxy-genase
IFN-γ	Interferon gamma

LIST OF ABBREVIATIONS

Ig	Immunoglobulin
IHLs	Intrahepatic lymphocytes
IL	Interleukin
ILCs	Innate lymphoid cells
iNKT	Invariant natural killer T (iNKT) cells
IP	Intraperitoneal
IU	International unit
IUIS	International Union of Immunological Societies
KC	Keratinocytes-derived chemokine
KCs	Kupffer cells
L	Liter
LPS	Lipopolysaccharides
LSECs	Liver sinusoidal endothelial cells
LTis	Lymphoid Tissue Inducers
MACS	Magnetic-activated cell sorting
MAIT	Mucosal-associated invariant T (MAIT) cells
MAPK1/ ERK1	Mitogen-activated protein kinase 3
MCP-1	Monocyte chemoattractant protein 1
MDSCs	Myeloid derived suppressor cells
MDSCs	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
mg	Milli-gram
MHC	Membrane histocompatibility complex
min	Minute
MIP-1	Macrophage inflammatory protein 1
ml	Milli-liter
MR	Mannose receptors
mRNA	messenger RNA
mTNF	membrane Tumor necrosis factor
MTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor kappa light-chain enhancer of activated B cells

LIST OF ABBREVIATIONS

NK	Natural Killer cells
NKG2D	C-type lectin-like receptor expressed on CD8+ T and NK cells
NKT	Natural Killer T (NKT) cells
Nlrp3	NOD-like receptors p3
nM	Nano-Molar
NPCs	Non-parenchymal cells
ns	Not significant
Ova	Ovalbumin
P-value	Probability value
P2RX7	P2X purinoceptor 7
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PDL-1	Programmed death-ligand 1
PLZF	Promyelocytic leukemia zinc finger
PPARα	peroxisome proliferator activated receptor α
PRR	Pattern recognition receptor
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RORγt	Related orphan nuclear receptor
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
RT	Reverse transcriptase
s / sec	Second
SARS	Severe acute respiratory syndrome
SD	Standard deviation
SEM	Standards error mean
sTNF	Soluble tumor necrosis factor
T-bet	T-box transcription factor
TCRs	T-cell receptors
TGF-β	Transforming growth factor - Beta
T_h	T helper cells
TLRs	Toll-like receptors

LIST OF ABBREVIATIONS

TNF	Tumor necrosis factor
TNFRs	TNF receptors
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
Treg	Regulatory T-cells
U	Units
vp	Viral particles
WHO	World health organization
wt / WT	Wild type
α-GalCer/ α-GC	α-Galactosyl Ceramide
γT	Gemma T lymphocytes
δT	Delta T lymphocytes
ζ-chain	Zeta chain
µg	Micro-gram
µl	Micro-liter
µm	Micro-meter
µM	Micro-Molar