Analysis of invariant natural killer T cells in health and disease: lessons from acute hepatitis C virus infection

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1.Zusammenfassung

Invariante natürliche Killer T-Zellen (iNKT-Zellen) vereinen die Eigenschaften von angeborenen und adaptiven Immunzellen und können die Immunantwort entscheidend modulieren. Da ihre Rolle bei humanen Virusinfektionen nur unzureichend verstanden ist, wurde eine longitudinale Analyse von iNKT-Zellen bei Patienten mit akuter Hepatitis C Virus (HCV) Infektion mit unterschiedlichem Krankheitsverlauf durchgeführt. In weiteren Analysen wurden die zugrundeliegenden Mechanismen der iNKT-Zellaktivierung und -Funktion detailliert untersucht.

Die Frequenz von aktivierten, $CD38^+$ und $CD69^+$ iNKT-Zellen war in der frühen Phase der akuten Infektion zunächst hoch, bevor sie in Patienten mit selbst-limitierender Infektion deutlich abnahm. Im Gegensatz dazu blieb die Zahl der aktivierten iNKT-Zellen bei Patienten, die eine chronische Erkrankung entwickelten, konstant erhöht. Die Häufigkeit von iNKT-Zellen, die Marker für Aktivierung, Zytotoxizität und Leber-„Homing"-Rezeptoren exprimieren, korrelierte signifikant mit der Leberentzündung, insbesondere bei Patienten, bei denen die Infektion ausheilte. Dies deutet auf eine Beteiligung von iNKT-Zellen an der Immunantwort gegen HCV während der akuten Infektion hin und legt weiterhin nahe, dass diese Zellen an der Entstehung der Leberentzündung während der HCV Eliminierung beteiligt sind. Die ex vivo Analyse von $CD38^+$ iNKT-Zellen zeigte eine allgemeine Disfunktionalität im Vergleich zu $CD38^-$ iNKT-Zellen auf. Dabei sekretierten $CD38^+$ Zellen weniger IFN-γ, TNFα oder produzierten Granzyme B. Darüber hinaus proliferierten sie weniger in Abwesenheit eines spezifischen T-Zell-Rezeptor Stimulus und wiesen zeigten eine geringere Expression der Transkriptionsfaktoren T-bet und PLZF auf. Mittels RNA-Sequenzierung wurde eine bisher unbekannte Transkriptionssignatur aktivierter menschlicher iNKT-Zellen nachgewiesen, wobei die Expression von Interleukin-32 herausragte. Eine detaillierte Analyse zeigte, dass die Expression von IL-32 auf iNKT-Zellen und anderen Populationen von angeborenen Lymphozyten beschränkt ist und unabhängig von TZR Signalen induziert wird. IL-32 produzierende iNKT-Zellen waren überwiegend in der CD4-Subpopulation vorhanden und sezernierten unverändert IFN-γ, TNFα und Granzyme B, was das proinflammatorische Potential von $CD4^-$ iNKT Zellen verdeutlicht.

Insgesamt erweitert diese Arbeit den Wissenstand über die Rolle von iNKT-Zellen in Zusammenhang mit der HCV-Immunität erheblich. Der einzigartige Phänotyp von $CD38^+$ iNKT-Zellen, der mit einer schlechten Effektor-Funktion verbunden ist, könnte auch bei anderen Viruserkrankungen eine Rolle spielen. Das hier beschriebene Genexpressionsmuster gibt Aufschluss über den allgemeinen Aktivierungsmechanismus menschlicher iNKT-Zellen und trägt damit wesentlich zu einem besseren Verständnis der von iNKT-Zellen abhängigen immunologischen Prozesse sowohl bei Krankheiten, als auch unter gesunden Bedingungen bei.

2. Summary

Invariant natural killer T (iNKT) cells show characteristics of innate and adaptive immune cells and potently modulate the downstream immune response. As their role during human viral infections is poorly understood, a longitudinal analysis of iNKT cells in patients with acute hepatitis C virus (HCV) infection with different outcome was conducted. In further analyses, the underlying mechanisms of iNKT cell activation and function were closely investigated.

The frequency of activated, $CD38^+$ and $CD69^+$ iNKT cells was initially high during the early phase of acute HCV infection before it significantly declined in patients with self-limited infection. In contrast, levels of activated iNKT cells remained consistently elevated in patients that progressed to chronic disease. The frequency of iNKT cells expressing markers of activation, cytotoxicity and liver homing receptors significantly correlated with liver inflammation, particularly in resolving patients. This indicates an involvement of iNKT cells in the anti-HCV immune response during acute infection and furthermore suggests that these cells contribute to liver inflammation during the clearance of HCV. Ex-vivo analysis of $CD38^+$ iNKT cells revealed a generalized functional deficiency in comparison to $CD38^-$ iNKT cells. Less $CD38^+$ cells secreted IFN-γ, TNFα or produced Granzyme B. Moreover, they proliferated less in the absence of a specific T cell receptor (TCR) stimulus, and showed low levels of the transcription factors T-bet and PLZF. RNA sequencing revealed a so far unknown transcriptional signature of activated human iNKT cells including the prominent expression of interleukin-32. Detailed analysis showed IL-32 expression is narrowly restricted to iNKT and other innate-like lymphocyte populations and is induced independently of TCR signalling. IL-32 producing iNKT cells were predominantly present in the $CD4^-$ subpopulation and stably secreted IFN-γ, TNFα and Granzyme B pointing out the proinflammatory potential of $CD4^-$ iNKT cells.

Taken together, this work significantly expands the knowledge about the role of iNKT cells in the context of HCV immunity. The unique phenotype of $CD38^+$ iNKT cells that is linked to poor effector function could also be implicated in other viral diseases. The gene expression pattern described here provides information on the general activation mechanism of human iNKT cells and thus significantly contributes to a better understanding of NKT cell-dependent immunological processes in health and disease.

3. Introduction

3.1. The immune system

The human immune system has evolved to protect the body against a plethora of foreign pathogens like bacteria and their toxins, parasites, and viruses. It consists of an innate and an adaptive branch each equipped with unique effector functions to fight off infections and other diseases. The innate immune system is the first line of defence against invading pathogens and can immediately respond to infection. The adaptive immune system is slower, yet highly specific, and needs several days before effectively clearing an infection. Another hallmark of the adaptive immune system is the generation of an immunological memory that confers long-lasting protection against the same antigen. Both arms of the immune system act in a highly coordinated manner in order to protect the host against a wide range of pathogens.

3.1.1. The innate immune system

The innate immune system comprises all entities that exist from birth on and have the ability to protect the host against environmental influences. This includes physical barriers like skin and mucosa, chemical barriers like gastric acid, as well as humoral factors such as the complement system or secretory antimicrobial peptides (Diamond et al. 2009; Kenneth Murphy 2018). A central dogma of the immune system is the discrimination between self and non-self. In the innate immune system, this task is fulfilled by germline encoded pattern recognition receptors (PRR) that recognize distinct non-self patterns in molecules derived from pathogens, so-called pathogen associated molecular patterns (PAMP) (Janeway and Medzhitov 2002; Iwasaki and Medzhitov 2010).

A crucial mediator of innate antiviral immunity is the interferon system. Interferons (IFN) were the first described cytokines (Pestka, Krause, and Walter 2004) and are induced by virus infection among a variety of other stimuli. Interferons can be classified into three subgroups. Type I interferons contain IFN-α and IFN-β which bind to their heterodimeric receptor composed of IFNAR1 and IFNAR2 subunits that is present on all nucleated cells. Thus, all cells are susceptible to type I IFN signalling which provides a potent protective mechanism against viral infection regardless of cell type. Type I IFNs are induced immediately after infection downstream of PRR signalling, e.g. by cytosolic RIG-I or by

endosomal toll like receptors (TLR) triggered by sensing of virus associated PAMPs, e.g. by cytosolic RIG-I or by endosomal toll like receptors (TLR). Even though virtually all cells are capable of producing type I interferons, plasmacytoid dendritic cells (pDC) exist as highly specialized type I IFN producers that secrete up to 1000-fold more IFN than any other cell type thereby strongly augmenting interferon dependent immune responses (Kenneth Murphy 2018). IFNs induce interferon-stimulated genes (ISG) downstream of IFN-α receptor ligation, which exert the antiviral mechanisms for instance by interfering with protein translation and viral replication as a consequence, stalling virus spreading (Kenneth Murphy 2018). Type III IFNs were discovered later than type I IFNs and encompass IFN-λ1 to IFN-λ4 (IFN-λ1-3 were formerly called IL-29, IL-28A and IL-28B). They have similar features as type I IFNs but differ in the tissue distribution of their receptor (Heim and Thimme 2014). The IFNLR1 subunit of the type III IFN receptor is expressed mainly on epithelial cells resulting in a tissue specific activity of IFN-λ (Hoffmann, Schneider, and Rice 2015). The type II IFN family comprises IFN-γ as its only member which is secreted by NK cells and effector T cells and thus, is triggered downstream of the immediate type I and III IFN response. The IFN-γ receptor is ubiquitously expressed and therefore transmits anti-viral and anti-tumorigenic activity in every cell type (Hoffmann, Schneider, and Rice 2015). Cellular effects of IFN-γ signalling are upregulation of MHC class I and II molecules, the activation of macrophages and regulation of leukocyte migration by upregulation of chemokines and adhesion molecules among many others (Schroder et al. 2004).

Cells assigned to the innate immune system encompass granulocytes, phagocytes such as macrophages and monocytes, as well as dendritic cells (DC) and natural killer (NK) cells. As the main effector cell of the innate cellular immune response, NK cells are crucial for the early response against viral infections (Abel et al. 2018). They are a productive source of IFN-γ and exert spontaneous cytotoxicity against infected cells and pave the way for the slower adaptive immune response which eventually clears the infection (Rehermann 2015).

NK cells arise from the same lymphoid progenitor as B- and T cells and consequently bear a striking similarity to $CD8^+$ T cells in terms of cytotoxicity and IFN-γ release but unlike T cells, they do not require priming upon antigen encounter. NK cell activity is tightly regulated by the integration of signals from a large array of activating and inhibiting surface

receptors. The absence of inhibitory ligands, e.g. certain MHC molecules ("missing-self" recognition) or upregulation of activating ligands, e.g. stress induced molecules ("induced-self" recognition), on the surface of a target cell will eventually lead to NK cell activation and killing of the target cell (Shifrin, Raulet, and Ardolino 2014). Most NK cell receptors are divided into three broader families, the activating natural cytotoxicity receptors (NCR), the killer-cell immunoglobulin like receptors (KIR) and c-type lectin receptors of the NKG2 family(Farag et al. 2002). Furthermore, NK cells express NK1.1 – the homolog to human CD161 – in mice and CD56 in humans but not the T cell lineage marker CD3 and account for about 10-15 % of all peripheral blood mononuclear cells. Based on CD56 expression levels, human NK cells are classified into different subgroups. $CD56^{dim}$ NK cells are believed to be mostly cytotoxic while $CD56^{bright}$ NK cells are predominant IFN-γ producers.

3.1.2. The adaptive immune system

Since the innate immune system is capable to inhibit but not always to completely eradicate certain pathogens, the adaptive immune system is required to confer sterile immunity and restore tissue homeostasis. Hallmarks of the adaptive immune system are variable, non-germline encoded antigen receptors that enable highly specific recognition of an almost infinite number of antigens. Moreover, the establishment of an immunological memory confers long-lasting protection to the host in case of re-exposure to the same pathogen. The adaptive immune response is slower than the innate immune response as it requires priming of naïve cells that differentiate into effector cells. After the first encounter with their cognate antigen, adaptive immune cells clonally expand and differentiate into effector cells, which are highly potent in clearing pathogens. After antigen clearance, a fraction of adaptive immune cells differentiate into long-lived memory cells that elicit a rapid secondary response upon antigenic rechallenge.

In accordance to the innate immune system, the adaptive immune system comprises a humoral and a cellular part, too. Humoral immunity is based mainly on antibodies that are secreted by B cells. Antibodies specifically bind to foreign epitopes and either neutralize or opsonize the antigen or activate the complement system in order to lyse an antibody tagged pathogen (Kenneth Murphy 2018). B cells are the key cells of the humoral arm of the adaptive immune system. They can recognize extracellular pathogens or other non-self-structures via their membrane bound B cell receptor (BCR). When activated by BCR

ligation, they secrete antibodies with the same antigen specificity as the BCR. B cells undergo two processes that enhance their efficacy. Antibody class switching gives rise to antibodies with specialized tissue localization and distinct functional properties. Affinity maturation enhances the affinity of antibodies to their respective epitope through somatic hypermutation.

The cellular arm of the adaptive immune system consists mainly of T cells. T cells also possess a highly specific antigen receptor called T cell receptor (TCR). In contrast to the BCR that can recognize structural antigens of any origin, the TCR of conventional T cells is limited to peptide antigens. T cells are coarsely classified into two groups according to their surface expression of CD4 and CD8. $CD4^+$ T cells are also called T helper cells and orchestrate cellular and humoral adaptive as well as innate immune responses as the main modulator of the immune system. $CD8^+$ T cells have cytotoxic activity and can efficiently kill cells that are either infected by an intracellular pathogen or malignantly transformed. T cells develop from common lymphoid progenitors from the bone marrow that migrate into the thymus where T cell differentiation and maturation occurs. T cell development is a complex process that is only briefly described here but is extensively reviewed elsewhere (Kenneth Murphy 2018). A central part of T cell development is the rearrangement of the T cell receptor alpha and beta chains from an array of gene segments. The alpha chain locus contains a set of variable and joining gene segments that rearrange to a functional alpha chain. The beta chain rearranges from variable, diversity and joining regions and couples with the rearranged alpha chain to form a fully functional TCR. The addition of randomly inserted n-nucleotides during rearrangement further enhances TCR diversity ultimately giving rise to a putative 10^{20} unique TCRs, a number that exceeds by far the amount of genes in a human genome and is the keystone for the huge antigen recognition diversity of adaptive immune cells (Godfrey et al. 2015).

A fundamental feature of T cells is the antigen recognition with restriction to major histocompatibility complex (MHC) molecules (in humans called human leukocyte antigen, HLA). In contrast to B cells, conventional αβ T cells are not able to bind to antigen unless it is complexed with MHC molecules. Classical MHC molecules can be subdivided into MHC class I and MHC class II. The MHC class I molecules are expressed on every nucleated cell and present endogenous peptides to patrolling $CD8^+$ T cells. If a cell is infected or transformed, it will present aberrant or non-self peptides to $CD8^+$ T cells that

will then respond by secreting IFN-γ or trigger apoptosis in the target cell. MHC class II molecules are expressed in professional antigen presenting cells (APC), like B cells, macrophages and dendritic cells. These cells constantly sample extracellular material, which is presented to $CD4^+$ T cells in a complex with MHC class II molecules (Kenneth Murphy 2018).

Besides conventional T cells that have been introduced so far, other T cells with unique immunological functions exist. These unconventional T cells can either express a TCR that is composed of a gamma and a delta chain, or express an αβ-TCR that has limited diversity and is thus called semi-invariant. A common feature of these unconventional T cells is their restriction to non-classical MHC molecules and their potential to recognize non-peptide antigens. Unconventional T cells are now known to prominently contribute to a multitude of immunological processes and have been reviewed in detail (Godfrey et al. 2015).

3.1.3. Linking the innate and the adaptive immune system

As mentioned before, the innate immune system is the first line of defence as it prevents the unrestricted growth of pathogens due to a rapid response. However, usually innate immunity only slows down pathogen spread until the adaptive immune system takes over to resolve the infection. The activation of adaptive immune cells depends on signals from the innate immunity. Professional antigen presenting cells, such as DCs are an exceptional example of cells that connect the two branches of the immune system. As innate cells, they sense the infection and then activate the downstream adaptive immune response by providing signals for T cell activation via presentation of antigen-MHC complexes (Palucka and Banchereau 1999; Steinman 2006; Novak et al. 2010). Bridging cells are located on the interface of the two immune compartments and can influence the downstream immune response by providing modulatory signals to other immune cells (Fig. 1). These can include cytokines, chemokines or cell-cell contact dependent signals. Another cell type that is able to bridge the innate and the adaptive immune system are NKT cells. NKT cells are unconventional T cells that phenotypically and functionally resemble both NK cells and T cells. Even though they possess many characteristics from the innate immune system like a rapid effector response and non-diverse receptors that recognize microbial derived structures, they are also considered T cells as they express

a rearranged TCR and develop in the thymus. NKT cells interact with many innate as well as adaptive immune cells and fine tune multifaceted immune responses by releasing many different cytokines (Van Kaer, Parekh, and Wu 2011). A detailed principal of iNKT cell function is described in section 3.2. Briefly, NKT cells respond to activated DCs by rapidly conveying activating or inhibitory signals to other immune cells (Figure 1). Hence, NKT cells act as key modulators between innate and adaptive immunity and are rightfully considered a bridge between these two compartments.

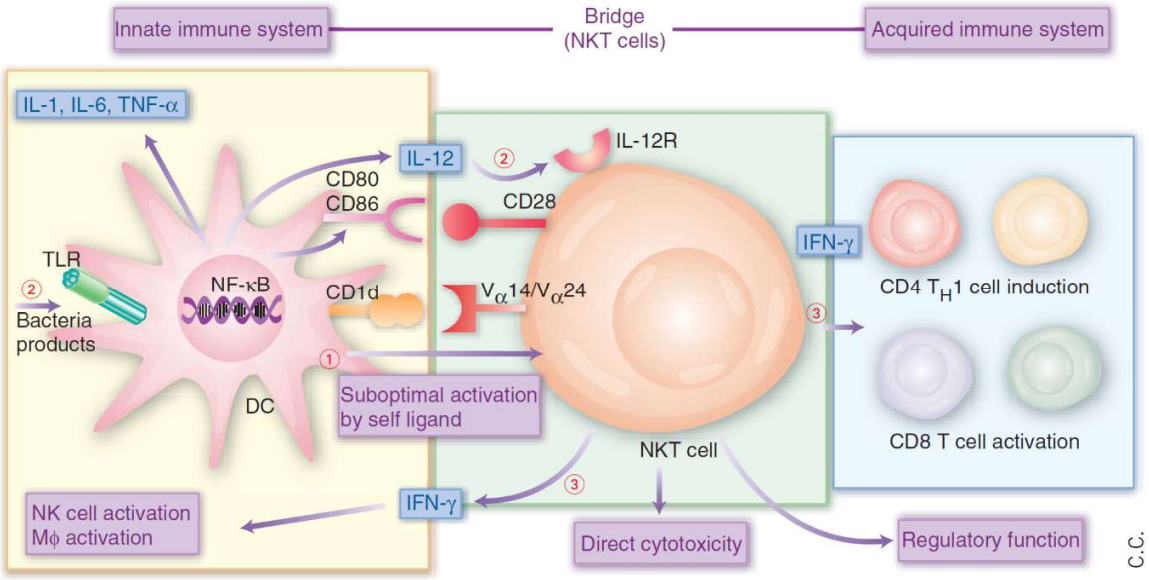

Figure 1. Invariant NKT cells bridge the innate and the adaptive immune system (Taniguchi, Seino, and Nakayama 2003). Invariant NKT cells can interact with cells from the innate immune system, such as DCs as well as with cell from the adaptive immune system such as conventional CD4+ and CD8+ T cells. They actively modulate both sites of the interaction and thus link adaptive immune cells with the signals provided by innate immune cells.

3.2. Type 1 invariant natural killer T cells

Natural killer T (NKT) cells are unconventional T lymphocytes that are restricted to the non-classical MHC class 1-like molecule CD1d. NKT cells recognize endogenous as well as exogenous lipid rather than peptide antigens and unify characteristics of the innate and adaptive immune system. While NKT cells are considered to be part of the T cell lineage as they express a TCR-CD3 complex and are matured in the thymus, they can also express markers that classically define NK cells like CD161 (NK1.1 in mice) and CD56.

This coexpression of CD3 and NK cell markers was often used to define NKT cells in the past, however recently a much more elaborate definition prevails. Nowadays, NKT cells are separated into two distinct classes based on their TCR repertoire: type 1 or invariant natural killer T cells (iNKT) and type 2 or non-invariant NKT cells. Type 1 NKT cells are the best characterized subset today. They express a semi-invariant TCR that is comprised of a Vα14 chain that is coupled with the Vβ8, Vβ7 or Vβ2 beta chain in mice. In humans, the NKT TCR is considered to be completely invariant as it is almost always comprised of a Vα24Jα18 alpha chain rearrangement combined with a Vβ11 beta chain in germline configuration (Bendelac, Savage, and Teyton 2007; Exley, Wilson, and Balk 2017). Invariant NKT cells are reactive to their prototypic antigen α-Galactosyceramide (αGalCer) and other glycolipids. Type 2 NKT cells use a more diverse TCR and recognize an array of glyco- and phospholipids and in some cases even hydrophobic peptides but not αGalCer. While in mice type 1 NKT cells are far more abundant than type 2 NKT cells, this ratio is reversed in humans. However, knowledge about human type 2 NKT cells is still limited, mostly due to the lack of specific staining reagents. As this work focuses on type 1 NKT cells, type 2 cells are only briefly introduced but have been reviewed in detail elsewhere (Dhodapkar and Kumar 2017; Singh, Tripathi, and Cardell 2018; Nishioka et al. 2018; Kato, Berzofsky, and Terabe 2018).

3.2.1. iNKT cell development

Like any other αβ-T cells, iNKT cells develop in the thymus. They originate from bone marrow derived common lymphoid progenitors that migrate into and mature in the thymus. Thymic maturation of conventional T cells is a complex process that is described in the literature in great detail and shows some similarities and a lot of differences to the maturation of iNKT cells (Kenneth Murphy 2018). Briefly, developing T cells are subject to positive and negative selection in the thymus. While positive selection ensures maturation of cells expressing a TCR that facilitates binding to peptide MHC complexes, negative selection eliminates cells that bind self-peptides with high affinity. This process is crucial for the maturation of functional T cells that are able to recognize peptides presented via MHC whilst prohibiting autoimmunity by T cells with high affinity to autoantigens. Conventional T cells exit the thymus as naïve cells. Upon the first encounter with their cognate antigen, they differentiate into effector and ultimately into memory T cells.

Different signals during this priming polarize e.g. the $CD4^+$ T helper (Th) cells into distinct effector subpopulations. Th1 cells secrete mainly IFN-γ and mediate inflammation in response to intracellular pathogens. Th2 cells release IL-4 and IL-13 and can act anti-inflammatory. TH17 are proinflammatory cells that are characterized by secretion of IL-17 and IL-22.

For obvious reasons of sample availability, most of the work on iNKT cell development was performed in mice. Very few studies analysed human thymic iNKT cells and uncovered that the number of thymic iNKT cells is highest during embryogenesis but already extremely low in the neonatal thymus (Sandberg et al. 2004). Even though the information gained by the study of human thymic iNKT cells is nowhere near that comprehensive as in mouse models, some similarities and differences between these two could be observed. Two other papers revealed that human thymic iNKT cell development generally proceeds similar to mice. However, some differences are also apparent, as CD161- iNKT cells, the human equivalent to NK1.1 that can be also used for classification of iNKT cell subsets, are far more abundant in the human than in the mouse thymus (Godfrey and Berzins 2007). Therefore, the following section focuses on the knowledge of iNKT cell developmental that was gained in mouse models.

The unique developmental trajectory of NKT cell development branches off from that of conventional T cells at the double positive stage, which follows the double negative stage and is characterised by the expression of both CD4 and CD8. Contrary to earlier believes, iNKT cells do not develop from a precursor committed to the iNKT lineage even before TCR expression (committed precursor model) but rather choose the path of the iNKT cell developmental program upon rearrangement of their invariant TCR (TCR instructive model) (Das, Sant'Angelo, and Nichols 2010). Similar to conventional αβ-T cells, iNKT cells are also subject to positive and probably also to negative selection in the thymus. However, the involved processes appear to be fundamentally different to conventional T cells. As soon as the invariant TCR is expressed during the stochastic process of TCR rearrangement, the cells start the lineage differentiation program of iNKT cells. This happens by interaction with other CD1d expressing double positive cortical thymocytes instead of cortical thymic epithelial cells (Das, Sant'Angelo, and Nichols 2010).

Positive selection of iNKT cells is dependent on recognition of endogenous lipids presented via CD1d. Even though these lipids are not unequivocally defined yet, recent

data hints towards the surprising existence of trace amounts of αGalCer and α-glucosylceramides (αGluCer) in the mouse thymus (Kain et al. 2014; Kain et al. 2015). Furthermore, the peroxisome- derived lysophospholipid antigens plasmalogen lysophosphatidylethanolamine and lysophosphatidic acid were suggested to be involved in iNKT cell positive selection (Facciotti et al. 2012).

Today, two models exist to describe thymic iNKT cell development. The linear model, describes a stepwise development of iNKT cells from stage 0 to stage 3. These stages are classified by the expression of CD24, CD44 and NK1.1, which is used as a maturity marker. According to this model, iNKT cells emigrate from the thymus at the immature stage 2 and mature in the periphery into NK1.1^{+}, stage 3 cells. However, it has now become clear that iNKT cells with a stage 2 phenotype found in the periphery are indeed mature NKT2 cells (Lee et al. 2013). Invariant NKT cells mature in the thymus into NKT1, NKT2 and NKT17 subsets from a common precursor following stage 0. Classification of iNKT subsets into NKT1, NKT2 and NKT17 is based on differential expression of master transcription factors promyelocytic leukaemia zinc finger (PLZF), T-box transcription factor TBX21 (T-bet) and RAR-related orphan receptor gamma t (RORyt) alongside the cytokine profile well known from T helper cell subsets. While NKT17 and NKT2 cells leave the thymus early as fully matured cells with a stage 2 phenotype, NKT1 cells resemble a stage 3 phenotype that is acquired in the periphery by NK1.1 upregulation. On the other hand, thymic NKT cells with a stage 3 phenotype are considered long lasting thymic residents (Pellicci, Koay, and Berzins 2020).

The positive selection of iNKT cells is accompanied by expression of transcription factor early growth response protein 2 (EGR2) and signalling via the signaling lymphocytic activation molecule (SLAM)-associated protein (SLAM-SAP) axis. This induces the expression of the master transcription factor PLZF that controls the development of innate T cells. PLZF signalling confers the poised effector phenotype on developing iNKT cells which is a hallmark of peripheral iNKT cells in contrast to conventional T cells that require priming in the periphery in order to acquire this effector phenotype (Gapin 2016; Savage, Constantinides, and Bendelac 2011; Kovalovsky et al. 2008).

3.2.2. iNKT cell activation

Invariant NKT cell activation can occur in either an antigen-dependent or an antigen-independent process. It has been proven that iNKT cells can directly recognize CD1d presented microbial glycolipids derived from *Sphingomonas* spp. and *Borrelia burgdorferi* (Mattner et al. 2005). Today, a variety of exogenous iNKT cell antigens derived from bacterial pathogens is described (Brigl and Brenner 2010). All of these bacterial lipid antigens provide a strong TCR signal to iNKT cells when presented via CD1d on the surface of APCs. This signal alone is sufficient for iNKT activation and does not require TLR signalling and subsequent cytokine release by DCs (Brigl and Brenner 2010). Interestingly, despite their ability to recognize these pathogen-derived antigens, iNKT cell activation during microbial infection is predominantly caused by APC-mediated IL-12 signalling while the TCR-dependent activation only plays a minor role (Brigl et al. 2003; Brennan, Brigl, and Brenner 2013). In this process, a weak TCR signal is delivered to the iNKT cells either by an exogenous antigen or by a lipid self-antigen presented by dendritic cells. In order to ensure that self-lipid mediated activation of iNKT cells does not occur under steady-state conditions, CD1d dependent activation is enhanced by several distinct ways during inflammation. Upon pathogen encounter, APCs can substitute CD1d binding glycolipids with very weak stimulatory capacity for self-lipids that are stronger TCR ligands(Brigl and Brenner 2010). Additionally, APCs can upregulate CD1d expression in response to innate stimuli like TLR or IFN signalling. Lastly, iNKT cells can also be activated independently of CD1d. Invariant NKT cells secrete IFN-γ in response to rigorous stimulation with proinflammatory cytokines IL-12 and IL-18 even in the absence of CD1d (Hou et al. 2003; Leite-De-Moraes et al. 1999). Collectively, iNKT cells respond to very diverse stimuli. Their activation either can rely solely on exogeneous TCR ligands or can be solely cytokine mediated. In between, iNKT cells are activated by recognition of endogenous lipids presented via CD1d in conjunction with DC-derived IL-12. This reflect the great functional heterogeneity of iNKT cells and explains their ability to modulate so many different immune processes.

3.2.3. iNKT cell subsets

Human iNKT cells can be divided into $CD4^+CD8^-$, double negative (DN) and additional $CD8^+CD4^-$ subgroups. Even though the purpose of CD4 and CD8 expression of iNKT cells is not clear, as they do not interact with classical MHC class I and II molecules, each subset display a unique phenotype and function (Godfrey and Berzins 2007). Human DN and $CD8^+$ iNKT cell subpopulations produce mainly Th1 cytokines, such as IFN-γ and TNFα. $CD4^+$ iNKT cells can produce Th1 as well as the Th2 cytokines IL-4 and IL-13 (Lee et al. 2002; Gumperz et al. 2002). Studies with in vitro expanded iNKT cells hint towards cytotoxic activity of iNKT cells that is mainly focussed on the $CD8^+$ subpopulation (Snyder-Cappione et al. 2010). There are also hints towards a human iNKT subtype that produces Th17 cytokines in a proinflammatory environment, however, this cannot be identified exclusively on the basis of CD4 and CD8 expression (Moreira-Teixeira et al. 2011). Besides their cytokine profile, human iNKT subsets also differ in their phenotype. $CD4^+$ and $CD4^-$ human iNKT cells each express a unique pattern of cytokine and chemokine receptors (reviewed in Kim, Butcher, and Johnston 2002). Furthermore, some cells within the $CD4^+$ subpopulation express CD62L which is completely absent in $CD4^-$ iNKT cells consistent with different tissue homing properties (Juno, Keynan, and Fowke 2012; Sandberg et al. 2002). This $CD62L^+$ iNKT cell subpopulation was shown to possess superior persistence and anti-tumour activity in vivo (Tian et al. 2016).

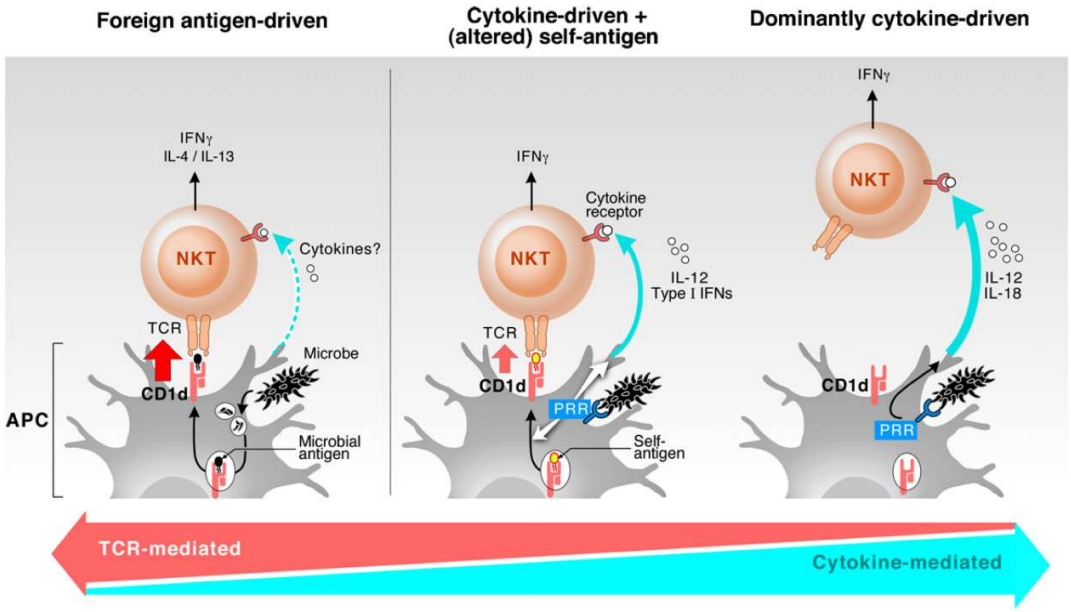

Figure 2. TCR-dependent and independent activation of iNKT cells is mediated by exogenous or endogenous lipid antigens presented by CD1d and IL-12 (Brigl and Brenner 2010). Invariant NKT cells can be activated by a strong TCR stimulus via CD1d bound foreign antigen, which is presented by DCs. Furthermore, activation of iNKT cells by weak TCR ligation from a CD1d restricted self peptide occurs in combination with a strong cytokine stimulus by DCs. A completely TCR-independent activation of iNTK cells also possible by cytokines IL-12 and IL-18.

3.2.4. Interaction of iNKT cells with other immune cells

In line with their unique functional properties, iNKT cell subsets differentially elicit activation of $CD4^+$ T cells, $CD8^+$ T cell, B cells and NK cells (Lin et al. 2006). Nevertheless, human iNKT cell crosstalk is not limited to other lymphocytes but includes a variety of immune cells to shape and fine tune innate and adaptive immune responses. Invariant NKT cell clones were shown to provide maturation signals to αGalCer presenting dendritic cells in a process involving the CD40/CD40L axis. This DC maturation is followed by IL-12 release from dendritic cells and cytokine secretion by iNKT cells (Liu et al. 2008; Vincent et al. 2002). The iNKT cell response to CD1d expressing dendritic cells strongly depends on the iNKT cell subtype. While $CD4^+$ iNKT cells release IL-4 and IL-13 in addition to IFN-γ after DC stimulation, DN iNKT cells kill the DC, posing a mechanism to reduce DC derived IL-12 signalling thereby fine tuning the downstream balance of the Th1/Th2 immune response (Liu et al. 2008). They were also shown to mediate crosstalk between plasmacytoid and myeloid dendritic cells via CD1d interaction in a mechanism

dependent on IFN-α and other soluble factors secreted by pDCs. This interaction results in IFN-γ secretion by iNKT cells and is believed to evoke iNKT cell responses during pDC sensed viral infections (Marschner et al. 2005; Montoya et al. 2006). Interaction of iNKT cells with professional APCs is not limited to dendritic cells. They also have been shown to provide survival signals to monocytes via the 4-1BB/4-1BBL axis. During inflammation, these survival signals are overridden by signals that trigger apoptosis of monocytes resulting in iNKT cell mediated control of an exuberant immune response caused by infiltrating monocytes at the site of inflammation (Cole et al. 2014). Moreover, B cells release IgM, IgA and IgG after in vitro co-culture with expanded iNKT cells even in the absence of an exogenous iNKT ligand. Co-culture with iNKT cells leads to B cell maturation as evidenced by upregulation of CD86 and CD40 on B cells. Reciprocally, B cells stimulate cytokine release from iNKT cells even though less effectively than dendritic cells (Zeng et al. 2013). Following αGalCer stimulation, iNKT cells release soluble factors that augment in vitro NK cell cytotoxicity (Moreno et al. 2008). Conversely, iNKT cells inhibit immune responses by amplifying regulatory T (T_{reg}) cell proliferation via IL-2 secretion (Jiang et al. 2005). In turn, T_{reg} cells can suppress cytokine production and cytotoxicity against tumour cell lines of Vα24$^+$ iNKT cells in a cell-cell contact dependent manner (Azuma et al. 2003).

3.2.5.iNKT cells in viral infections

Invariant NKT cells are implicated in the immune response against various microbial infections. Their role in bacterial infection has been excellently described and is underlined by the numerous bacterial iNKT cell antigens already identified (Kinjo, Kitano, and Kronenberg 2013). Despite lipid antigens are not found in viruses, multiple studies in mice and humans have highlighted the role of iNKT cells as critical regulators in the antiviral immune response. The phenotype and function of human iNKT cells during viral infection is best characterized in human immunodeficiency virus (HIV) infection. In HIV infected patients, iNKT cells are severely depleted which is especially apparent in the CD4$^+$ iNKT cell fraction (Sandberg et al. 2002). Not only the predominantly depleted CD4$^+$ subset but also CD4$^-$ iNKT cells are functionally impaired during HIV infection marked by reduced proliferation as well as cytokine secretion (Juno, Keynan, and Fowke 2012). Whether iNKT cells can actively contribute to viral control during HIV infection is not completely clear but

it has been shown that supernatants of αGalCer stimulated, iNKT cell enriched peripheral blood mononuclear cells (PBMCs) suppressed HIV replication in vitro (Vasan et al. 2007). Another hint at a crucial involvement of iNKT cells in the control of HIV infection is the downregulation of CD1d by the HIV protein Nef (Chen et al. 2006). The Nef protein is well known to downregulate MHC class I molecules as an immune escape mechanism (Schwartz et al. 1996; Mwimanzi et al. 2012) highlighting the importance of the CD1d-iNKT cell axis in the anti-HIV immune response. Of note, downregulation of CD1d was also described in Kaposi sarcoma–associated herpesvirus (KSHV) infection and on herpes simplex virus type I (HSV-1) infected APCs, speaking in favour of CD1d as a general target of viral immune escape strategies (Sanchez, Gumperz, and Ganem 2005; Yuan, Dasgupta, and Cresswell 2006).

Activation of iNKT cells with αGalCer during influenza A virus (IAV) infection reduces viral tires in the early phase of infection in mice (Ho et al. 2008). After inoculation with a sublethal dose of IAV, CD1d knockout (KO) mice are unable to clear the virus from the lungs and are more likely to die compared to wild type animals. This failure to clear the virus in CD1d KO mice is caused by low levels of IFN-γ in the lungs resulting in decreased NK cell and antigen-specific $CD8^+$ T cell mediated cytotoxicity (Ishikawa et al. 2010). Besides viral suppression, iNKT cells also dampen overshooting immunopathogenesis caused by infiltrating, inflammatory monocytes in the lungs of influenza infected mice thus ameliorating disease outcome. IAV infected human monocytes were in vitro killed by iNKT cells in a CD1d dependent manner providing an explanation for the observed phenotype in mice (Kok et al. 2012). In humans, in vitro activated iNKT cells inhibit immunosuppressive myeloid derived suppressor cells (MDSC) from IAV infected individuals. Moreover, this data was conferred to mice showing that iNKT cell mediated inhibition of MDSC caused reduction in IAV titres and restoration of the anti-IAV T cell response (De Santo et al. 2008).

Studies of iNKT cells in hepatitis B virus (HBV) infection are almost completely limited to mouse models of transgenic or adenoviral HBV expression. αGalCer activated iNKT cells augment the generation of HBV-specific cytotoxic T cells in HBsAg immunized mice (Ito et al. 2008). In another study, iNKT cell activation by αGalCer injection directly abrogated transgenic HBV expression. This HBV suppression was $CD4^+$ and $CD8^+$ T cell independent and based on the induction of type I and II IFNs and mobilisation of NK cells

(Kakimi et al. 2000). In 2012, Zeissig et al. showed in a mouse model of adenoviral HBV expression that iNKT cells are induced as early as two days post infection by sensing HBV modified host-lipids on the surface of infected hepatocytes. Activated iNKT cells recruited NK cells and $CD8^+$ T cells, resulting in control of HBV replication, which was delayed in NKT cell deficient mice (Zeissig et al. 2012). These results are only partially transferrable to the human system as shown in a phase I/II clinical trial of experimental αGalCer injection in a cohort of HBV infected patients. Even though αGalCer injection led to vigorous iNKT cell activation, a sustained decrease of HBV was observed in only one of 20 participants (Woltman et al. 2009). Different observational studies in human cohorts consistently reported decreased iNKT cell frequencies in chronic HBV patients compared to healthy controls accompanied by hyperactivation of the remaining cells and CD1d upregulation on infected hepatocytes (Jiang et al. 2011; Tan et al. 2018). In one study, this decrease was limited to patients with additional liver cirrhosis where it was concomitant with secretion of IFN-γ, IL-4 and IL-13 by iNKT cells (Wei et al. 2019).

Invariant NKT cells are abundantly present in the mouse but not in the human liver (Kenna et al. 2003). Until recently, when a mouse model for acute and chronic hepacivirus infection was established (Billerbeck et al. 2017), appropriate small animal models for immunological studies of HCV infection were missing. Hence, most studies of iNKT cells in HCV infection were conducted in cohorts of patients with chronic or self-limiting HCV infection that are compared to healthy controls. Most of these studies observed comparable frequencies between patients with persistent and self-limiting infection or healthy controls in the periphery (Inoue et al. 2006; van der Vliet et al. 2005) as well as in the liver (Deignan et al. 2002). On the functional level, iNKT cells from chronically HCV infected patients express higher levels of CXCR3 and produce more IL-13 indicating a shift towards a Th2 cytokine polarization (Inoue et al. 2006). In line with this, in a thesis carried out in the same institute as this work, iNKT cell frequencies of HCV-RNA positive and HCV-RNA negative people who inject drugs (PWID) were comparable. According to this prior cross-sectional analysis, the levels of $CD38^+$ iNKT cells were significantly higher in the HCV-RNA positive group (Senff 2018). Diverging from this, Lucas et al. reported significantly decreased levels of $Vα24^+Vβ11^+$ iNKT cells in chronically HCV infected patients compared to HCV-RNA negative and healthy controls (Lucas et al. 2003). Interestingly, intrahepatic iNKT cells are strongly activated even in the absence of

detectable HCV (Lucas et al. 2003). Additionally, iNKT cells likely contribute to liver pathology as they switch to a Th2 cytokine expression pattern in cirrhotic livers of patients with viral hepatitis (de Lalla et al. 2004).

These studies propose iNKT cells to be involved in HCV immunity, even though their exact contribution remains elusive. As iNKT cells are part of the innate immune system, it is reasonable to assume that they are implicated in the early immune response during acute infection rather than in the chronic phase when the infection is already established and the adaptive immune system dominates the immune response. Invariant NKT cells were analysed in a study that investigated immune alterations during acute HCV infection in health care workers exposed to HCV after accidental needlestick injury. As early as 2-6 weeks post exposure, NKG2D expression on iNKT cells was significantly elevated compared to baseline expression after 6 months (Werner et al. 2013). Due to the scarcity of samples from acute HCV infection, a comprehensive longitudinal analysis covering the entire acute phase in patients with different outcomes will greatly contribute to the understanding of iNKT cell mediated HCV immunity.

3.3. Hepatitis C virus

Hepatitis C is a human hepatotropic virus that was originally described in 1975 as non-A non-B hepatitis (Feinstone et al. 1975) before it was successfully cloned and renamed to HCV in 1989 (Choo et al. 1989). Currently, approximately 58 million people are infected with HCV and each year there are 1.5 million new infections (WHO 2021). HCV is an enveloped, single stranded RNA virus and belongs to the hepaciviruses of the flaviviridae family (Modrow et al. 2013). The 9.6 kilobases long HCV genome in positive orientation contains a single open reading frame that encodes for a large polyprotein comprising structural (C, E1, E2) and non-structural proteins (p7,NS2-NS5B). The molecular virology of HCV has been reviewed extensively elsewhere and thus is only briefly introduced here (Bartenschlager, Lohmann, and Penin 2013; Shi and Suzuki 2018). Since HCV is a blood-borne pathogen, transmission via contaminated blood products has been a major route of infection in the past. After HCV was discovered as the cause for transfusion associated hepatitis in industrialized countries, systematic screening of blood products was introduced leading to a drastic reduction in transfusion-associated transmissions.

However, in low-income countries, infections related to hospitalisation and blood transfusions are still a major cause of HCV transmission. In industrialized countries, the main HCV transmission route today is injection drug use (Thursz and Fontanet 2014).
Infection with HCV is followed by an incubation period of 8-12 weeks before the onset of symptoms. HCV viremia can be detectable 2 weeks post infection but is often not recognized, as HCV is a clinically inapparent disease in most of the cases. However, about 15-30 % of patients develop acute hepatitis including typical symptoms like jaundice and elevation of the liver enzyme alanine transaminase (ALT) (Westbrook and Dusheiko 2014; Marcellin 1999). Only 15-45 % of the acutely HCV infected individuals are able to spontaneously clear the infection without any treatment within the first 6 months (73-86%) to 12 months (87-95 %) defined by a sustained virological response (SVR) and no detectable HCV viremia (Grebely et al. 2012). The remaining patients develop chronic hepatitis C, which is characterized by continuous viral replication. Eventually, chronic HCV infection can slowly progress to liver cirrhosis in 10-20 % of those affected and 1-5 % of those with cirrhosis will develop hepatocellular carcinoma each year (Westbrook and Dusheiko 2014; Hajarizadeh, Grebely, and Dore 2013). This makes HCV a leading cause of liver failure and transplantation in the western world and thus poses a serious public health risk (Westbrook and Dusheiko 2014). In the past, the standard HCV therapy consisted of a combination of pegylated IFN-α and Ribavirin with success rates of 45 % up to 80 % depending on the HCV genotype with a SVR, defined as undetectable HCV-RNA for at least 12 weeks after treatment was stopped. In 2011, directly acting antivirals (DAA) against HCV were introduced into therapy with substantially improved treatment outcomes (Feeney and Chung 2014). Across all HCV genotypes, SVR rates of 95 % and more are typically achieved within 8-12 weeks of therapy (Terrault and Hassanein 2016; Holmes, Rutledge, and Chung 2019). Encouraged by this success, the WHO has formulated the goal to eliminate hepatitis C until 2030; elimination is defined as reduction of HCV transmission by 90 % and of HCV-associated mortality by 60 % (WHO 2016). Despite this enormous success of therapy, there still is a medial need for a prophylactic vaccine against HCV for full eradication of HCV as DAA therapy is expensive and thus not publicly available in the majority of low-income countries that are plagued by a high HCV prevalence (Bartenschlager et al. 2018). Furthermore, most of HCV infected individuals do not know they are infected.

3.3.1. Innate immune responses during acute HCV infection

Shortly after infection of the liver parenchyma by HCV, innate immunity is triggered by double stranded RNA intermediates generated during HCV replication. This replicative intermediates serve as PAMPs and thus are sensed by PRRs in hepatocytes and sentinel cells of the innate immune system, such as pDCs (Li and Lemon 2013; Horner and Gale 2013; Takahashi et al. 2010; Heim and Thimme 2014; Thimme, Binder, and Bartenschlager 2012). HCV derived PAMPs are mainly sensed by the cytosolic PRRs retinoic acid inducible gene-I (Rig-I) and melanoma differentiation antigen 5 (MDA5), the endosomal toll-like receptor TLR-3 and the non-traditional PRR protein kinase R (PKR). Downstream signalling of these PRRs induces expression of type I and type III IFNs and other proinflammatory and immunomodulatory genes causing the induction of an antiviral state and eventually activation of the adaptive immune system. In HCV infected livers, interferons act in an autocrine and paracrine manner, protecting neighbouring uninfected cells against infection (Li and Lemon 2013). Upon HCV infection, ISGs are rapidly and strongly induced in the liver. Which specific interferon subclass mediates this ISG induction is controversially discussed. Since the ISG signature of type III IFNs remarkably overlap with type I ISGs, it was proposed that the type III IFNs might be responsible rather than type I IFNs as IFN-λ receptor expression is increased in infected liver biopsy samples. IFN-λ mRNA and IFN-λ1 (IL-29) protein were the main IFNs detected in the liver and serum of experimentally infected chimpanzees, respectively (Thomas et al. 2012; Park et al. 2012). However, IFN-α potently induces IFN-λ receptor expression on the surface of primary human hepatocytes rendering them responsive to IFN-λ signalling. Thus, ISG induction in the liver during early acute HCV infection is likely a combined effect of type I and type III interferon signalling (Heim and Thimme 2014). The importance of IFN-λ in HCV infection is also underlined by the association of single nucleotide polymorphisms (SNP) in the IFN-λ3 and IFN-λ4 locus that are associated with spontaneous clearance and better response to treatment (Heim and Thimme 2014).

In about half of the patients with chronic HCV infection, activation of the IFN system prevails which is marked by elevated ISG expression but is ineffective in viral clearance. When treated with IFN-α, patients with an pre-activated IFN system usually respond poorly to treatment with IFN-α and ribavirin while patients with baseline levels of ISG

expression achieve SVR more frequently. Hence, elevated IFN signalling during viral persistence represents a risk factor for treatment failure (Sarasin-Filipowicz et al. 2008). Natural killer cells are significantly increased in the human liver compared to the peripheral blood and are involved in the anti-HCV immune response at all stages of HCV infection (Cheent and Khakoo 2011; Kokordelis et al. 2014). NK cells are highly activated during acute HCV infection marked by increased degranulation. Thereby, NK cell degranulation correlated with the strength of subsequent T cell responses but was irrespective of infection outcome (Pelletier et al. 2010). Later, it was shown by Alter et al. that a reduced frequency of NKp30, NKp46, NKG2D and CD161 expressing NK cells during the acute phase was associated with viral clearance, further supporting an important role of the innate cellular immune response on HCV infection outcome (Alter et al. 2011). Genetic association studies supported that the NK cell response is important for clearance of HCV. It was observed that patients with certain combinations of KIRs and their respective ligands on HLA-molecules had a higher likelihood to spontaneously clear the HCV infection. Precisely, patients with KIR2DL3^{+} NK cells who are also homozygous for the a1 domain of the Cw motif on the HLA-C molecule had a higher likelihood of spontaneous resolution (Khakoo et al. 2004) and SVR after treatment with pegylated IFN-α and ribavirin (Vidal-Castineira et al. 2010; Knapp et al. 2011). Mechanistically, it was shown that protection against HCV prior to seroconversion was conferred by KIR2DL3^{+}NGK2A- NK cells in PWID that are exposed to HCV but do not develop acute infection (Thoens et al. 2014). In the same PWID cohort, an association between spontaneous clearance and a KIR3DL1/HLA-Bw4(T) genotype was observed, indicating that NK cells from patients with specific receptor ligand constellation contribute to protection against HCV (Thons et al. 2017).

3.3.2. Adaptive immune responses during acute HCV infection

While the innate immune response is immediately activated by HCV, as seen by ISG induction in the liver and the involvement of NK cells during viral clearance, the adaptive response is markedly delayed after HCV infection. This becomes clear by the long incubation time of several weeks before seroconversion and the absence of detectable T cell activity in the liver shortly after infection. The humoral adaptive immune response during acute HCV infection is only incompletely understood. In principal, HCV specific

antibodies against structural and non-structural antigens are generated in the majority of all acutely HCV infected patients. However, only a minority of these antibodies is directed against the E1 and E2 glycoproteins and thus could exert neutralizing activity by inhibiting virus entry and preventing viral spread. Analysis of patient cohorts and experimentally infected chimpanzees revealed that neutralizing antibodies were also present in patients with viral persistence. In turn, neutralizing antibodies were undetectable in some patients with resolved infection indicating that they are no requirement for successful clearance (Logvinoff et al. 2004; Shin, Sung, and Park 2016; Heim and Thimme 2014). The characterization of the antibody response has proven cumbersome in the past as the diversity of HCV isolates hampers the capture of all HCV specific antibodies in different patients. In order to overcome this challenge, Pestka et al. analysed samples from a single source outbreak, showing that the timing of the antibody response does indeed contribute to the outcome of acute HCV infection (Pestka et al. 2007). In contrast to T cells, antibodies are not long lasting and wane over time even in patients that successfully cleared HCV infection (Takaki et al. 2000). In patients that developed a high titre of neutralizing antibodies early during acute infection, the likelihood of spontaneous resolution was high, while the occurrence of neutralizing antibodies at later time points was associated with viral persistence (Pestka et al. 2007). Newer studies made tremendous progress in identifying broadly neutralizing antibodies that are associated with viral clearance and also confer protection in animal challenge experiments (Law 2021; Brasher et al. 2020; Kinchen et al. 2019) suggesting antibodies to be more importanted than previously appreciated (Kemming, Thimme, and Neumann-Haefelin 2020). Immune responses and immunopathology in acute and chronic viral hepatitis).

While the impact of the antibody response on the outcome of acute HCV infection was controversially discussed until recently, the essentiality of the $CD4^+$ and $CD8^+$ T cell response for virus clearance is clearly established. However, even though the innate immune system is activated hours after HCV infection, the T cell response cannot be detected until 6-8 weeks post infection (Heim and Thimme 2014). This T cell delay is caused by impaired induction of HCV specific T cells rather than impaired recruitment as seen by the early induction of chemokines in the liver (Shin et al. 2011). The first occurrence of CD8β mRNA in the liver – indicating the presence of $CD8^+$ T cells –is associated with a drop in viral load and the onset of liver inflammation indicated by

elevation of serum ALT levels. Viral clearance is associated with a vigorous $CD4^+$ and $CD8^+$ T cells response targeting a broad range of epitopes. Experimental depletion of either $CD4^+$ or $CD8^+$ T cells by antibodies in HCV infected chimpanzees resulted in viral persistence indicating the indispensability of both cell types for clearance of HCV (Grakoui et al. 2003; Shoukry et al. 2003). The $CD4^+$ T cell response in patients that clear HCV is directed against a broad range of epitopes and show better proliferation next to production of the cytokines IFN-γ, TNF-a and IL-2 (Park and Rehermann 2014). During acute infection, $CD4^+$ T cell responses are mounted in all patients regardless of outcome. However, while in resolving patients, the $CD4^+$ T cells responses are long lasting and of a memory phenotype, in chronically evolving HCV infection, the HCV specific $CD4^+$ T cells quickly disappear from the circulation (Schulze Zur Wiesch et al. 2012; Chen et al. 2020). Eventual clearance of HCV requires $CD8^+$ T cell mediated cytolytic as well as non-cytolytic activity against HCV infected hepatocytes (Klenerman and Thimme 2012). Both mechanisms suppress HCV with high efficacy; however, in vitro studies using an HCV replicon model showed that non-cytolytic effector mechanisms, preferentially IFN-γ release, dominated the $CD8^+$ T cell mediated HCV suppression (Jo et al. 2009). These finding were further corroborated by the concomitance of IFN-γ mRNA upregulation in the liver and viral clearance in experimentally infected chimpanzees (Klenerman and Thimme 2012; Thimme et al. 2002). Notably, viral clearance also occurred when ALT levels remained unchanged indicating the absence of cytotoxic immune reaction. In patients with acute HCV infection, $CD8^+$ T cells display a stunned phenotype which is characterized by diminished cytotoxicity, proliferation and IFN-γ release. However, this phenotype is mostly present during early acute infection and is reversed in patients that resolve the infection. Thereby, HCV-specific $CD8^+$ T cells start to secrete IFN-γ after emergence of a HCV-specific $CD4^+$ T cell response, which eventually leads to viral control. These $CD4^+$ and $CD8^+$ T cell responses do not clear the virus during the acute phase which is followed by viral persistence in the majority of all patients. This failure of the $CD8^+$ T cell response is caused by viral escape mutations or by T cell exhaustion (Klenerman and Thimme 2012).

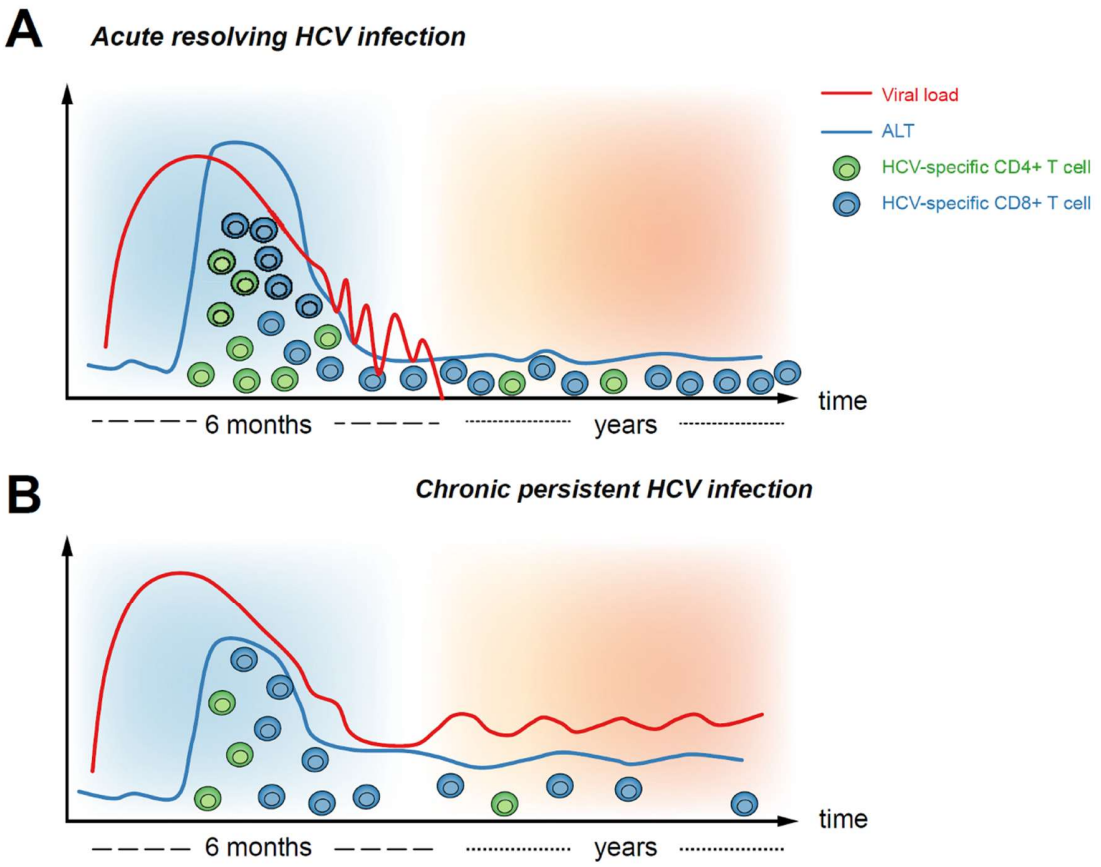

Figure 3. Natural course of self-limited and persisting HCV infection (Thimme 2021). (A) The course of a self-limiting HCV infection and (B) a persisting HCV infection is depicted. The disappearance of HCV-specific T cells despite continuous viral replication is a hallmark of chronic hepatitis C.

3.3.3. Immune responses in chronic HCV infection

In patients with self-limited infection, HCV specific $CD4^+$ T cell responses are long-lasting and can be detected for years after resolution. In chronic patients, T cell responses are temporary and HCV specific T cells disappear from the circulation soon (Chen et al. 2020; Schulze Zur Wiesch et al. 2012). The high replicative fitness of HCV allows for the production of up to 10^{12} viral particles per day (Park and Rehermann 2014). Paired with an error prone RNA dependent RNA polymerase (10^{-4} substitutions per site and round of replication)(Echeverria et al. 2015) (Bartenschlager and Lohmann 2000), this leads to a pool of a myriad of viral quasispecies that are produced until the onset of the adaptive immune response (Timm and Walker 2015). Substitutions in sequences that are targeted

by the host cell TCR or that are crucial for epitope processing and MHC loading lead to functional immune escape.

T cells directed against such escaped sequences are characterized by expression of CD127 and high functional capability, however, they do not contribute to viral clearance. On the other hand, T cells directed against epitopes that do not allow for mutations often exhibit an exhausted phenotype during viral persistence characterized by the expression of programmed cell death protein 1 (PD-1) and multiple other exhaustion markers alongside impaired functionality and proliferation (Holz and Rehermann 2015).

As mentioned before, delayed induction of the adaptive immune response is a hallmark of HCV. It is still not understood why adaptive immunity takes so long to respond to HCV infection despite rapid induction of innate immunity and a strong IFN response. It was speculated that the linkage between innate and adaptive immunity is disturbed in HCV infected individuals (Heim and Thimme 2014). One hypothesis is insufficient priming of $CD8^+$ T cells by defective professional antigen presenting cells such as macrophages and dendritic cells (Thimme, Binder, and Bartenschlager 2012; Rehermann and Nascimbeni 2005; Rehermann 2009). Given the well described role of iNKT cells in bridging innate with adaptive immune responses, a likewise impairment of iNKT cells during acute HCV infection might be a reasonable explanation for immune failure that precede viral persistence.

3.4. Aims of the study

Human iNKT cells bridge the innate with the adaptive immune system and modulate the immune response by release of a multitude of cytokines and by interaction with various other immune cells. Invariant NKT cells have been shown to be critically involved in anti-viral immunity against HIV, HBV and HCV among others. While their role in chronic HCV infection has been investigated in the past, knowledge of iNKT cell mediated immunity in the acute phase of HCV infection is very limited. The acute phase of HCV infection is characterized by a delayed induction of adaptive immune responses despite activated innate immunity suggesting perturbation in the activity of bridging cells such as iNKT cells. Therefore, in this study, the phenotype of human iNKT cells is characterised in detail in a cohort of patients with acute HCV infection that either resolved or progressed to chronic

infection. Differences in the iNKT cell phenotype could hint at mechanisms that are linked to viral persistence during acute HCV infection. Furthermore, an association of the iNKT cell phenotype with parameters that define HCV replication and liver damage is investigated. This will answer the question, if iNKT cells contribute to differential outcome of HCV infection and if they are directly involved in viral suppression and immunopathogenesis. In a second step, the phenotype of human iNKT cells observed during acute HCV infection is comprehensively analysed to investigate a potential association with an altered functionality, which could hint at mechanisms that are responsible for viral persistence.

It has been shown in a thesis conducted in this working group that iNKT cells are significantly more activated in patients with chronic compared to patients with resolved hepatitis C (Senff 2018). However, this activation was not linked to an obvious difference in effector functions. In order to characterize the functionality of activated iNKT cells in more detail, RNA-sequencing of activated and resting iNKT cells is performed. The resulting gene expression profile of human activated iNKT cells facilitates the identification of target genes critical for effector function and anti-viral immunity by iNKT cells.

4. Material

4.1. Patient Samples

Peripheral blood samples of patients and healthy donors were obtained from the following listed sources.

Buffy coats of healthy donors were collected at the blood donation centre of the University Hospital Düsseldorf. Ethylenediaminetetraacetic acid (EDTA)-blood samples from healthy donors that were compared with liver perfusate samples were collected at the Institute of Virology of the University Hospital Düsseldorf.

Whole blood samples from PWID that were treatment-naïve, were collected from the ward for inpatient detoxification treatment of drug addicts or the clinic for opioid maintenance treatment at the Department for Addiction Medicine and Addictive Behaviour of the LVR-Hospital Essen at the University Hospital Essen and kindly provided by Prof. Dr. Norbert Scherbaum. Samples were serologically tested at the Institute of Virology of the University Hospital Düsseldorf for anti-HCV antibodies. Patients with detectable anti-HCV antibodies were divided into chronic hepatitis C patients and patients with resolved hepatitis C according to the presence of detectable HCV-RNA in the plasma.

PBMC samples from patients with acute HCV infection were kindly provided by Prof. Georg M. Lauer from the Massachusetts General Hospital and Harvard Medical School in Boston, USA and Lia L. Lewis-Ximenez from the Laboratory of Viral Hepatitis at the Oswaldo Cruz Institute, FIOCRUZ in Rio de Janeiro, Brazil.

All samples were collected under approval of the local ethics committees and written informed consent was given by all participants.

4.2. Patients with acute HCV infection

In this study, 30 patients with acute hepatitis C from North America and Brazil covering various transmission routes were analysed. In case of an unknown exposure event, the estimated time of infection (ETI) was calculated as 7 weeks prior to peak ALT levels. Longitudinal samples from the first year post infection were analysed and the patients were grouped into “resolvers” (n=15), that spontaneously cleared the infection within the first 12 months post estimated time of infection (ETI) and “progressors” (n=15) which were defined as patients with ongoing viremia at 12 month post ETI. The patients had no history

of HBV infection and were excluded from the study as soon as they received anti-HCV treatment.

Table 1 Patient characteristics of the acute hepatitis C cohort

	Complete cohort	Chronic progression	Resolving
Nmber of patients	30	15	15
Female (%)	16 (53.3 %)	8 (53.3 %)	8 (53.3 %)
Male (%)	14 (46.7 %)	7 (46.7 %)	7 (46.7 %)
Mean peak ALT (range)	433 (19 – 1844)	619 (54 – 1844)	234 (19 – 875)
Mean peak viral load (range)	19405798 (282 – 394631560)	29920462 (228 – 394631560)	1005135 (282 – 6040000)

4.2. Media, buffers and cell culture additives

Table 2 Media and buffers

Buffer	Supplier	Cat. No.
RPMI 1640 Medium, GlutaMAX™	Thermo Fisher Scientific	72400021
Fetal Calf Serum (FCS) Supreme	Pan Biotech	P30-3031
Penicillin-Streptomycin	Thermo Fisher Scientific	15140-22
Dulbecco's phosphate buffered saline (PBS)	Thermo Fisher Scientific	14190144
Alpha-Galactosylceramide (KRN7000)	Funakoshi	KRN7000
Human Interleukin-12, recombinant	Reprokine	RKP29460
Human Interleukin-15, recombinant	Reprokine	RKP40933
Human Interleukin-18, recombinant	Reprokine	RKQ14116
Interleukin-2, human	Roche	11147528001
Brefeldin A (BFA)	Sigma	B5936
Phorbol-12-myristat-13-acetat (PMA)	Sigma	P8139
Ionomycin	Sigma	407952
CpG ODN	Invivogen	
Poly(I:C)	biomol	IAX-200-021

EDTA	Thermo Fisher Scientific	AM9261
Dimethyl sulfoxide (DMSO)	Carl Roth	4720.1

Table 3 Medium composition

R10	RPMI 1640 Medium + 10 % FCS + 1 % Penicillin-Streptomycin
Freezing medium	FCS + 10 % DMSO
FACS buffer	PBS + 2 % FCS
MACS buffer	PBS + 0.5 % FCS + 2mM EDTA

4.3. Reagents and Chemicals

Table 4 Reagents and chemicals

Reagent	Supplier	Cat. No.
TRIzol™ Reagent	Thermo Fisher Scientific	15596026
eBioscience™ IC Fixation Buffer	Thermo Fisher Scientific	00-8222-49
eBioscience™ Permeabilization Buffer (10X)	Thermo Fisher Scientific	00-8333-56
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	00-5523-00
Brilliant Stain Buffer	BD Biosciences	566349
Pancoll	Pan Biotech	P04-60500
Compensation Beads	BD Biosciences	552843
Ultracomp Beads	Thermo Fisher Scientific	01-2222-42
Alexa Fluor® 647 Streptavidin	Biolegend	405237

4.4. Antibodies for flow cytometry

Table 5 Antibodies for flow cytometry

Antigen	Fluorochrome	Clone	Supplier	Cat. No.
CD244	PeCF594	2-69	BD Biosciences	564881

TCR Va24Ja18	BV421	6B11	Biolegend	342916
TCR Va24Ja18	PE	6B11	Biolegend	342904
TCR Va24Ja18	APC	6B11	Biolegend	342908
aGalCer loaded CD1d Dextramer	APC	n/a	Immudex	XD8002
CD127	PE	A019D5	Biolegend	351304
CD127	BV711	HIL-7R-M21	BD Biosciences	563165
CD127	APC	eBioRDR5	Thermo Fisher Scientific	17-1278-42
CD14	BV711	63D3	Biolegnd	367140
CD14	eF506	61D3	Thermo Fisher Scientific	69-0149-41
CD14	Biotin	Tük4	Miltenyi Biotec	130-113-145
CD161	AF700	HP-3G10	Biolegend	339942
CD161	BV605	HP-3G10	Biolegend	339916
CD19	APCeF780	HIB19	Thermo Fisher Scientific	47-0199-42
CD19	PerCP-Cy5,5	HIB19	Thermo Fisher Scientific	45-0199-42
CD19	PeCy7	HIB19	Thermo Fisher Scientific	25-0199-42
CD19	eF506	HIB19	Thermo Fisher Scientific	69-0199-42
CD19	Biotin	LT19	Miltenyi Biotec	130-113-167
CD3	PerCP-Cy5.5	OKT3	Thermo Fisher Scientific	45-0037-42

CD3	PE/Dazzle™ 594	HIT3a	Biolegend	300336
CD3	BV605	HIT3a	BD Biosciences	564712
CD3	BV650	SK7	BD Biosciences	563999
CD3	APCeF780	OKT3	Thermo Fisher Scientific	47-0037-42
CD3	AF700	HIT3a	Biolegend	300324
CD38	PeCy7	HIT2	Thermo Fisher Scientific	25-0389-42
CD38	PeCy7	HB-7	Biolegend	356608
CD38	PE			
CD4	BV786	RPA-T4	BD Biosciences	740962
CD4	PE	RPA-T4	Thermo Fisher Scientific	12-0049-42
CD4	BV510	SK3	Biolegend	344634
CD56	BV570	HCD56	Biolegend	318330
CD56	PerCP-eFluor 710	CMSSB	Thermo Fisher Scientific	46-0567-42
CD69	FITC	FN50	Biolegend	310903
CD69	APC	FN50	Biolegend	310910
CD69	eF450	FN50	Thermo Fisher Scientific	48-0699-42
CD8a	AF700	OKT8	Thermo Fisher Scientific	56-0086-41
CD8	APC	RPA-T8	Thermo Fisher Scientific	17-0088-42
CD8	PE	RPA-T8	BD Biosciences	555367
CD8	FITC	RPA-T8	eBio	11-0088-42
CD8	BV510	RPA-T8	Biolegend	301047
CD8	BV650	RPA-T8	Biolegend	301042
CXCR6	APC	K041E5	Biolegend	356006

eBioscience™ Fixable Viability Dye	eFluor™ 506	n/a	Thermo Fisher Scientific	65-0866-14
eBioscience™ Fixable Viability Dye	eFluor™ 780	n/a	Thermo Fisher Scientific	65-0865-18
Gd T cells	PeCF594	B1	Biolegend	331226
Granzyme B	PE/Dazzle™ 594	QA16A02	Biolegend	372215
CD4	BUV737	RPA-T8	BD Biosciences	563795
CD8	BUV395	SK3	BD Biosciences	564306
IFNg	FITC	4S.B3	Thermo Fisher Scientific	11-7319-82
IL-32	AF647	KU32-52	Biolegend	513503
PD-1	BV785	EH12.2H7	Biolegend	329930
PD-1	BV650	EH12.1	BD Biosciences	564104
PD-1	BV711	EH12.2H7	Biolegend	329928
PLZF	PE	R17-809	BD	564850
Tbet	BV786	4B10	Biolegend	644835
TNFa	PE	MAb11	Thermo Fisher Scientific	12-7349-82
TNFa	APC	MAb11	Thermo Fisher Scientific	17-7349-82
TNFa	PeCy7	MAb11	Biolegend	502929
TCR Vα24	FITC	C15	Beckman Coulter	IM1589
TCR Vβ11	PE	C21	Beckman Coulter	IM2290
LIVE/DEAD™ Fixable Dead Cell Stain Kit	Near infrared/APC-Cy7	n/a	Thermo Fisher Scientific	L34976

4.5. Peptides

Table 6 Peptides used in CMV Pool

Peptide	Sequence	CMV Protein
1	VTEHDTLLY	pp50
2	VLEETSVML	IE-1
3	NLVPMVATV	pp65
4	IMREFNSYK	gB
5	AYAQKIFKIL	IE-1
6	QYDPVAALF	pp65
7	DIYRIFAEL	pp65
8	TPRVTGGGAM	pp65
9	QIKVRVDMV	IE-1
10	ARVYEIKCR	pp28
11	CPSQEPMSIYVY	pp65
12	ELRRKMMYM	IE-1
13	FEQPTETPP	IE-2
14	QEFFWDANDI	pp65
15	QAIRETVEL	pp65
16	QMWQARLTV	pp65
17	PTFTSQYRIQGKL	pp65
18	GPISGHVLK	pp65
19	DALPGPCI	pp65
20	KMQVIGDQY	pp65
21	CEDVPSGKL	pp65
22	TRATKMQVI	pp65

4.6. Kits

Table 7 Kits

Kit	Supplier	Cat. No.
Anti-Biotin MicroBeads UltraPure	Miltenyi Biotec	130-105-637

LS columns	Miltenyi Biotec	130-042-401
Pan T Cell Isolation Kit, human	Miltenyi Biotec	130-096-535
Anti-iNKT MicroBeads, human	Miltenyi Biotec	130-094-842
Gibco™ Dynabeads™ Human T-Activator CD3/CD28	Thermo Fisher Scientific	111.61D
CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit	Thermo Fisher Scientific	C10427
CellTrace™ violet proliferation kit	Thermo Fisher Scientific	C34557

4.7. Instruments and devices

Table 8 Instruments

BD Fortessa	BD Biosciences
BD LSR II	BD Biosciences
BD Canto	BD Biosciences
XP-300 cell counter	Sysmex
Mr. Frosty™ freezing container	Thermo Fischer Scientific
Leucosep tubes	Greiner (#227290)
QuadroMACS™ Separator	Miltenyi Biotec

4.8. Software

Table 9 Software

Prism 9	GraphPad Software
FlowJo V10.7	BD Biosciences
Microsoft Office 365	Microsoft Corporation

5. Methods

5.1.Purification and cryopreservation of peripheral PBMCs

PBMCs were isolated from EDTA-blood or buffy coats by density gradient centrifugation. The blood was diluted with PBS in a 1:1 ratio and layered over a density gradient (Pancoll) in a 50ml Leucosep tube. After centrifugation at 1100 x g for 10min, the PBMC ring was resuspended, transferred into a new tube and washed three times with 50ml PBS. Cells were then adjusted to 10^7 / ml in freezing mediumand frozen in a Mr. Frosty freezing container at -80°C before transferred to liquid nitrogen until further use.

5.2. Preparation of intrahepatic lymphocytes

Intrahepatic lymphocytes were isolatedprepared from perfusates of liver specimens, obtained from fresh tumour resections of uninfected individuals. The liver perfusates were collected in the Department of Gastroenterology and Hepatology of the University Hospital Essen and kindly provided by Dr. Ruth Broering. Mononuclear cells were isolated from the perfusates according to the PBMC purification described in 5.1. The study was approved by the local ethics committee and written informed consent was given by all participants.

5.3. Thawing of PBMCs

PBMCs were thawed at 37°C in a water bath and transferred into 10ml warm PBS. Cells were centrifuged at 524 x g for 5 minutes and washed twice in PBS. Cells were then stained for flow cytometry or if cultured, cells were counted with a XP-300 cell counter and seeded in the required concentration in R10 medium.

5.4. Flow cytometry surface staining

Up to $5x10^6$ cells per staining reaction were transferred to each well of a 96-well round bottom plate or up to 10^7 cells were transferred to a 5 ml FACS tubes. Cells were centrifuged at 524 x g for 5 minutes and the supernatant was discarded. The cell pellets were resuspended in PBS containing αGalCer loaded CD1d dextramer and incubated for 20 minutes at room temperature in the dark. When the dextramer was used to stain iNKT cells, an anti-CD19 antibody was included into the surface staining to exclude

unspecifically bound B cells. Afterwards, cells were washed with PBS, resuspended in 100 µl viability dye master mix and incubated for 15 minutes at 4°C in the dark. The cells were then washed once with FACS buffer followed by staining with antibodies directed against surface molecules in FACS buffer for 15 minutes at 4°C in the dark. If multiple antibodies conjugated to a Brilliant Violet™ fluorochrome were used, FACS buffer was mixed in a 1:1 ratio with Brilliant Stain Buffer. After incubation, cells were washed in FACS buffer and resupended in IC Fixation Buffer for at least 15 minutes at 4°C in the dark. After a final wash step, cells were resuspended in FACS buffer and acquired on a BD FACS Canto II, BD LSR II or BD LSR Fortessa flow cytometer. Fluorescence minus one (FMO) controls were used where applicable to define positive and negative populations. Single stained compensation beads and single stained cells were used to compensate spectral overlap. Automatic compensation in the BD FACS Diva software was applied to calculate the compensation matrix. The compensation matrix was reviewed and adjusted if necessary during analysis using the compensation tool built in the FlowJo V10 software.

5.5. Staining of intracellular antigens

Cells were stained with viability dye and antibodies against surface markers and fixed according to section 5.4. Afterwards, cells were washed twice in 1x permeabilization buffer and antibodies directed against intracellular antigens were added in 1x permeabilization buffer for 20 minutes in the dark at 4°C. Afterwards, cells were washed once with 1x permeabilization buffer and once with FACS buffer before they were resuspended in FACS buffer and acquired on a flow cytometer. If costaining of intranuclear antigens was required, the FoxP3 staining buffer kit was used according to the manufacturer's instructions.

5.6. In vitro activation of iNKT cells

PBMCs were thawed and adjusted to a concentration of $2x10^6$ /ml in prewarmed R10 medium. Cells were seeded in a multiwell flat bottom plate and αGalCer or a cocktail of IL-12, IL-15 and IL-18 was added in a concentration of 1 µg/ml or 10 ng/ml, 100 ng/ml and 50 ng/ml, respectively for the indicated periods of time. CpG ODN was added in a concentration of 1mM for 24 hours and Poly(I:C) was added in a concentration of 5 µg/ml

for 24. Additional stimuli were added as indicated in the figures. PMA (10 ng/ml) and Ionomycin (1 µg/ml) served as a positive control for cytokine secretion. If cytokines were stained, BFA was added to the culture for the last 4 hours in a concentration of 10 ng/ml. Incubation occurred for the indicated time at 37°C, 5 % CO_2 in a humidified atmosphere.

5.7. Magnetic activated cell sorting (MACS)

In order to magnetically enrich iNKTor $CD3^+$ T cells from human PBMCs, PBMCs were thawed and counted as described above and resuspended in 500 µl of MACS buffer per 10^8 cells . The iNKT microbead kit was used for labelling and positive enrichment of the iNKT cell fraction according to the manufacturer's protocol. Cells were separated by LS columns and the negative fraction was applied to another LS column to further increase yield of positively isolated iNKT cells. Aliquots of pre separated PBMCs next to the positive and negative fraction were collected and analysed for purity and yield of iNKT cells in a flow cytometer. Cells were then stained for flow cytometry or adjusted to a concentration of $2x10^6$ /ml in R10 for cultivation or in vitro activation. The pan T cell isolation kit was used for the negative selection of $CD3^+$ T cells per manufacturer's instructions. $CD14^+$ and $CD19^+$ cells were depleted from PBMCs by labelling with biotin labelled anti-CD14 and anti-CD19 antibodies followed by depletion with the anti-biotin microbead kit as indicated by the manufacturer. In order to maximize iNKT yield and minimize loss of iNKT that remain unspecifically bound on the column, columns were eluted twice.

5.8. Fluorescence activated cell sorting (FACS) of iNKT cells

$5x10^8$ PBMCs were thawed and adjusted to 10^8 cells per 500 µl in MACS buffer. After negative enrichment of $CD14^-$ and $CD19^-$ cells as described in section 5.7., the unlabelled cell fraction was stained with viability dye and anti-Vα24Jα18 antibody for subsequent FACS analysis of iNKT cells. Invariant NKT cells were sorted at the Core Flow Cytometry Facility at the Institute for Transplantation Diagnostics and Cell Therapeutics Düsseldorf into pre-warmed R10. Afterwards cells were washed once with R10 and seeded into two wells of a 96-well flat bottom in 100 µl R10 each followed by incubation for 24 hours at 37°C, 5 % CO_2 in a humidified atmosphere.

5.9. RNA sequencing

FACS purified iNKT cells were stimulated with IL-12 (10 ng/ml), IL-15 (100 ng/ml) and IL-18 (50 ng/ml) or left untreated for 24 hours before they were pelleted by centrifugation for 5 minutes at 524 x g. The supernatant was aspirated and the pellet was resuspended in 100 µl TRIzol. Cells were frozen at -80°C until further use. RNA isolation, library preparation and sequencing were performed at the Primate Genetics Laboratory of the German Primate Centre in Göttingen in the laboratory of Prof. Dr. Lutz Walter according to the total RNA-sequencing protocol by Illumina including removal of ribosomal RNA. 50 base pair single read sequencing was performed with an Illumina HiSeq4000. The GO term enrichment analysis of the differentially expressed genes revealed by RNA-sequencing was performed with the use of the Panther database (Ashburner et al. 2000; Gene Ontology 2021; Mi et al. 2019).

5.10. Proliferation assay

Cryopreserved PBMCs were thawed as described above and up to 40mio cells were labelled with cell trace violet according to the manufacturer's instructions. The staining reaction was quenched with R10 for 5 minutes at room temperature before cells were washed with R10. Cells were then stained with anti-Vα24Jα18 and anti-CD38 antibody before they were resuspended in FACS buffer and sorted at the Core Flow Cytometry Facility at the Institute for Transplantation Diagnostics and Cell Therapeutics Düsseldorf. A population of either $CD38^+$ iNKT cells or $CD38^-$ iNKT cells were sorted into prewarmed R10. Subsequently they were mixed with $2x10^6$ autologous, irradiated (30Grey), unlabelled PBMCs which served as feeder cells and washed with R10. The cells were then resuspended in 1ml R10 supplemented with 25 U/ml human recombinant IL-2 and seeded in two wells of a 48-well plate. One of the cultures was additionally stimulated with 1 µg/ml aGalCer. In the following, the cells were incubated for 12 days at 37°C, 5 % CO_2 in a humidified atmosphere and fed with half of the initial volume R10 and 25 U/ml IL-2 after 8 days. Subsequently, the cells were harvested and stained with fixable viability dye, anti-CD3, anti-CD38, anti-Va24Ja18 antibody and their proliferation was analysed by flow cytometry according to the dilution of violet cell trace.

5.11. Staining of apoptotic cells

PBMCs were stimulated with 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 or left untreated for 24 hours and BFA was added in the last 4 hours. As a marker for apoptosis, cleaved caspase 3 and caspase 7 were stained with the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit according to the manufacturer's instructions. Subsequently, surface markers and IL-32 were stained like described above and cells were analysed by flow cytometry.

5.12. Expansion of cytomegalovirus (CMV)-specific CD8+ T cells from PBMCs

PBMCs were thawed and seeded in a concentration of $2x10^6$ /ml in R10 in a flat bottom well plate. The cells were stimulated with 25 U/ml human recombinant IL-2, anti-CD28/49d and a pool of 22 immunodominant CMV peptides (table 2) that were restricted to a broad range of HLA types. This facilitates potent expansion of antigen-specific CD8+ T cells in in patients with diverse HLA genotypes. The CMV pool was added in a concentration of 1 µg/ml of total peptide or 45.5 ng/ml of each peptide. Afterwards, the cultures were incubated at 37°C, 5 % CO2 in a humidified atmosphere for 10 days. After 5-7 days, half of the volume R10 and 25 U/ml IL-2 were added. After 10 days, cells were resuspended and 200 µl of the culture were transferred into a 96-well round-bottom plate. The cells were restimulated with the CMV peptide pool in a concentration of 10 µg/ml or left untreated. BFA was added to all wells in a concentration of 10 ng/ml and the cells were incubated for 4h. Afterwards, intracellular cytokine staining (ICS) was used as described in section 5.5 to detect IFN-γ secreting cells.

5.13. Expansion of iNKT cells

PBMCs were thawed and seeded in R10 in a concentration of $2x10^6$ /ml in flat bottom well plates. Subsequently, 1 µg/ml αGalCer and 25 U/ml human recombinant IL-2 were added. Cells were incubated at 37°C, 5 % CO_2 in a humidified atmosphere for at least 10 days. After 5-7 days half of the volume of R10 and IL-2 were additionally added. Following the expansion, cells were harvested, stained as described above and analysed by flow cytometry.

5.14. T-distributed stochastic neighbor embedding (t-SNE) analysis

T-SNE analysis was performed with the FlowJo V10 software. All samples with more than 100 NKT cells were downsamples to 100 NKT cells and concatenated into a single file. T-SNE analysis with this file was performed with standard settings including the markers Vα24Jα18, PD-1, CD127, CD4, Vα24, NKG2D, CD49a, 2B4, CD38, CD69, CD8 and CD3. The resulting cell clusters were gated and analysed for the expression of individual markers and the over representation of certain cell populations.

5.15. Statistics

Graphpad Prism V9 was used for statistical analysis and visualisation of the data. Data were testes for normal distribution by Shapiro-Wilk test. Grubbs test was used to detect significant outliers. Statistical tests were performed as indicated in the figure legends. Pearson or non-parametric spearman correlation tests were used for correlation analysis. A p-value of $p \leq 0.05$ was considered as significant. Simple linear regression analysis was used to detect trends in the longitudinal data. Two groups were compared by nonarametrical or parametrical t-test.

6. Results

6.1. Staining protocol of invariant natural killer T cells

In the past, invariant natural killer T cells were often defined as CD3 and CD56 double positive cells. As the understanding of these cell type increased and a more sophisticated classification of various NKT cells emerged, characterization of these cells changed. Several strategies have been described to specifically detect human iNKT cells by flow cytometry. One of the most common uses an antibody (clone 6B11) directed against the CDR3 loop of the Vα24Jα18 chain of the invariant TCR (Exley et al. 2008). In analogy to the detection of other antigen specific T cells, iNKT cells can also be stained with antigen loaded multimers (Karadimitris et al. 2001). Here, an αGalCer loaded CD1d dextramer was used. This multimer can detect all αGalCer specific T cells restricted to CD1d and thus possibly a small fraction of αGalCer reactive non-iNKT cells, too (Exley et al. 2017). A third method relies on the co-staining of the Vα24 alpha chain with the Vβ11 beta chain of the TCR (Fig. 4A). This staining method also marks the vast majority of human iNKT cells, albeit a small subset that uses another beta chain might exist and thus would remain undetected with this staining method (Exley et al. 2017; Exley, Wilson, and Balk 2017). In order to evaluate the best strategy to specifically stain human iNKT cells, all three protocols were tested simultaneously in three healthy donor PBMC samples with either low, intermediate or high iNKT cell frequencies. The numbers of iNKT cells stained by all three methods were highly reproducible (Fig. 4B–C) and costaining with the various reagents revealed a high overlap in the iNKT populations (Fig. 4D–F). As all three staining protocols worked similarly well, iNKT cells were stained with 6B11 antibody in this work unless stated otherwise.

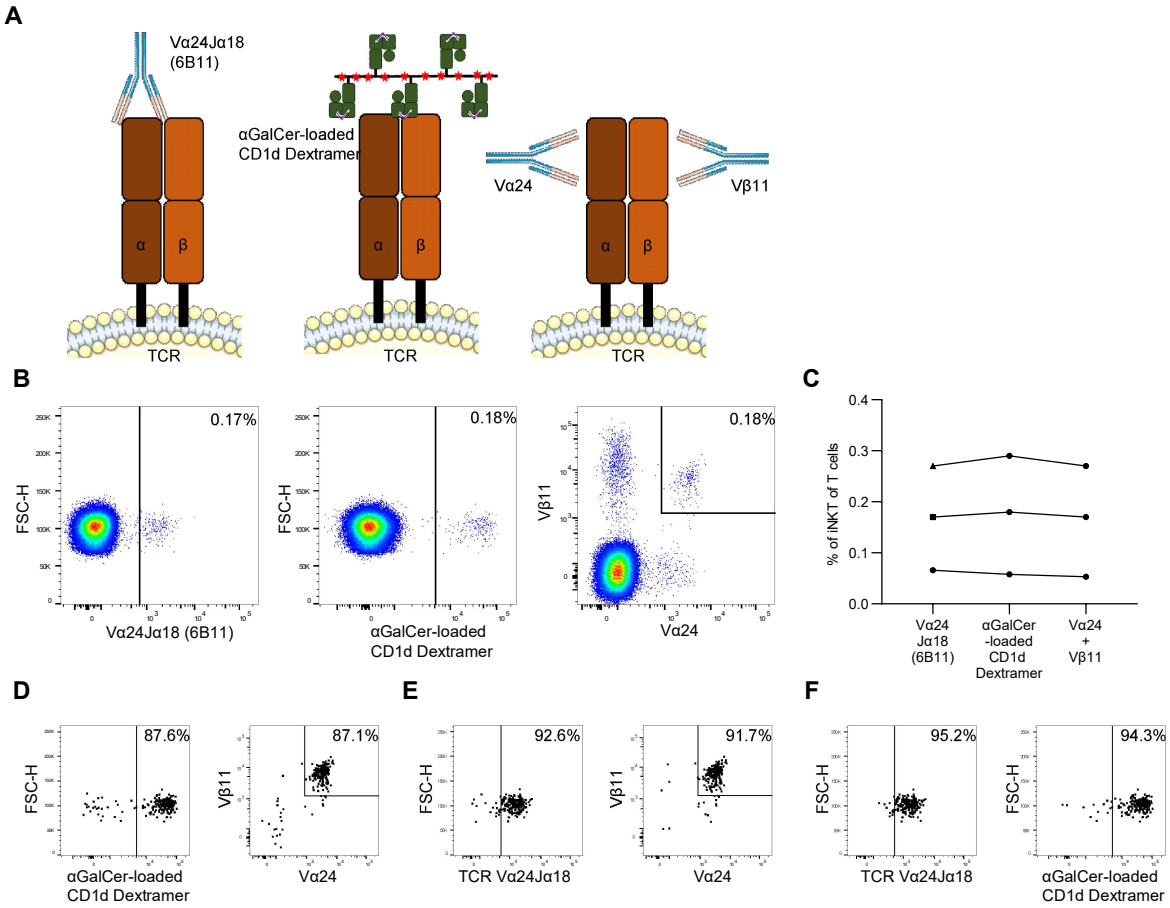

Figure 4. Comparison of three different staining strategies for human iNKT cells. The frequency of iNKT cells in PBMCs from three healthy donors was analysed. (A) Cells were either stained with a monoclonal antibody directed against the invariant alpha chain of the TCR (Va24Ja18), an αGalCer loaded CD1d dextramer or a combination of antibodies directed against Va24 and Vb11 chain of the TCR. (B) Representative flow cytometry plot of iNKT cells stained with the three different approaches. (C) iNKT cell frequencies as detected with the indicated staining methods. (D–F) PBMCs were costained with the staining reagents described in (A) and gated on TCR Va24Ja18 positive (D), αGalCer loaded CD1d multimer positive (E) or Va24 Vb11 double positive cells. Frequency of cells positive for the indicated markers are depicted for one representative donor. (A) parts of the image were modified from smart.servier.com

6.2. Longitudinal analysis of iNKT cell phenotype during acute HCV infection

Unlike the well described role of T and NK cells in acute HCV infection, the role iNKT cells play during the early immune response against HCV is still ill defined (Gardiner 2015; Heim and Thimme 2014; Holz and Rehermann 2015; Rehermann 2009). Here, a comprehensive phenotypical analysis of peripheral blood iNKT cells from acutely HCV infected patients (Table 1) was performed over the time course of one year post ETI). Patients were binned into two separate groups according to their disease status at 12 months post ETI. The first group contained all patients that developed a chronic HCV infection (chronic progression) and the second group comprised all patients that spontaneously cleared the infection (resolving).

The early phase of acute HCV infection is characterised by dynamic and complex interactions of the immune system and the virus (Rehermann 2000). Hence, a longitudinal follow up was conducted that facilitated a detailed evaluation of iNKT cells during the entire acute phase of HCV infection. Thereby, transient changes in receptor expression that might occur during the initial phase of infection can readily be detected. All patients with more than two samples from different time points available were included in the longitudinal analysis. Linear regression analysis of all available time points in each patients was used to estimate the overall trend of the frequency of cells positive for a certain marker over time. In addition, the earliest available time point of each patient prior to 10 weeks post ETI was compared between resolvers and progressors in a cross-sectional analysis to estimate the average receptor expression levels during early acute HCV infection. Figure 5 shows a representative gating strategy for the flow cytometric evaluation of iNKT cell frequency and phenotype in PBMC samples from acutely HCV infected patients.

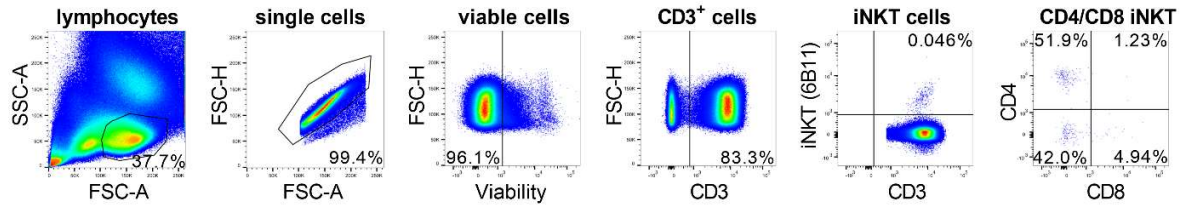

Figure 5. Invariant NKT cell gating strategy. Representative gating strategy of one acutely HCV infected patient shows the characterization of iNKT cells from PBMCs.

6.2.1. Invariant natural killer T cell activation is associated with the outcome of acute HCV infection

The frequency of iNKT cells prior to 10 weeks post ETI ranged from 0.007 % to 0.17 % (median 0.035 %) in resolvers and from 0.002 % to 0.37 % (median 0.061 %) in progressors. While iNKT cell frequencies varied greatly between individuals, they were remarkably similar between groups (Fig. 6A, p=0.7146). Longitudinal analysis showed that iNKT cell levels remained stable over the first year in the majority of samples indicated by linear regression analysis (Fig. 6B-C, mean slope 0.00215 with 95 % CI -0.0012 to 0.0055 in resolvers and mean slope 0.00007 with 95 % CI -.00002 to 0.0003 in progressors).

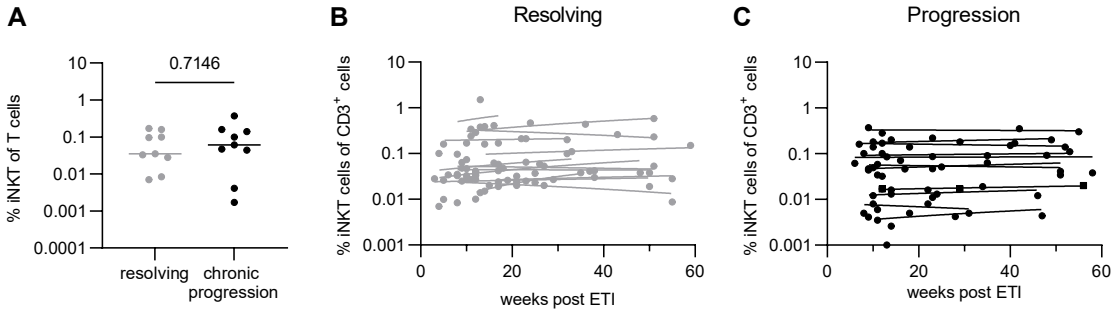

Figure 6. Invariant NKT cell frequency during acute HCV infection. (A) The frequency of iNKT cells at the earliest available time point of each patient that was collected before 10 weeks post ETI was analysed. (B+C) The iNKT cell frequency was analysed longitudinally in each patient with more than two time points available during the first 60 weeks of infection.

The majority of iNKT cells comprises $CD4^+$ iNKT cells or double negative iNKT cells while a smaller fraction of iNKT cells are $CD8^+$ (Krovi and Gapin 2018). The relative frequency of $CD4^+$ (mean 40.90 % and 38.07 %), $CD8^+$ (mean 23.02 % and 16.58 %) and DN (mean 33.80 % and 44.30 %) iNKT cells during early acute infection was similar in resolvers and progressors (Fig. 7A) and did not change significantly during the first year (Fig. 7B–G).

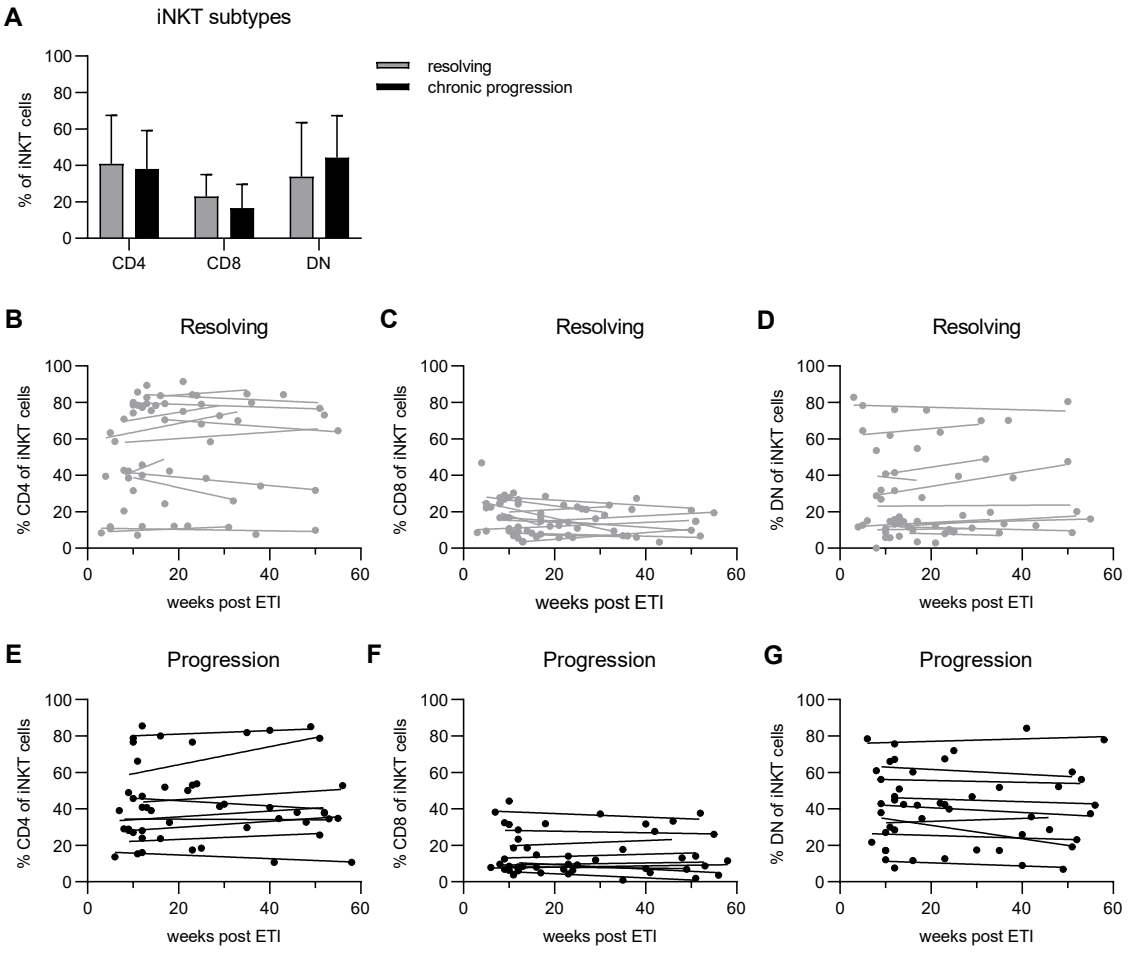

Figure 7. Invariant NKT cell subset distribution during acute HCV infection. (A) $CD4^+$, $CD8^+$ and double negative (DN) iNKT subtypes were analysed at the earliest available time point prior to 10 weeks post ETI. Mean with standard deviation is shown. (B–G) The frequency of iNKT subsets $CD4^+$ (B+E), $CD8^+$ (C+F) and DN (D+G) was analysed longitudinally in each patient with more than two time points available during the first 60 weeks of infection. Patients were divided into resolvers (B–D) and chronic progressors (E–G). Each line represents the trend of the frequency in an indivdual patient determined by linear regression analysis. (A–G) Only samples with more than 20 iNKT cells were included into the analysis.

Besides frequency and CD4 and CD8 expression, a detailed iNKT cell phenotype during acute HCV with regard to different infection outcomes was determined next. Therefore, a multitude of lymphocyte surface markers that are either associated with general iNKT cell function (Geissmann et al. 2005; Germanov et al. 2008; Loh et al. 2014) or T cell and NK cell activation and differentiation some of which reported to be implicated in HCV infection (Bengsch et al. 2010; Golden-Mason et al. 2006; Kasprowicz et al. 2008; Northfield et al.

2008; Urbani et al. 2006) were analysed at the earliest available time point and during the first year of infection.

Acute HCV infection can come together with strong immune activation (Amadei et al. 2010; Larrubia et al. 2014; Park and Rehermann 2014; Shin, Sung, and Park 2016). Hence, iNKT cell activation was determined by expression of CD38 and CD69. At the earliest analysed time point of the acute infection, CD38 was expressed in a median of 20.65 % of iNKT cells in resolvers and 10.90 % in progressors while CD69 was detectable in a median of 16.60 % of iNKT cells in resolvers and 15.20 % in progressors (Fig. 8A+D). Even though cross sectional analysis could not detect a significant difference prior to 10 weeks post ETI, longitudinal analysis revealed that the majority of resolvers had declining levels of CD38 (Fig. 8B, mean slope of linear regression -0.4345 with 95 % CI -0.719 to -0.150) and, albeit less pronounced, CD69 (Fig. 8E, mean slope of linear regression -0.4388 with 95 % CI -0.818 to -0.060) during the first year of infection. In turn, progressors showed, despite similar initial levels, sustainably high levels of $CD38^+$ (Fig. 8C, mean slope of linear regression -0.029 with 95 % CI -0.292 to 0.234) and $CD69^+$ (Fig. 8F mean slope of linear regression -0.028 with 95 % CI -0.180 to 0.124) iNKT cells. This clearly indicates a divergence in the activation status of iNKT cells during acute hepatitis C dependent on the infection outcome.

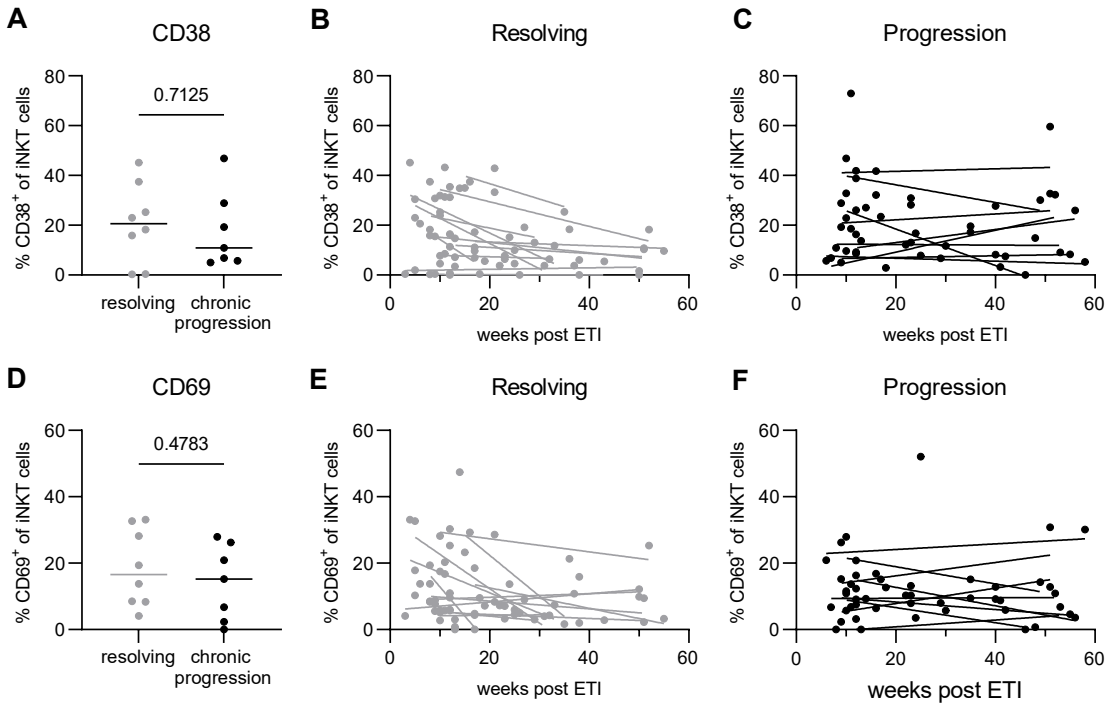

Figure 8. Frequency of CD38⁺ and CD69⁺ iNKT cells during acute HCV infection. (A–C) The frequency of CD38⁺ iNKT cells was analysed at the earliest available time point prior to 10 weeks post ETI (A) and in each patient with more than 2 time points available during the first 60 weeks of infection in resolvers (B) and chronic progressors (C). Each line represents the trend of the frequency in an individual patient determined by linear regression analysis. (D–F) The frequency of CD69⁺ iNKT cells was analysed at the earliest available time point prior to 10 weeks post ETI (D) and in each patient with more than two time points available during the first 60 weeks of infection in resolvers (E) and chronic progressors (F). Each line represents the trend of the frequency in an individual patient determined by linear regression analysis. (A–F) Only samples with more than 20 iNKT cells were included into the analysis.

As next to CD38 upregulation a concomitant decrease of CD127 is described during in vitro iNKT cell activation (Senff 2018) (Senff, Menne et al. in submission), expression of this marker was analysed as well. Furthermore, early occurrence of CD127 expressing HCV-specific T cells is a strong predictor of HCV clearance (Shin 2013). Virtually all iNKT cells expressed CD127 (Fig. 9A, median 92.80 % in resolvers and 93.70 % in progressors), as described before (Senff, Menne et al. in submission), however, levels of CD127⁺ iNKT cells remained high over time and showed no differences between groups (Fig. 9B–C).

While the liver homing receptor CXCR6 was expressed in a median of 30.30 % of iNKT cells in resolvers and 14.90 % of iNKT cells in progressors (Fig. 9D), the T cell exhaustion

marker PD-1 was expressed on substantial, yet lower numbers of iNKT cells during early acute HCV infection (Fig. 9G, median 7.90 % in resolvers and 14.50 % in progressors). Both markers remained stable during the first year of infection in progressors and resolvers (Fig. 9E–F and Fig. 9H–I). CD56 which can act as a marker for cytotoxicity on conventional T cells (Van Acker et al. 2017) was expressed in a median of 28.50 % and 14.10 % of all iNKT cell in resolvers and progressors, respectively, remaining constant over time (Fig. 9K–L). On a side note, this underscores the necessity to use iNKT-specific staining reagents rather than rely on surrogate markers like CD56, which were described before to be expressed by less than half of all true iNKT cells (Montoya et al. 2007). The iNKT cell differentiation marker CD161 which is also linked to cytotoxicity in conventional memory T cells (Konduri et al. 2020) was expressed by high amounts of iNKT cells in resolvers (median 50.35 %) as well as progressors (median 46.90 %) in constant numbers during the first year (Fig. 9M–O).

In conclusion, while iNKT cell frequencies and subtypes remain constant in resolving as well as in chronic progressing acute hepatitis C, iNKT cell activation is associated with HCV infection outcome. This suggests a possible functional role of activated iNKT cells during acute HCV infection.

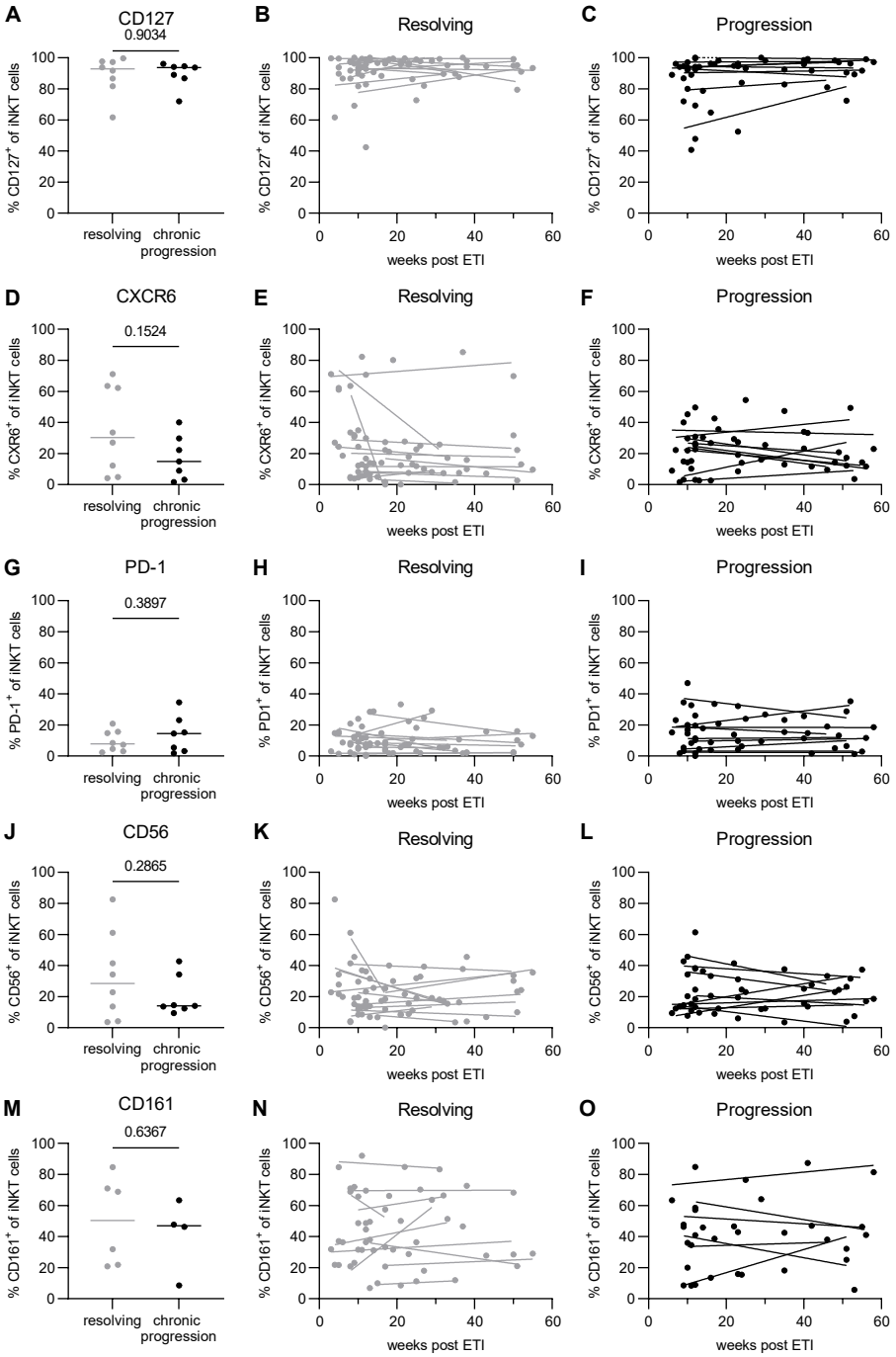

Figure 9. Analysis of surface marker expression on iNKT cells during acute HCV infection. (A–O) The frequency of CD127$^+$ (A – C), CXCR6$^+$ (D – F), PD-1$^+$ (G – I), CD56$^+$ (J – L) and CD161$^+$ (M – O) iNKT cells was analysed at the earliest available time point prior to 10 weeks post ETI (left panels). Furthermore, each patient with more than two time points available during the first 60 weeks of infection with resolving (middle panels) and chronic progressing infection (right panels) was analysed. Each line represents the trend of the frequency in an individual patient determined by linear regression analysis. Only samples with more than 20 iNKT cells were included into the analysis.

6.2.2. Activation of iNKT cells during acute HCV infection correlates with liver inflammation

In order to evaluate a possible relationship between the observed activated iNKT cell phenotype and clinical manifestations of HCV, ALT serum levels were determined. ALT is released by perishing hepatocytes and thus can be used as a surrogate marker for liver damage during acute hepatitis. Hepatocyte damage and subsequent ALT release is thereby mostly mediated by immunopathologic mechanisms, as HCV itself is a non-cytopathic virus (Larrubia et al. 2014; Shin, Sung, and Park 2016; Sung, Racanelli, and Shin 2014).

ALT levels positively correlated with the frequency of activated iNKT cells as marked by CD38 and CD69 expression. Interestingly, this correlation was particularly strong in resolvers indicating an involvement of iNKT cells in liver inflammation during HCV clearance (Fig. 10A, r=0.3729, p=0.0065 for CD38 and Fig. 10C, r=0.4475, p=0.0009 for CD69).

While no strong correlation of surface markers with serum ALT levels were present in samples from chronic progressors (Fig. 10B, 10D, 11B, 11D, 11F, 11H), ALT levels from patients with resolving HCV infection showed an association with a distinct iNKT cell phenotype (Fig. 10 and Fig. 11).

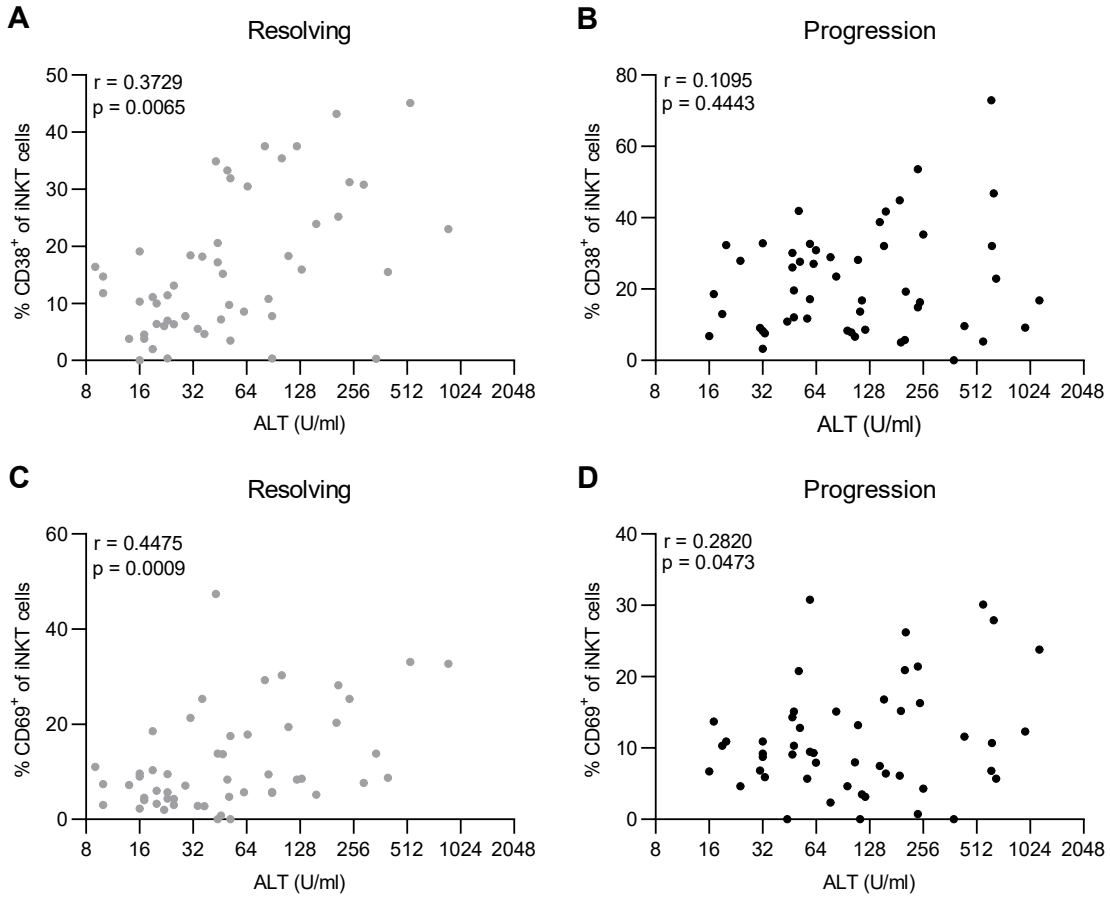

Figure 10. Correlation of serum ALT levels with iNKT cell activation in acute HCV infection. (A+B) The frequency of CD38⁺ iNKT cells was correlated with serum ALT levels from the same time point. All available time points during the first 60 weeks post ETI in resolvers (A) or chronic progressors (B) were grouped into one graph. (C+D) The frequency of CD69⁺ iNKT cells was correlated with serum ALT levels from the same time point. All available time points during the first 60 weeks post ETI in resolvers (C) or chronic progressors (D) were grouped into one graph. Pearsons correlation coefficient was calculated and r-value and p-value are depicted. Only samples with more than 20 iNKT cells were included into the analysis.

Serum ALT levels positively correlated with frequencies of iNKT cells expressing CXCR6 (Fig. 11A, r=0.4130, p=0.0023), CD56 (Fig. 11C, r=0.3432, p=0.0113) and CD8 (Fig. 11G, r=0.4299, p=0.0015), whereas levels of CD4⁺ iNKT cells negatively correlated with serum ALT level (Fig. 11E, r=-0.3043, p=0.0283). This indicates an enrichment of activated iNKT cells biased towards a CD8⁺ cytotoxic phenotype that are capable of homing to the liver via CXCR6 receptor upregulation in resolving patients with high liver damage. Ultimately, these cells might participate in the inflammatory response during acute resolving HCV infection.

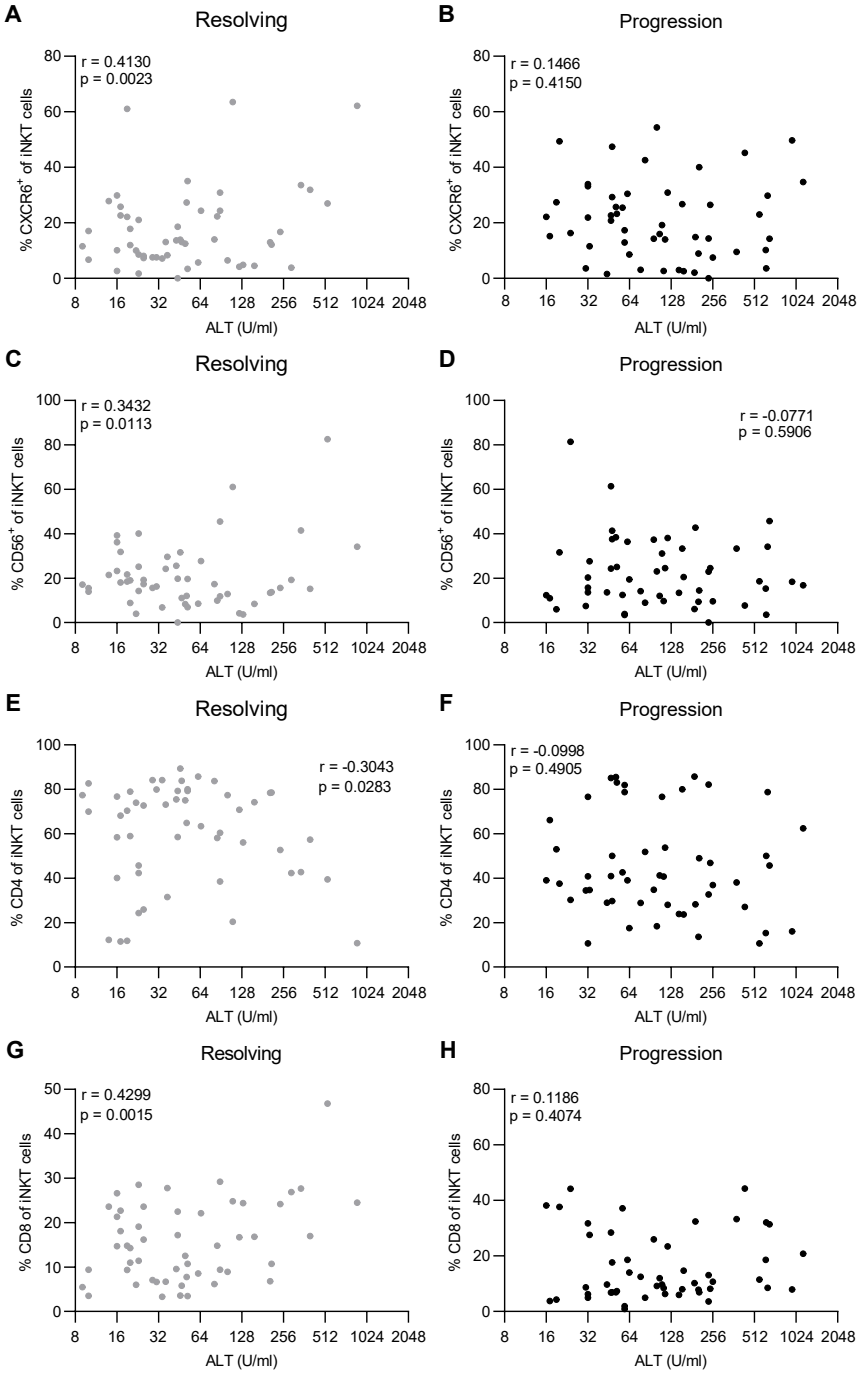

Figure 11. Correlation of iNKT cell surface marker expression with serum ALT levels in all samples analysed during the first year post ETI. (A–H) The frequency of CXCR6$^+$ (A+B), CD56$^+$ (C+D), CD4$^+$ (E+F) and CD8$^+$ (G+H) iNKT cells was correlated with serum ALT levels from the same time point. All available time points during the first 60 weeks post ETI in resolvers (left panels) or chronic progressors (right panels) were grouped into one graph. Pearsons correlation coefficient was calculated and r-value and p-value are depicted. Only samples with more than 20 iNKT cells were included into the analysis.

6.3. Characterization of intrahepatic iNKT cells

This association of liver inflammation with activation of peripheral blood iNKT cells raised the question if iNKT cells in the liver substantially differ from iNKT cells in the periphery. Therefore, mononuclear cells isolated from perfusates of surgically resected liver tissue and healthy donor PBMCs were analysed by flow cytometry (Fig. 12A).

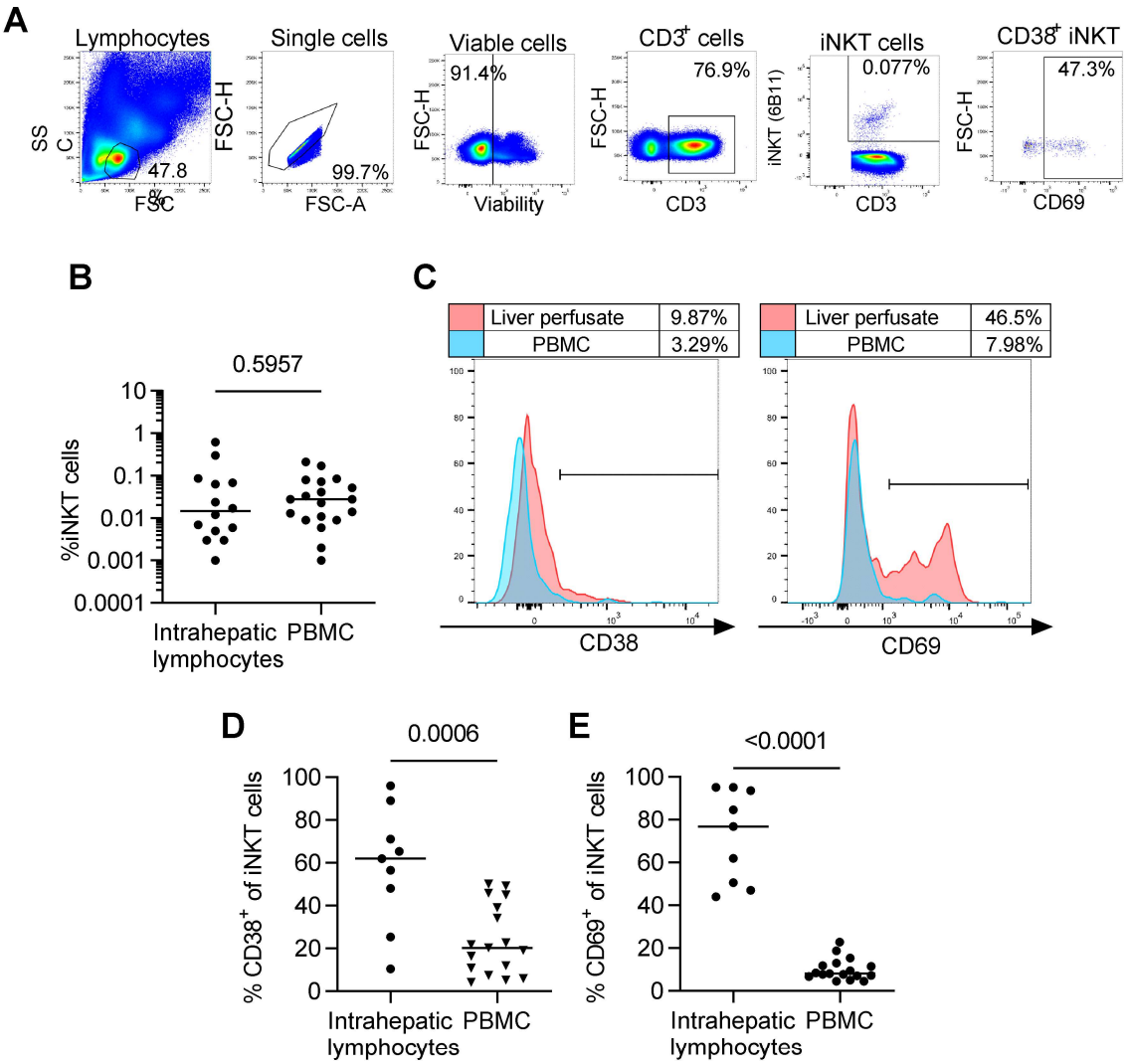

Figure 12. Frequency and activation status of intrahepatic and peripheral iNKT cells. (A) Representative gating strategy for intrahepatic iNKT cells obtained from liver perfusates and peripheral iNKT cells. (B) The frequency of iNKT cells in the liver (n=14) and the periphery (n=19) was analysed. P-value was calculated with the Mann-Whitney test. (C) Representative histogram of CD38 and CD69 expression by intrahepatic and matched PBMC derived iNKT cells. (D+E) The frequency of $CD38^+$ (D) and $CD69^+$ iNKT cells perfusates of resected livers (n=9) and in the periphery of unmatched healthy donors (n=17) was analysed. Unpaired t-test was used for comparison of the groups and p-value is depicted. Only samples with more than 20 iNKT cells were included into the analysis.

Hepatic iNKT cells represented a median of 0.0145 % of lymphocytes with a large range from 0.001 % to 0.62 %. This frequency was comparable with iNKT cells from peripheral blood that accounted for a median of 0.028 % of all lymphocytes (Fig. 12B, range 0.001 % to 0.21%, p=0.5957). This is in striking contrast to what is known about iNKT cells in the rodent liver where they can make up to 30 % of all intrahepatic lymphocytes.
Comparison of hepatic with matched and non-matched circulatory iNKT cells showed elevated levels of activated iNKT cells marked by CD38 and CD69 expression in the liver (Fig. 12C–E). PBMCs comprised a median of 20.3 % $CD38^+$ iNKT cells (range 4.23 %–50 %) and 8.11 % $CD69^+$ iNKT cells (range 4.44 %–22.8 %). In contrast, CD38 and CD69 were expressed on a median of 61.50 % (range 10.50 %–96.10 %, p=0.0006) and 76.90 % (range 44 %–95.20 %, p<0.0001) of all intrahepatic iNKT cells, respectively.
To further investigate the phenotype of hepatic iNKT cells, a t-SNE analysis was performed with 11 PBMCs derived from healthy donors and four unmatched liver samples based on the expression data of 12 surface markers. Liver derived iNKT cells thereby clustered distinctly from circulatory iNKT cells indicating their unique phenotype (Fig. 13). This hepatic iNKT cell cluster was enriched not only in activation markers CD38 and CD69 but also other markers associated with T cell activation and differentiation like PD-1 and 2B4 (Kroy et al. 2014) (Fig. 13).
Overall, this shows that iNKT cells are highly activated in the liver and resemble the phenotype observed during chronic progression of HCV, indicating a unique phenotype of intrahepatic iNKT cells even under steady-stae conditions.

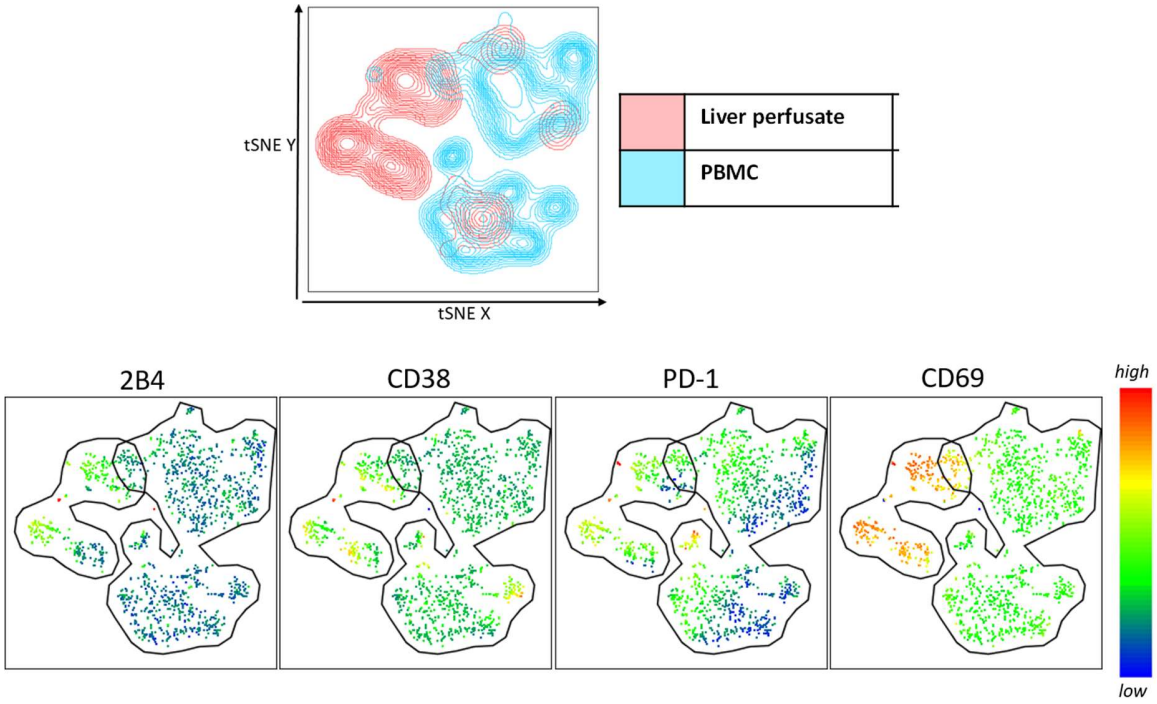

Figure 13. TSNE analysis of intrahepatic and peripheral iNKT cells. Samples with more than 100 iNKT cells (n=4 intrahepatic, n=11 PBMC) were used for tSNE analysis. The surface markers Vα24Jα18 TCR, PD-1, CD127, CD4, Va24, NKG2D, CD49a, 2B4, CD38, CD69, CD8, CD3 were included in the analysis. Liver samples and PBMC samples were gated in the tSNE plot and the populations containing the majority of these cells were gated. The liver and PBMC gates were then analysed for their expression of the depicted marker.

6.4. Functional analysis of iNKT cells in health and disease

6.4.1. IFN-γ release of iNKT cells ex-vivo

As iNKT cells are activated during chronic progression of HCV, it was determined if they are also functionally different depending on the disease outcome. Previous data from our group showed that iNKT cells from HCV-RNA positive or negative PWID secreted similar amounts of IFN-γ when unspecifically stimulated with PMA and ionomycin (Senff 2018). In order to employ a more physiological stimulus, an iNKT cell specific stimulation protocol was established. Therefore, PBMCs from healthy donors were stimulated with αGalCer or a cocktail of IL-12, IL-15 and IL-18 for 10 days to activate and expand iNKT cells. Subsequently, the expanded cells and freshly thawed cells from the same donor were restimulated with either αGalCer or IL-12, IL-15 and IL-18 cocktail or PMA and Ionomycin. Invariant NKT cells secreted high levels of IFN-γ and TNFα and low levels of IL-4 when ex-vivo stimulated with PMA and ionomycin while no other stimulus did induce a robust cytokine response (Fig. 14A–B). Interestingly, restimulation with IL-12, IL-15 and IL-18 of αGalCer preactivated iNKT cells led to IFN-γ secretion in 27.15 % of all iNKT cells whereas only 2.05 % of ex-vivo IL-12, IL-15 and IL-18 stimulated iNKT cells released IFN-γ (Fig. 14D). Surprisingly, and in contrast to this, IL-12, IL-15 and IL-18 preactivated iNKT cells that were restimulated with αGalCer showed almost no IFN-γ release (data not shown). This suggests an additive effect of specific TCR stimulation and the presence of proinflammatory cytokines IL-12, IL-15 and IL-18 on the capacity of iNKT cells to release IFN-γ.

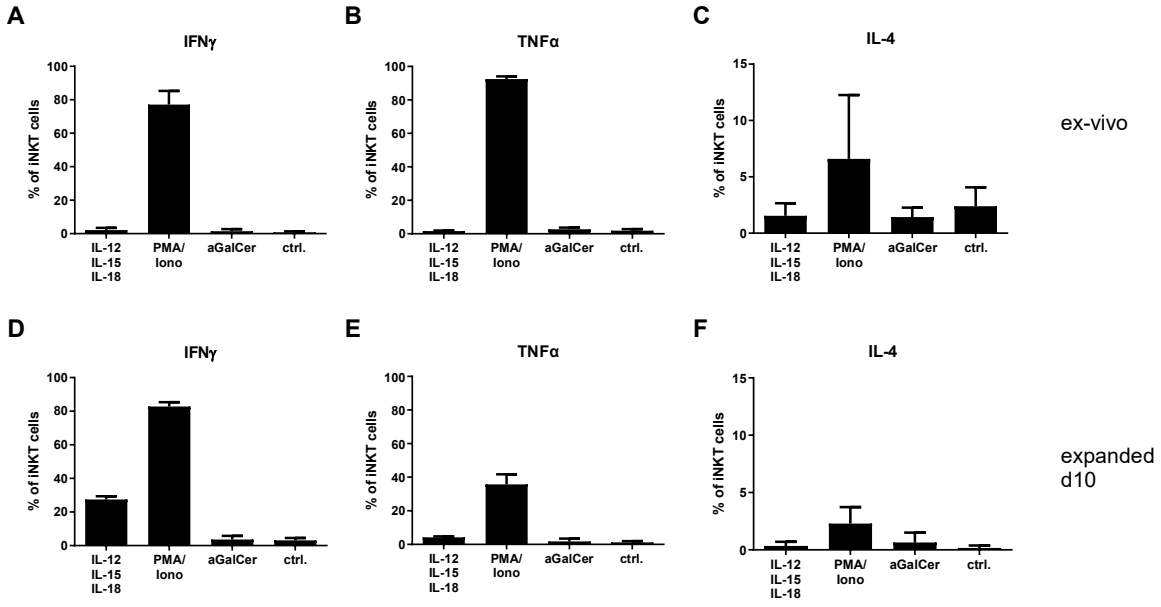

Figure 14. Release of cytokines by iNKT cells ex vivo and after 10 days expansion. (A–C) PBMCs from healthy donor buffy coats were stimulated with the indicated substances and BFA for 4 hours and the frequency of IFN-γ$^+$ (A), TNFα$^+$ (B) and IL-4$^+$ (C) iNKT cells was determined by intracellular cytokine staining. (D – F) Invariant NKT cells were expanded from PBMCs of the same donors as above for 10 days with 1 µg/ml αGalCer and 25 U/ml IL-2 before restimulation with the indicated substances and in the presence of BFA for 4h. The frequency IFN-γ$^+$ (D), TNFα$^+$ (E) and IL-4$^+$ (F) iNKT cells was determined by ICS. The mean and standard deviation of 2 donors is shown.

Next, PBMCs were stimulated with αGalCer or IL-12, IL-15 and IL-18 or a combination of both for 24 hours to evaluate if IFN-γ release could also be evoked ex-vivo and to rule out that it was an effect caused by in vitro cultivation and expansion of iNKT cells. IL-12, IL-15 and IL-18 cocktail and αGalCer activated iNKT cells in an additive manner marked by an 1.82-fold and 1.68-fold increase in CD38$^+$ iNKT cells compared to αGalCer or the interleukin cocktail alone, respectively (Fig. 15A). While after αGalCer (mean 11.20 %) or interleukin (mean 25.50 %) treatment only few iNKT cells secreted IFN-γ, combination of both augmented IFN-γ secretion dramatically (Fig. 15B, mean 54.07 %).

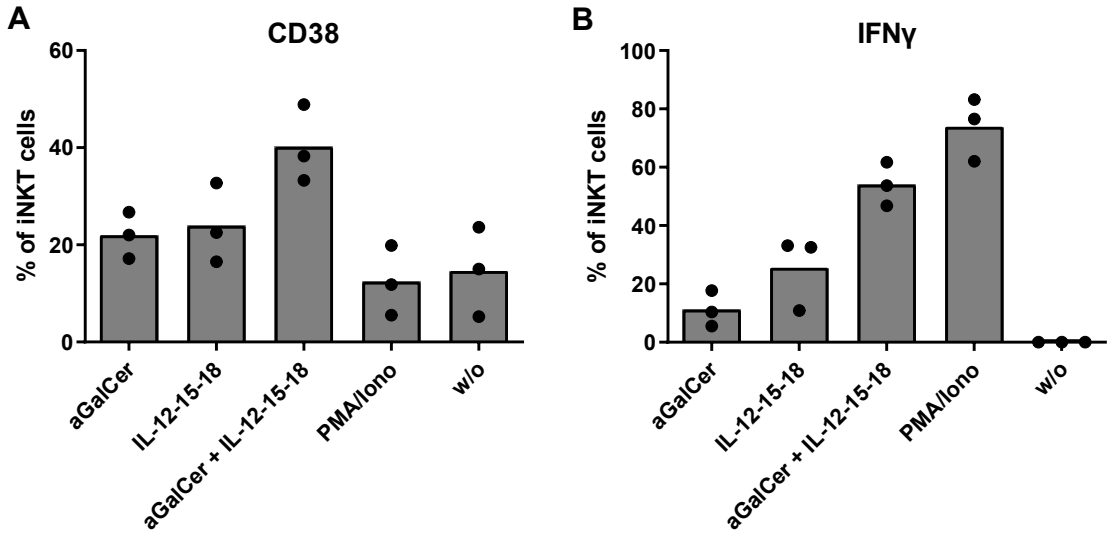

Figure 15. CD38 and IFN-γ expression by in vitro activated iNKT cells. (A+B) PBMCs from healthy donors were stimulated for 24 hours with the indicated stimuli in the presence of BFA for the last 4 hours and the frequency of $CD38^+$ (A) and IFN-γ^+ (B) iNKT cells was analysed by flow cytometry and ICS. Each point represents one donor and bars represent the mean of three individual donors.

6.4.2 Influence of TLR triggers on iNKT cell activation and IFN-γ secretion

As iNKT cells are part of the innate immune response, a possible effect of innate stimuli on the functionality of iNKT cells was determined. In a former analysis, stimulation of PBMCs with type I and type III interferons had no effect on the expression of CD38 on iNKT cells, however type I interferon slightly induced CD1d on monocytes (Senff 2018). Additionally, one study found an increase in the TCR mediated activation of enriched iNKT cells by addition of CpG oligodeoxynucleotides (ODN). In order to determine a possible influence of TLR ligands on iNKT cell activation and cytokine release, PBMCs were stimulated with the TLR9 agonist CpG ODN or the TLR3 agonist Poly(I:C) alone or in combination with αGalCer or IL-12, IL-15 and IL-18 for 24h. The TLR ligands alone neither activated iNKT cells in terms of CD38 upregulation (Fig. 16A+C) nor induced an IFN-γ response by iNKT cells (Fig. 16B+D). Moreover, they did not augment the effects of αGalCer or IL-12, IL-15 and IL-18 on CD38 expression and IFN-γ secretion in iNKT cells (Fig. 16A–D).

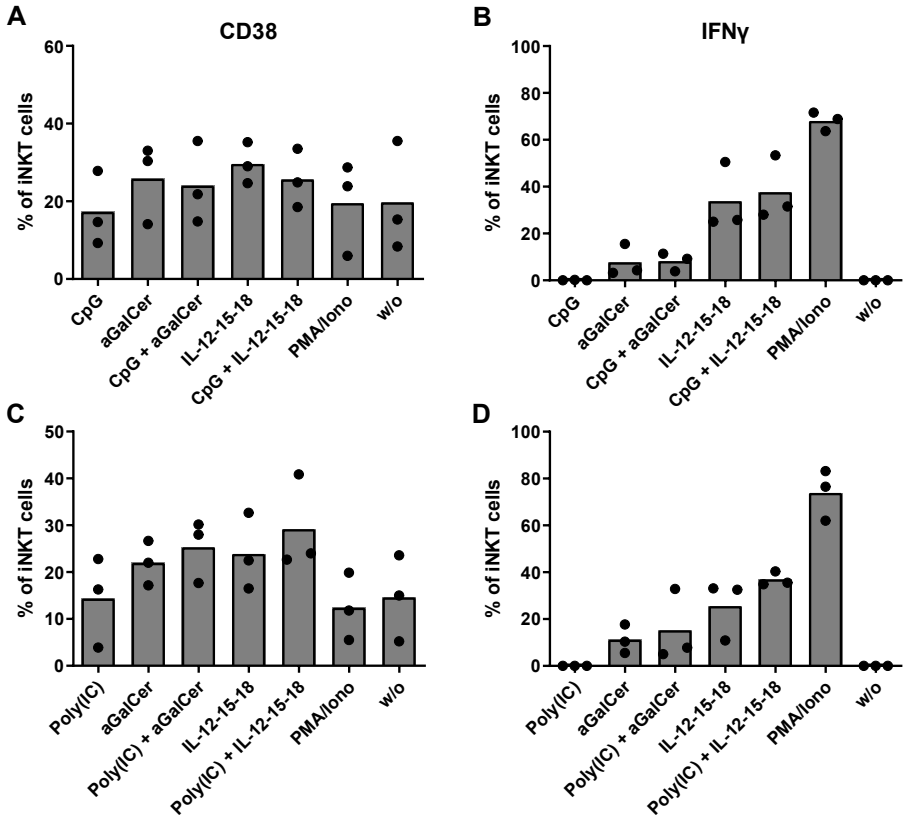

Figure 16. Influence of TLR triggers on iNKT activation and IFN-γ secretion. (A–D) PBMCs from healthy donors were treated for 24 hours with the indicated stimuli in the presence of BFA for the last 4 hours. The frequency of $CD38^+$ (A+C) and IFN-γ^+ (B+D) iNKT cells was determined by flow cytometry and ICS. Dots represent the individual donors and bars the mean of three donors.

6.4.3 IFN-γ release by iNKT cells in HCV-RNA positive and HCV-RNA negative PWID

In order to test, if iNKT cells from HCV-RNA posititve PWID show altered functionality when activated with a more physiological stimulus, PBMCs from HCV-RNA positive and HCV-RNA negative anti HCV positive PWID were stimulated for 24 hours with αGalCer and IL-12, IL-15 and IL-18 and the IFN-γ response was determined by flow cytometry. In HCV-RNA positive PWID an average of 15.05 % of iNKT cells stained positive for IFN-γ while a mean frequency of 13.00 % of iNKT cells in HCV-RNA negative PWID were IFN-γ positive (Fig. 17, p=0.8004). Overall, a protocol for rapid and robust IFN-γ induction in iNKT cells from PBMCs was established that relied on αGalCer mediated TCR ligation combined with stimulation with proinflammatory cytokines IL-12, IL-15 and IL-18. Additional stimulation of the innate immunity by TLR ligands did not seem to augment the IFN-γ response of iNKT cells.

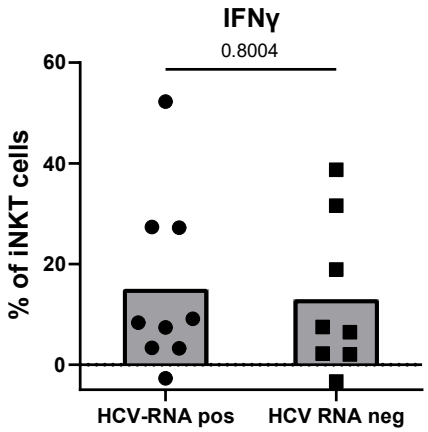

Figure 17. IFN-γ secretion by iNKT cells from HCV-RNA positive and negative PWID. PBMCs from HCV-RNA positive and HCV-RNA negative PWID were stimulated for 24 hours with 1 µg/ml αGalCer, 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 in the presence of BFA for the last 4 hours. The frequency of IFN-γ$^+$ iNKT cells was determined by ICS. The value of the unstimulated negative controls were subtracted from the stimulated samples. Each dot shows one donor while the bars represent the mean of nine HCV-RNA positive and 8 HCV-RNA negative donors. Unpaired t-test was used to calculate the depicted p-value.

This protocol facilitated an antigen dependent stimulation and thus activated iNKT cells in a physiological way. This optimised protocol for iNKT cell activation did not reveal any differences in IFN-γ secretion between HCV-RNA positive and negative PWID.

6.4.4 Influence of iNKT cell activation on CD8$^+$ T cell response

Since the IFN-γ response from iNKT cells seemed to be unaltered in chronically HCV infected patients, it was next evaluated if iNKT cells might have an indirect effect on the immune response by modulating the downstream T cell response. CMV specific CD8$^+$ T cells were expanded from PBMCs for 10 days with a pool of CMV peptides covering different HLA restrictions. Half of the cultures were substituted with αGalCer to specifically activate iNKT cells. Expectedly, iNKT cells strongly proliferated to a mean frequency of 2.67 % and were significantly higher in CD38 (mean 98.23 %) and lower in CD127 (mean 27.35 %) after 10 days of αGalCer stimulation but not when cultured without αGalCer (Fig. 18A–C).

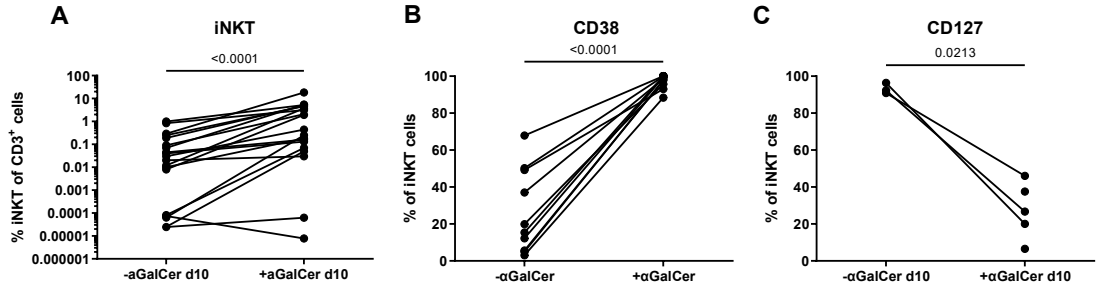

Figure 18. Phenotype of iNKT cells after co-culture with CMV-specific CD8⁺ T cells. Buffy coat derived PBMCs from CMV positive donors were stimulated for 10 days with 1 µg/ml CMV peptide pool and 25 U/ml IL-2. Simultaneously, iNKT cells were expanded in the same culture with 1 mg/ml αGalCer or left untreated. (A) Invariant NKT cell frequency, (B) frequency of CD38⁺ and (C) CD127⁺ iNKT cells was determined after 10 days by flow cytometry. Invariant NKT cells were defined as Vα24 and Vβ11 double positive. Each dot represents one individual of 20 donors. Only samples that contained more than 20 iNKT cells were included in the analysis. Groups were compared by Wilcoxon matched-pairs signed rank test (A) or paired t-test (B+C)

CMV specific T cells were defined via IFN-γ ICS after restimulation with the CMV peptide pool. Without αGalCer addition an average of 22.92 % of CD8⁺ T cells stained positive for IFN-γ and with αGalCer addition a mean of 22.36 % of CD8⁺ T cells secreted IFN-γ indicating robust CMV specific T cell expansion regardless of simultaneous iNKT cell activation (Fig. 19A). Moreover, there was a significant albeit very slight increase in CD38⁺ T cells when αGalCer was added to the culture during T cell expansion (66.81 % vs. 62.81 %, p=0.0381) whereas CD127 expression on T cells was unaltered (Fig. 19B-C). Overall, iNKT cell stimulation showed hardly any effect on the expansion of CMV-specific T cells.

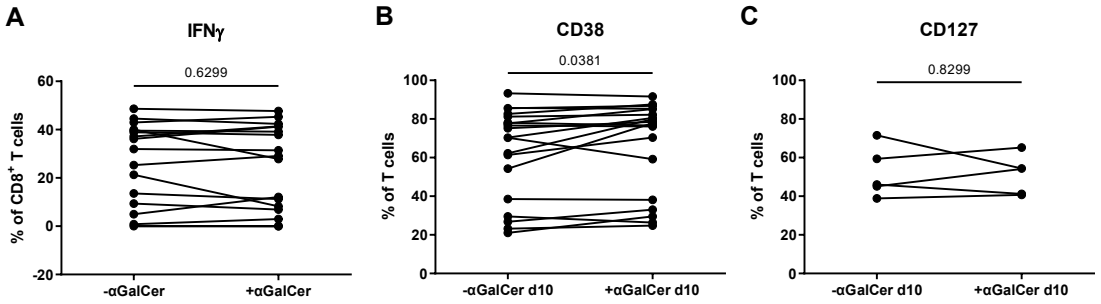

Figure 19. IFN-γ secretion and phenotype of T cells after iNKT costimulation. Buffy coat derived PBMCs from CMV positive donors were stimulated for 10 days with 1 µg/ml CMV peptide pool and 25 U/ml IL-2. Simultaneously, iNKT cells were expanded in the same culture with 1 mg/ml αGalCer or left untreated. (A) IFN-γ⁺ CMV-specific CD8⁺ T cell frequency after restimulation for 4 hours with CMV peptide pools and BFA was analysed by ICS. (B) Frequency of CD38⁺ and (C) CD127⁺ T cells was determined after 10 days by flow cytometry. Each dot represents one individual of a total of 20 donors. Groups were compared by paired t-test and p-value is shown.

6.5. CD38 defines an iNKT cell subset with a distinct phenotype and reduced effector functions

Since iNKT cells with an activated $CD38^+$ phenotype were enriched during progression to chronic hepatitis C, it was investigated if these cells phenotypically and functionally differ from $CD38^-$ iNKT cells.

6.5.1. Establishment of a protocol for functional analysis of $CD38^+$ and $CD38^-$ iNKT cells ex vivo

Like conventional T cells, human iNKT cells upregulate CD38 after activation. In order to focus on iNKT cells that express CD38 ex vivo and to avoid induction of CD38 skewing the analysis, an in vitro activation protocol was established that facilitated the analysis of ex vivo $CD38^+$ iNKT cells.

PBMCs from healthy donors were in vitro stimulated with αGalCer and IL-12, IL-15 and IL-18 for various time periods and the IFN-γ secretion, CD38 upregulation and TCR downmodulation of iNKT cells was analysed by flow cytometry. At 14 hours post activation, despite high levels of IFN-γ secretion (Fig. 20C, mean 30.73 %), iNKT cell frequency as detected by Va24Ja18 TCR staining was reduced by 3.45-fold (Fig. 20A, $p=0.0017$). In addition, $CD38^+$ iNKT cells increased from 10.61 % to 21.45 % (Fig. 20B, $p=0.0058$).

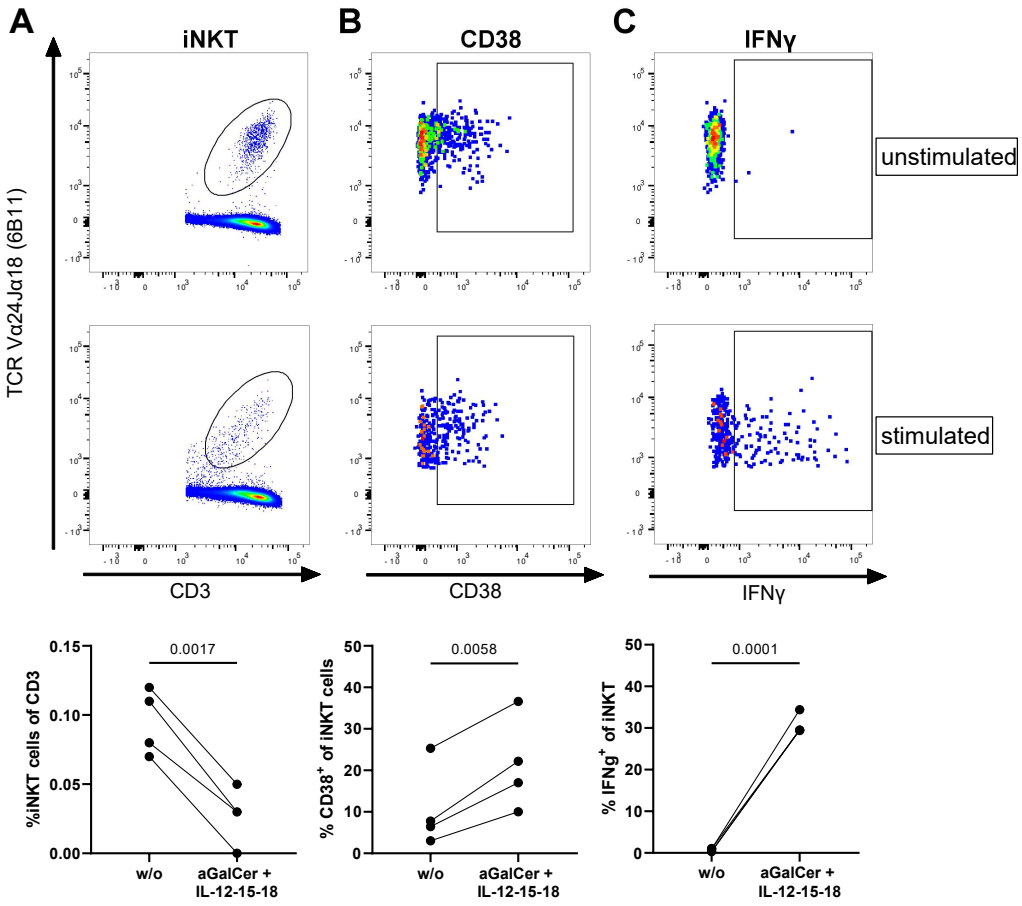

Figure 20. Invariant NKT activation 14 hours post stimulation. Healthy donor buffy coat derived PBMCs were stimulated for 14 hours with 1 µg/ml αGalCer, 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 or left untreated in the presence of BFA for the last 4h. (A) Frequency of iNKT cells as determined by Vα24Jα18 TCR staining, (B) CD38 expression of iNKT cells and (C) IFN-γ secretion of iNKT cells were analysed by flow cytometry and ICS. A representative flow cytometry plot is shown in the upper panels. In the lower panels, each dot represents one of 4 individual donors. Paired t-test was used to compare groups and the p-value is depicted.

High levels of IFN-γ positive iNKT cells were also detectable after 8h of activation (Fig. 21C, mean 47.08 %). Even though the MFI of the iNKT TCR was slightly lower than ex vivo, the frequencies of total (Fig. 21A, mean 0.0775 % and 0.090 %) and CD38⁺ iNKT cells were not significantly altered (Fig. 21B, mean 20.86 % and 17.92 %). In contrast, 6h post stimulation less iNKT cells secreted IFN-γ (Supplemental Fig. 1). Therefore, the ex-vivo functionality of CD38⁺ iNKT cells was analysed after 8h of stimulation with αGalCer and a cocktail of IL-12, IL-15 and IL-18.

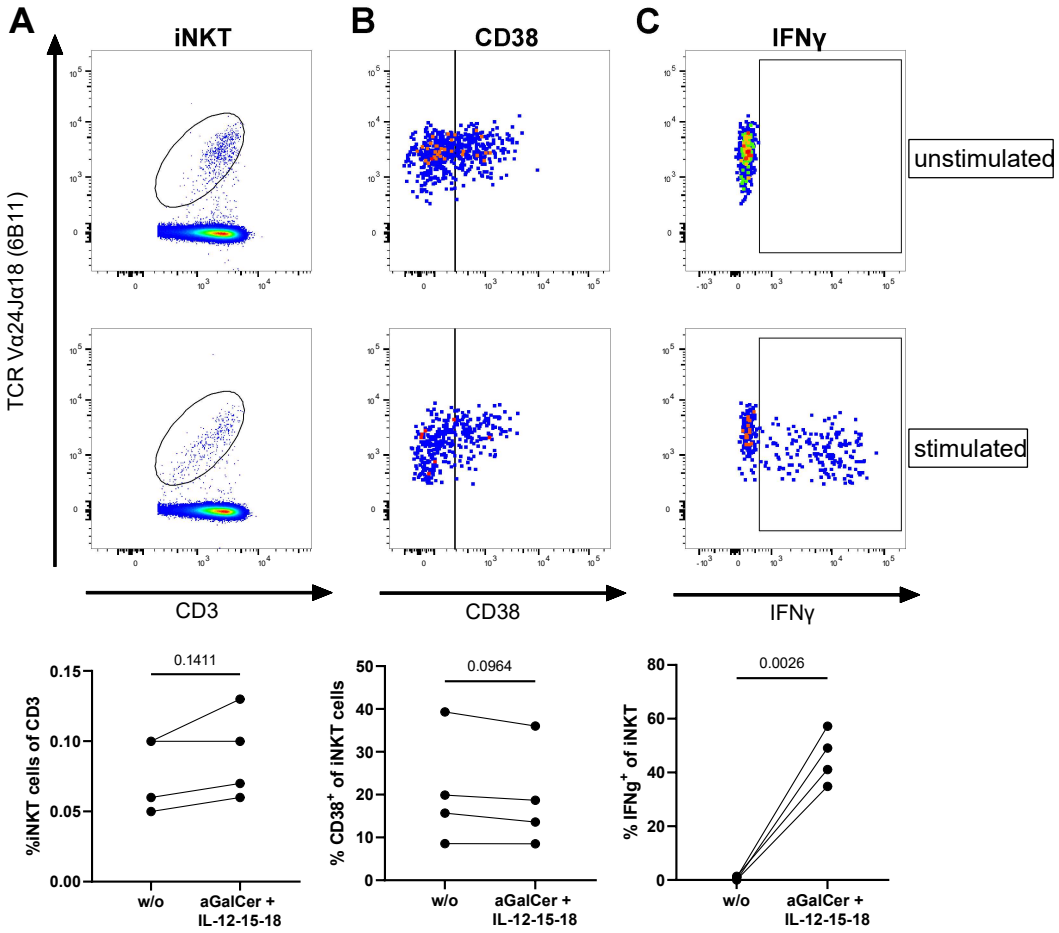

Figure 21. Invariant NKT activation 8 hours post stimulation. Healthy donor buffy coat derived PBMCs were stimulated for 8 hours with 1 µg/ml αGalCer 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 or left untreated in the presence of BFA for the last 4h. (A) Frequency of iNKT cells as determined by Vα24Jα18 TCR staining, (B) CD38 expression of iNKT cells and (C) IFN-γ secretion of iNKT cells were analysed by flow cytometry and ICS. A representative flow cytometry plot is shown in the upper panels. In the lower panels, each dot represents one of four individual donors. Paired t-test was used to compare groups and the p-value is depicted.

6.5.2. CD38 marks a population of iNKT cells with a unique phenotype and effector functions

In order to define a distinct phenotype and cytokine profile of the CD38$^+$ and CD38$^-$ iNKT cell compartments, cytokine production as well as surface markers expression in these subtypes were analysed. Ex-vivo stimulation of PBMCs for 8 hours and subsequent analysis of the CD38$^+$ and CD38$^-$ iNKT cell subpopulations (Fig. 22A) revealed reduced IFN-γ secretion in the CD38$^+$ iNKT cells (Fig. 22B, mean 16.26 %). CD38$^-$ iNKT cells contained a 3.3-fold higher frequency of IFN-γ positive cells (mean 53.60 %, p=0.0048).

Interestingly, reduced IFN-γ expression was not observed in PD-1$^+$ iNKT cells, a marker that was traditionally defined as an exhaustion marker and thus can be linked to a diminished functional T cell response under certain conditions (Fig. 22C). This led to the hypothesis that CD38 marks a distinct subset of iNKT cells that might be phenotypically different and functionally impaired. Thus, expression of other markers and cytokines was examined on CD38$^+$ compared to CD38$^-$ iNKT cells. CD38$^+$ iNKT cells showed significantly decreased levels of activation after αGalCer and IL-12, IL-15 and IL-18 stimulation, marked by CD69 expression (Fig. 22D, p=0.0171). Analysis of CD4 and CD8 expression revealed enrichment of CD4$^+$ iNKT cells within the CD38$^+$ iNKT population (Fig. 22E, mean 71.08 % vs. 27.42 %, p=0.0029). In contrast, double negative iNKT cells were significantly diminished in the CD38$^+$ population (Fig. 22G, mean 10.88 % vs. 49.06 %, p=0.0110) while CD8 levels remained constant (Fig. 22F).

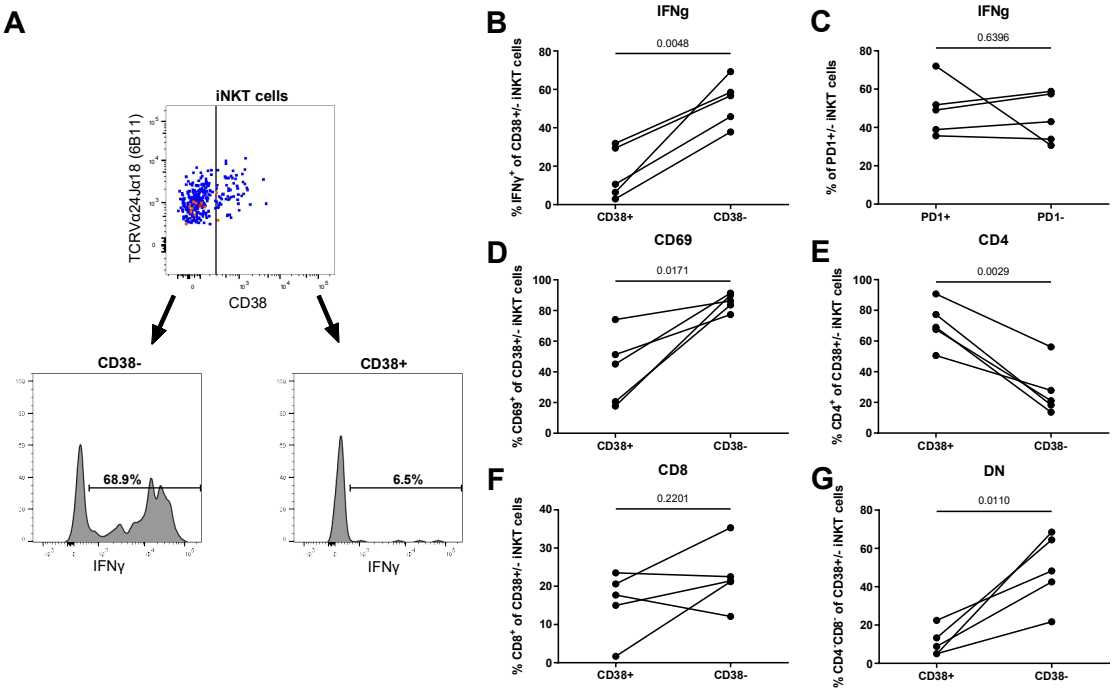

Figure 22. CD38$^+$ iNKT cells show reduced activation and IFN-γ secretion and are CD4 biased. PBMCs from healthy donor derived buffy coats were stimulated for 8 hours with 1 µg/ml αGalCer, 10 ng/ml IL-12, 100ng/ml IL-15 and 50 ng/ml IL-18 in the presence of BFA for the last 4h. (A) Invariant NKT cells were gated into a CD38$^+$ and CD38$^-$ population (iNKT cells were gated into a PD-1$^+$ and PD-1$^-$ population for (C)). (B–G) Frequency of IFN-γ$^+$ (B+C), CD69$^+$ (D), CD4$^+$ (E), CD8$^+$ (F) and DN (G) cells in the respective iNKT-parent population indicated on the x-axis. Each dot represents an individual donor (n=5). Paired t-test was used to calculate the indicated p-value.

6.5.3. CD38$^+$ iNKT cells have diminished proinflammatory and cytotoxic effector functions

As CD38 expression on iNKT cells was associated with reduced IFN-γ secretion in in vitro stimulated iNKT cells, association of CD38 with other functional cytokines was assessed. PBMCs of healthy donors were in vitro stimulated with PMA and ionomycin and the expression of TNFα and intracellular expression of Granzyme B in iNKT cells was analysed. Again, less TNFα and Granyzme B positive cells were observed in the CD38$^+$ than in the CD38$^-$ iNKT cell population. While an average of 10.99 % of all CD38$^-$ iNKT cells stained positive for Granzyme B, the CD38$^+$ iNKT cell fraction contained only 2.05 % of Granzyme B$^+$ cells (Fig. 23A, p=0.0018). TNFα positive iNKT cells showed a significant, almost 2-fold increase in the CD38$^-$ (mean 30.40 %) compared to the CD38$^+$ population (mean 56.54 %) (Fig. 23B, p=0.0013). Furthermore, the presence of multifunctional cells which secrete multiple cytokines simultaneously, was investigated. Interestingly, the CD38$^+$ iNKT cell population contained also significantly less IFN-γ$^+$TNFα$^+$ double positive cells (Fig. 23C, p=0.0094). In order to test if this phenotype is CD38 specific, cytokine expression was also tested in the PD-1 positive and negative population which is associated with T cell activation but also exhaustion. Surprisingly, there was a strong tendency towards elevated levels of cytokine producing iNKT cells in the PD-1 positive population (Fig. 23D–F). This is in contrast to the observed CD38 phenotype and indicates that CD38 marks an iNKT cell phenotype that is associated with reduced cytokine secretion after in vitro stimulation that might be independent of markers that are classically used to define T cell populations with altered functionality, like PD-1.

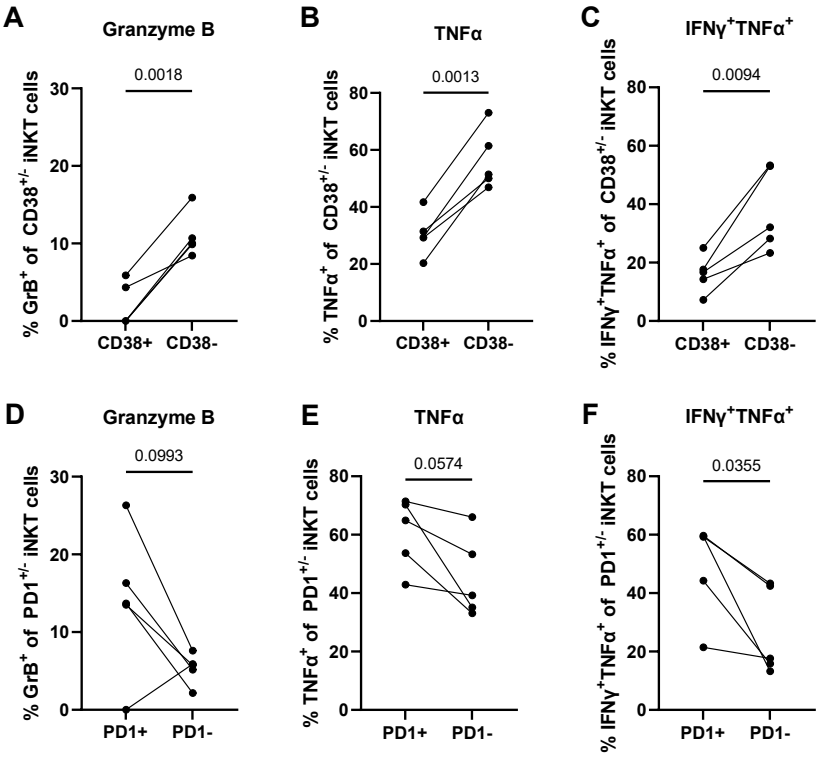

Figure 23. CD38⁺ iNKT cells have diminished functional cytokine secretion and cytotoxicity. PBMCs from healthy donors were stimulated for 4 hours with PMA and ionomycin in the presence of BFA and the cytokine expression of iNKT cells was analysed by flow cytometry and ICS. Invariant NKT cells were gated into a CD38⁺ and a CD38⁻ or a PD-1⁺ and a PD-1⁻ population. (A – C) The frequency of Granzyme B⁺ (A), TNFα⁺ (B) and IFN-γ⁺TNFα⁺ (C) cells in the CD38⁺ or CD38⁻ iNKT cell population is depicted. (D – F) Frequency of Granzyme B⁺ (A), TNFα⁺ (B) and IFN-γ⁺TNFα⁺ (C) cells in the PD-1⁺ or PD-1⁻ iNKT cell population is depicted. (A – F) Each dot represents one of 5 individual donors. Groups were compared with a paired t-test and calculated p-values are shown.

6.5.4. CD38⁺ iNKT cells display a unique transcription factor expression

Invariant NKT cells express high levels of the transcription factor PLZF which acts as their master (Kovalovsky et al. 2008). As CD38⁺ iNKT cells were significantly reduced in IFN-γ and TNFα secretion, expression of PLZF and T-bet, the transcription factor linked to a Th1 response in CD4⁺ T cells were examined. According to the up to this point observed altered phenotype of the CD38⁺ population, a remarkable fraction of CD38⁺ iNKT cells was devoid of PLZF expression (Fig. 24A, p=0.0006). At the same time they also expressed significantly less T-bet in comparison to CD38⁻ iNKT cells (Fig. 24B, p=0.0165) indicating that CD38 marks a subset of iNKT cells with less capability to mount a Th1 cytokine response and potentially altered iNKT cell specific effector functions.

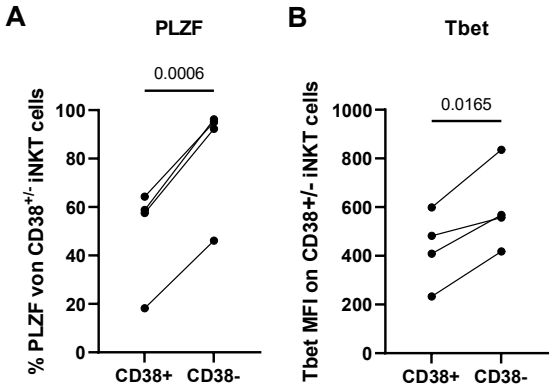

Figure 24. Expression of transcription factors by CD38 iNKT cell subsets. Healthy donor buffy coat dervied PBMCs were analysed for expression of transcription factors (A) PLZF and (B) T-bet (mean fluorescence intensity) in iNKT cells by flow cytometry and intranuclear cytokine staining. Each dot shows an individual of 4 donors. P-value was calculated by paired t-test. Samples with less than 20 iNKT cells were excluded from the analysis.

6.5.5. CD38$^+$ iNKT cells show impaired proliferation

In order to determine the proliferative capacity of CD38$^+$ and CD38$^-$ iNKT cells, PBMCs of two healthy donor buffy coats were stained with a cell tracing dye and an anti-CD38 antibody. Cells were then sorted into a CD38$^+$ and negative population and afterwards co-cultured with feeder cells. Half of the cells were stimulated with αGalCer and IL-2 while the other half was treated with IL-2 only. After 12 days, proliferation of the iNKT cells was assessed by dye dilution. They were divided into two groups according to the fluorescence intensity of the remaining dye. All cells that divided at least once and thus showed signs of dye dilution were considered as proliferated cells which was the case for almost all cells after 12 days. If on the other hand, the cells proliferated until the cell tracing dye was completely diluted, the cells were categorised as highly proliferated (Fig. 25A). While virtually all iNKT cells were highly proliferated after 12 days when activated with αGalCer regardless of CD38 expression (Fig. 25B), in the absence of iNKT specific stimulation the majority of cells had only moderately proliferated. Thereby, the iNKT cells that were CD38$^-$ at day 0 seemed to contain a higher amount of highly proliferated cells with 46.30 % after 12 days while only 10.25 % of cells that were CD38$^+$ at d0 were highly proliferated (Fig. 25C). On a side note, all CD38$^-$ cells induced CD38 during the stimulation with αGalCer suggesting that CD38 expression after αGalCer stimulation of bulk iNKT cells is due to CD38 upregulation and not due to an expansion of the CD38$^+$ population.

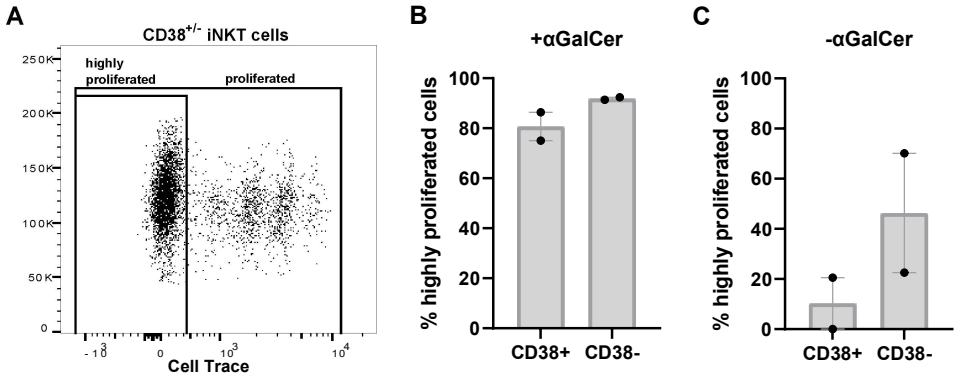

Figure 25. Proliferation of CD38$^+$ and CD38$^-$ iNKT cells. PBMCs from healthy donor buffy coats were stained with a cell trace dye and FACS sorted into a CD38$^+$ and CD38$^-$ iNKT cell population. The isolated cells were then cultured together with irradiated, autologous, unlabelled PBMCs as feeder cells for 12 days in the presence of αGalCer and IL-2 or IL-2 alone before proliferation was assessed by dye dilution. (A) Representative flow cytometry plot of proliferated iNKT cell with at least on cell division event and highly proliferated cells that completely diluted the cell trace dye. (B) Frequency of highly proliferated iNKT cells i shown in the cultures that were initially CD38$^+$ or CD38$^-$ at d0 and cultured in the presence of αGalCer. (C) Frequency of highly proliferated iNKT cells in shown in the cultures that were initially CD38$^+$ or CD38$^-$ at d0 and cultured in the absence of αGalCer. Each dot represents an individual donor and bars show the mean frequency of two donors.

In conclusion, ex vivo CD38 expression on iNKT cells can mark a subpopulation of functionally defective cells with reduced ability to secrete IFN-γ, TNFα, Granzyme B and proliferative capacity.

6.6. Transcriptome profiling of activated iNKT cells

As iNKT cells with an activated, CD38⁺ phenotype were enriched during chronic progression of acute HCV, gene expression of activated iNKT cells was analysed to reveal functional insights into this iNKT subpopulation. Therefore, RNA-sequencing was conducted to define the complete transcriptome of activated iNKT cells in detail.

In order to define the optimal time point for transcriptome analysis, the activation kinetics of iNKT cells was examined. Expression of CD38 and CD69 was measured at various time points following stimulation of healthy donor PBMCs with αGalCer or a combination of IL-12, IL-15 and IL-18.

The early activation marker CD69 started to increase 6h post stimulation to levels of 58 % of all iNKT cells after 72h (Fig. 26C). CD38 was unaltered for the first 24 hours before expression levels started to increase. At 72h post stimulation, 40.55 % of iNKT cells were CD38⁺ and continued to rise afterwards (Fig. 26B). Off note, a substantial proportion of iNKT cells became undetectable after in vitro stimulation due to TCR downregulation that is well described for αGalCer activated iNKT cells (Van Kaer, Parekh, and Wu 2015; Wilson et al. 2003) (Fig. 26A). The kinetics of CD38 and CD69 expression as well as TCR downregulation were similar after stimulation with αGalCer and IL-12, IL-15 and IL-18 (Fig. 26A-C).

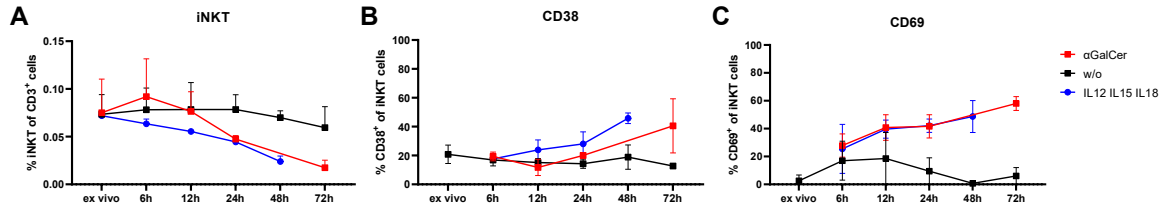

Figure 26. Activation kinetics of in vitro stimulated iNKT cells. Buffy coat derived healthy donor PBMCs were in vitro activated with the indicated stimuli and (A) iNKT cell frequency and activation as marked by (B) CD38 and (C) CD69 expression was analysed by flow cytometry.at various time points. (A) Each dot represents the mean of 2 – 4 individuals. Error bars represent the standard deviation.

This suggested the analysis of iNKT cell functionality at a time point of 24 hours post activation, when iNKT cells start to actively upregulate CD38 expression.

An important feature of iNKT cells is their ability to become activated in a TCR-independent way (Reilly, Wands, and Brossay 2010) (Fig. 15, Fig. 26). This cytokine dependent mechanism also likely plays an important role for iNKT cell activation during

chronic progression of HCV infection (Senff, Menne et al. in submission). Therefore, the transcriptome of iNKT cells was analysed after in vitro activation with IL-12, IL-15 and IL-18 after 24h. A pure population of isolated iNKT cells was used to focus solely on intrinsic activation pathways stimulated by cytokine treatment and to exclude any pathways that were indirectly kickstarted by cytokine release of other immune cells in the culture. Additionally, isolated iNKT cells are preferentially stimulated by cytokines rather than TCR ligands as CD1d is not expressed by iNKT and T cells and is thus absent from the culture. Hence, this ruled out any CD1d-TCR mediated activation via endogenous iNKT ligands. This was confirmed by analysis of CD69 expression on iNKT cells from either whole PBMCs or MACS purified $CD3^+$ cells after 24 hours stimulation with IL-12, IL-15 and IL-18 or αGalCer. While iNKT cells within whole PBMCs upregulated CD69 with both stimuli, iNKT cells from the $CD3^+$ fraction were only responsive to the interleukin cocktail (Supplemental Fig. 2)

6.6.1. Establishment of an isolation protocol of iNKT cells from PBMCs

Since iNKT cells are rare in peripheral blood, up to $3.5x10^8$ PBMCs from buffy coats were used for FACS based iNKT cell isolation with the anti-Vα24Jα18 antibody. Plate bound anti-Vα24Jα18 antibody was reported to specifically activate iNKT cells as it binds to the CDR3 region of the iNKT cell invariant TCR (Exley et al. 2008; Exley, Wilson, and Balk 2017). To rule out any TCR mediated activation of the cells by soluble antibody during isolation, PBMCs were stained with the anti-Vα24Jα18 antibody and cultured for 72h. Afterwards, they were stained with αGalCer loaded CD1d dextramer and CD38 expression was analysed. Remarkably, after 72h of stimulation, CD38 expression was unaltered on cells pretreated with 6B11 antibody indicating that staining with this antibody does not preactivate iNKT cells (Fig. 27).

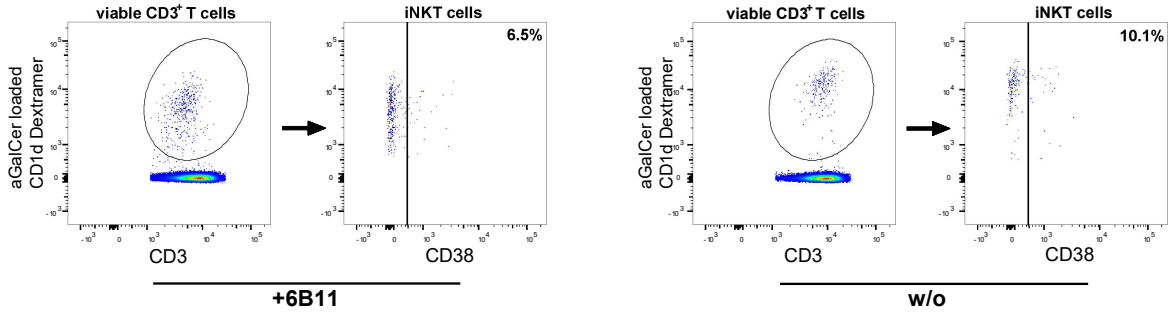

Figure 27. Activation of iNKT cells with soluble 6B11 antibody. PBMCs from healthy donor buffy coats were stained with the anti- Vα24Jα18 antibody and cultured for 72h before stained with 6B11 antibody or αGalCer loaded CD1d dextramer. Activation of iNKT cells was analysed by flow cytometry and CD38 expression. One representative of 5 donors that were pre selected for sufficient iNKT cell frequency is shown.

In order to reduce sorting time and thus enhance the viability of the recovered cells, a MACS based pre-enrichment protocol was established.

First, iNKT cells were positively isolated via anti-iNKT microbeads directed against the invariant TCR. This kit showed up to 93-fold enrichment of iNKT with high yield (Fig. 28A).

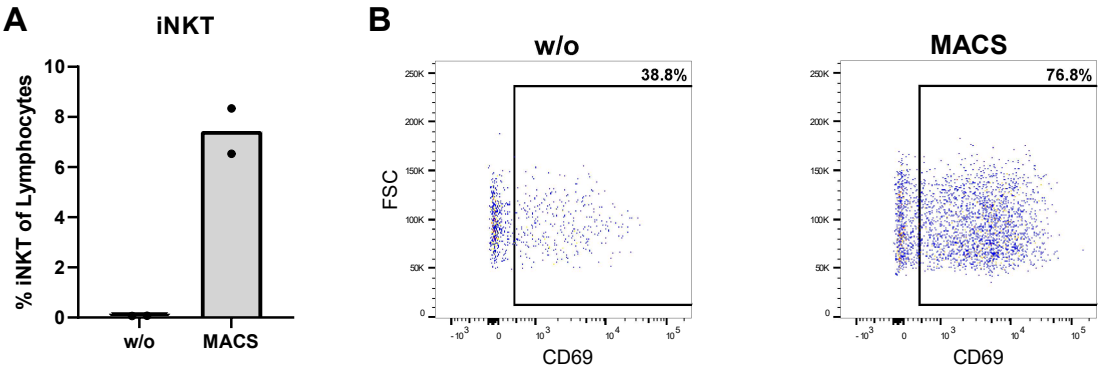

Figure 28. Positive selection of iNKT cells by anti-iNKT microbeads. Invariant NKT cells from PBMCs of healthy donor buffy coats were positively isolated via magnetic activated cell sorting with iNKT specific microbeads or left untreated and cultured for 24h. (A) Frequency of iNKT cells and (B) activation of iNKT cells as marked by CD69 expression was analysed by flow cytometry. (A) Each dot represent one individual donor and bars show the mean of 2 donors. (B) One representative sample is shown.

However, analysis of the recovered iNKT cells after 24 hours of culture revealed iNKT cell activation in the absence of IL-12, IL-15 and IL-18 indicated by CD69 upregulation in comparison to the unsorted population. This was most probably caused by TCR cross-linking by the microbeads directed against the invariant TCR (Fig. 28B).

Next, a negative isolation of $CD3^+$ cells was utilised to pre-enrich iNKT cells. This approach showed substantial depletion of CD3- cells and enriched $CD3^+$ cells to almost

100 %. However, a great amount of iNKT cells remained on the column after purification likely because of retention of CD56⁺ cells that are depleted in this protocol (Fig. 29).

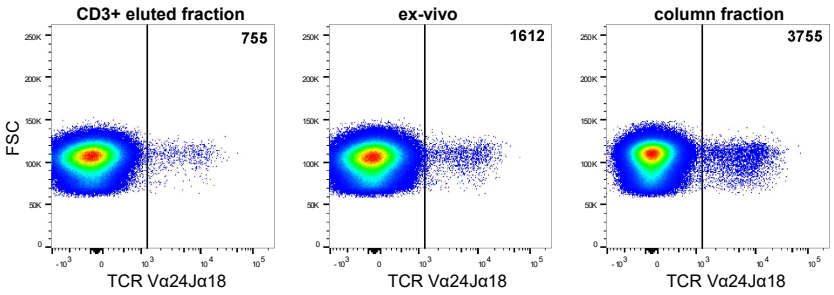

Figure 29. Negative isolation of CD3⁺ cells by CD3 negative isolation kit. CD3⁺ cells were negatively isolated from PBMCs from healthy donor buffy coats with the pan T cell isolation kit. Absolute numbers of iNKT cells in the eluted fraction, ex-vivo before MACS and in the column fraction are depicted. A representative experiment of two samples is shown.

Therefore, CD14⁺ and CD19⁺ cells were specifically depleted to exclude monocytes and B cells from the culture whilst retaining NK cells and thus the CD56⁺ iNKT subpopulation in the culture.

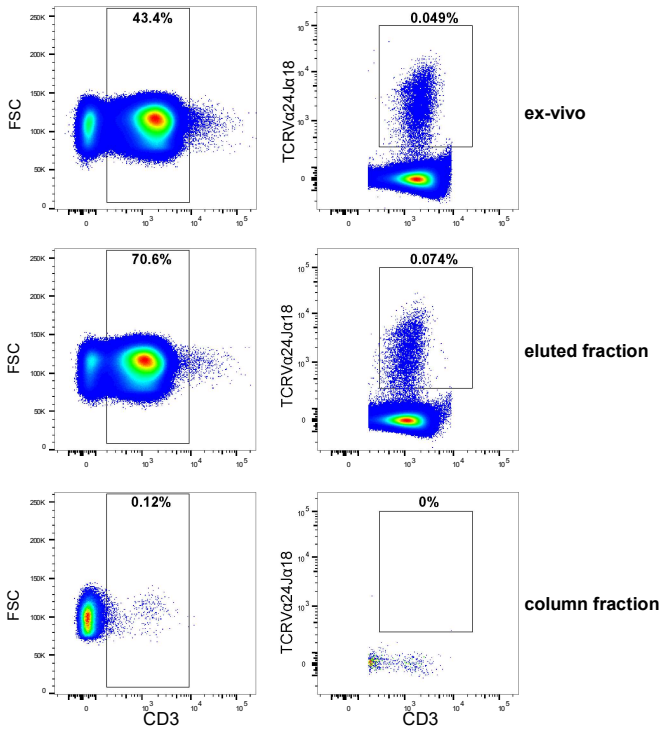

Figure 30. Negative isolation of CD3⁺ cells by depletion of CD14⁺ and CD19⁺ cells. CD3 positive cells were negatively isolated from PBMCs from one healthy donor buffy coat by MACS depletion of CD14⁺ and CD19⁺ cells. Frequency of CD3⁺ (left panels) and iNKT cells (right panels) of all events measured by flow cytometry are depicted.

This protocol facilitated up to 1.63-fold and 1.51-fold enrichment of $CD3^+$ cells and iNKT cells, respectively whilst minimizing loss of these cells (Fig. 30). Hence, it facilitated a profound decrease in sorting time and was used for pre enrichment of $CD3^+$ T cells from whole PBMCs.

6.6.2. Identification of differentially expressed genes in activated versus resting iNKT cells by RNA-sequencing

Invariant NKT cells defined by Va24Ja18 TCR staining were FACS sorted from PBMCs derived from buffy coats of six different donors of Va24Ja18 TCR and stimulated for 24 hours with IL-12, IL-15 and IL-18 or left untreated. Subsequently, RNA-sequencing was performed and the expression of mRNA analysed at the Primate Genetics Laboratory, German Primate Centre in Göttingen as described in the methods section.

Transciptome analysis of purified, IL-12, IL-15 and IL-18 activated vs resting iNKT cells revealed 258 differentially expressed genes (DEG). The majority of these were upregulated in activated iNKT cells (226) and only a few were downregulated (32) (Fig. 31)

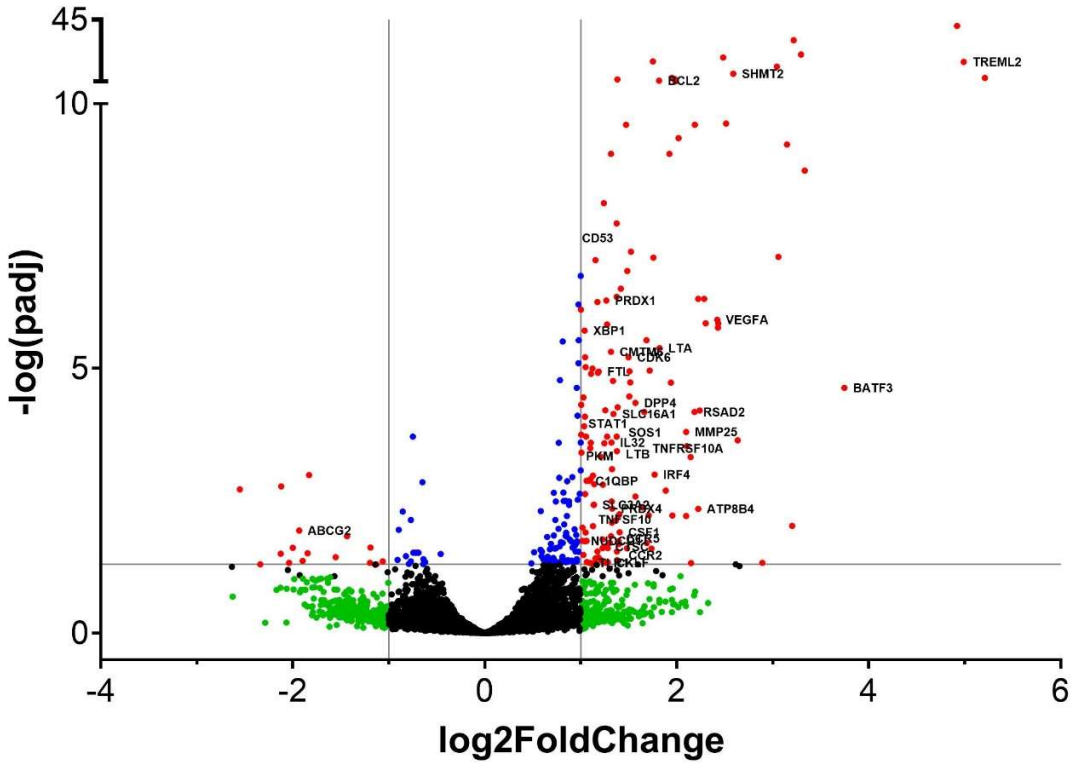

Figure 31. Transcriptome analysis of activated versus resting iNKT cells. Fold change and adjusted p-value of all analysed genes are shown in a volcano plot. Isolated iNKT cells from 6 donors were either in vitro activated or left untreated and their transcriptome analysed by RNA-seq. Significantly differentially regulated genes in in vitro activated compared to resting iNKT cells (adjusted p-value <0.05) are shown in blue or red if genes are more than 2-fold differentially expressed. Green dots represent genes that had a more than 2-fold differential expression but were not significantly altered between groups. Genes that were annotated with a immunological GO Term from the „biological Processes“ GO Terms are highlighted.

A comprehensive list of all DEGs is attached in the supplementary files (Supplementary Table 1). Enrichment analysis of GO terms assigned to “Biological Processes” within the 226 upregulated DEGs revealed a large array of pathways that were associated with iNKT cell activation. Many of these pathways reflected an altered metabolic state while some pathways also showed iNKT cell activation after cytokine stimulus (Fig. 32A, red squares). Next, all upregulated DEGs were annotated with GO terms from the collection “Biological Processes” and filtered for genes that comprised GO Terms containing the phrase “immun” to narrow down the pathway analysis on immunologically relevant genes. Again, GO Term enrichment analysis was conducted and revealed a large set of immunological pathways that were associated with immune cell activation and effector responses upregulated after iNKT cell activation (Fig. 32B).

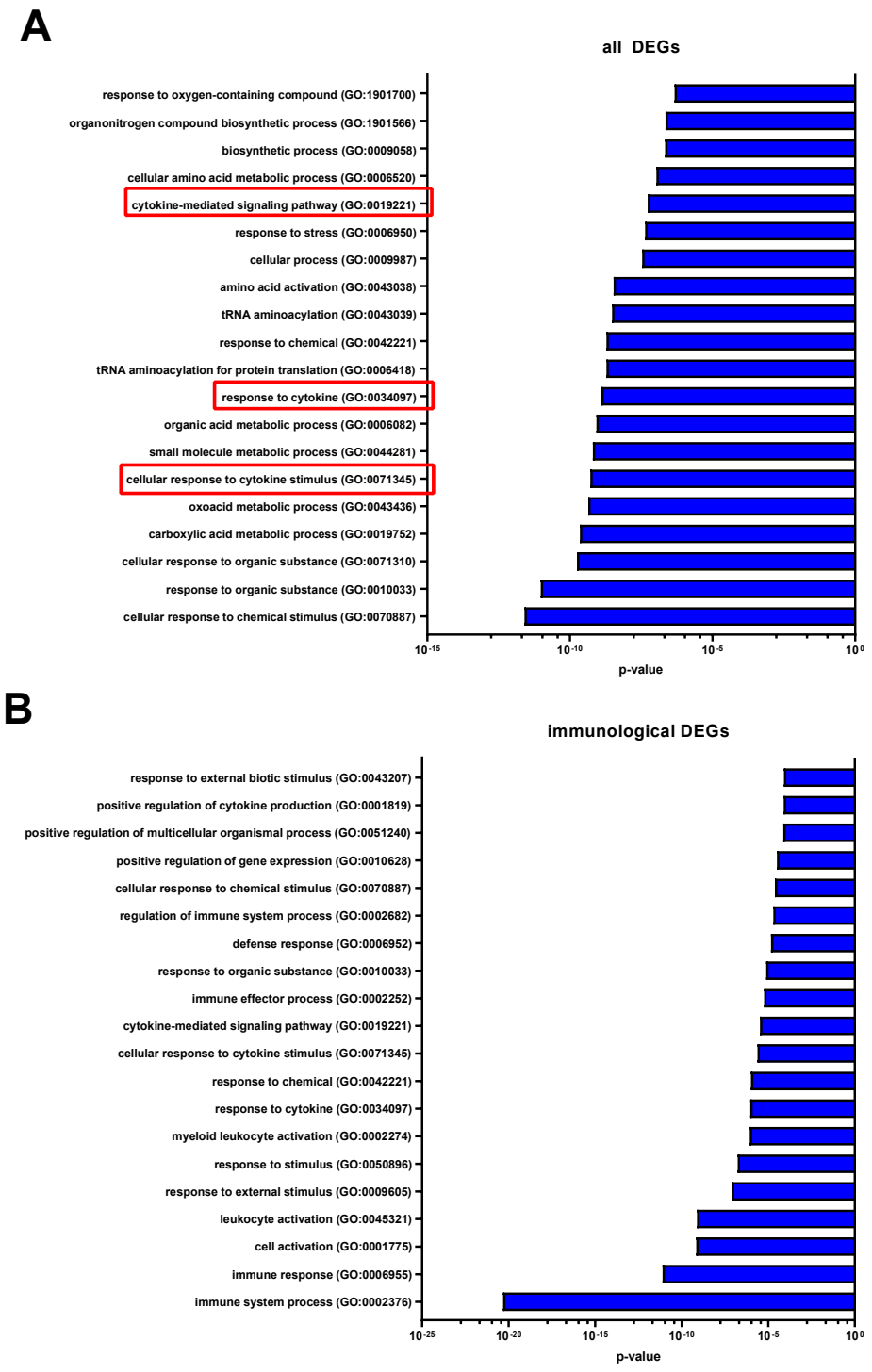

Figure 32. GO Term enrichment analysis of all upregulated DEGs. (A) All significantly upregulated DEGs were analysed for enrichment of GO TERMS „Biological Processes“ with the Panther database. (B) DEGs were filtered for genes that were assigned to a GO Term that contained the phrase „immun“ to filter all immunological genes before GO Term enrichment analysis was performed with the Panther database. The 20 GO Terms with the lowest p-value are depicted.

6.7. Characterization of IL-32 as an innate effector molecule in iNKT cells

In order to further characterise the functionality of activated iNKT cells, all significantly DEGs were screened for effector molecules like cytokines and interleukins as key mediators of the immune response. Interleukin-32 (IL-32) expression was increased by 2.49-fold (adjusted p=0.00025) in iNKT cells after in vitro stimulation with IL-12, IL-15 and IL-18. This interleukin can act proinflammatory and induces release of further proinflammatory mediators from other immune cells (Moschen et al. 2011). As the expression of IL-32 was prominent among an array of interleukins in activated iNKT cells, it was characterised in detail.

6.7.1. IL-32 expression in response to IL-12, IL-15 and IL-18 does not associate with apoptosis in iNKT and T cells

IL-32 was described to be expressed upon activation induced cell death in anti-CD3 activated T cells (Goda et al. 2006). Hence, it was determined if IL-32 upregulation after IL-12, IL-15 and IL-18 stimulation is linked to apoptosis or is also present in non-apoptotic cells. PBMCs were in vitro activated with IL-12, IL-15 and IL-18 and their expression of IL-32 and the apoptosis markers cleaved caspase 3 and 7 as well as the incorporation of a fixable viability dye were analysed. As shown in Fig. 33, less than 10 % of iNKT and T cells were apoptotic regardless of IL-32 expression. Expression of IL-32 neither unambiguously marked apoptotic nor necrotic iNKT cells (Fig. 33A) or T cells (Fig. 33B) after activation with the interleukin cocktail. After all, there was a modest decrease in viability in the IL-32 negative population albeit not statistically significant (95.90 % versus 74.67 % in IL-32$^+$ and IL-32$^-$ iNKT cells and 93.367 % versus 86.533 % in IL-32$^+$ and IL-32$^-$ T cells). On a side note, CD3- negative lymphocytes slightly tended to be apoptotic when expressing IL-32, however this cell population is highly heterogenic and consists of NK cells, B cells and other non-T cell types, so a more detailed definition would be required if these cells were to be studied (data not shown).

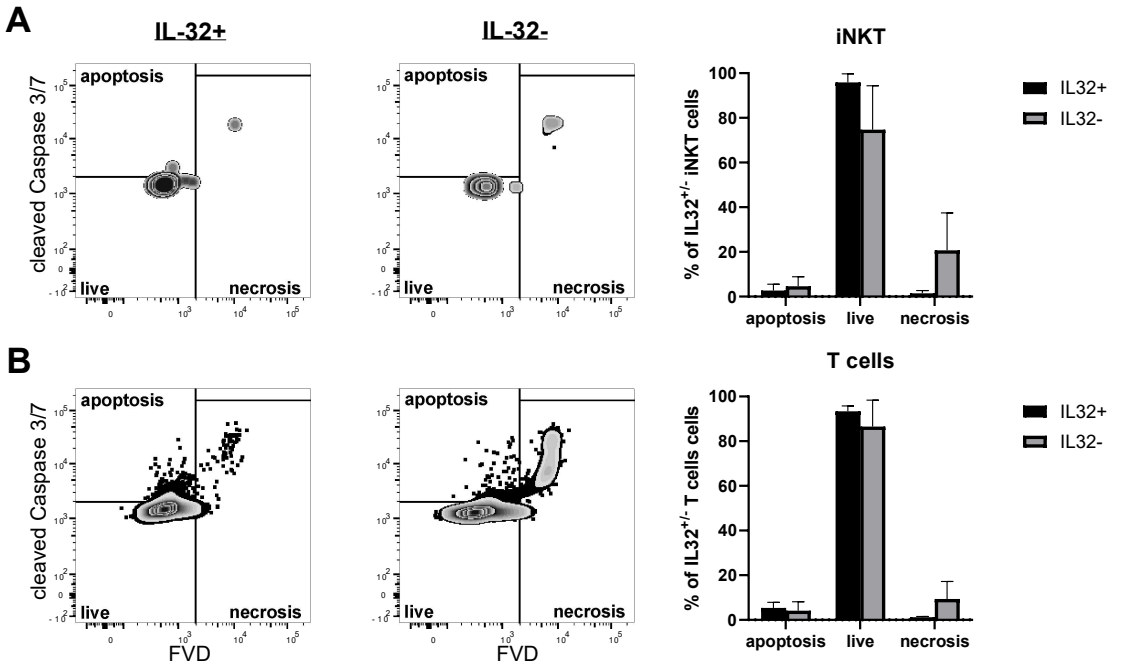

Figure 33. IL-32 is not linked to apoptosis in iNKT cells after in vitro stimulation with interleukins. PBMCs from healthy donor buffy coats were stimulated with 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 or left untreated for 24 hours and BFA was added for the last 4 hours. Apoptosis was determined by staining cleaved caspase 3 and 7 in addition to fixable viability dye. Live, apoptotic and necrotic (A) iNKT cells and (B) T cells in the IL-32 positive and IL-32 negative parent population are depicted in a representative plot and the mean of three donors is indicated by the bars with standard deviation. At least 16 iNKT cells were present in each analysed sample.

6.7.2. IL-32 is preferentially expressed by innate-like cells

Originally, IL-32 was discovered to be upregulated in NK cells and $CD4^+$ T cells after IL-2 or IL-12 and IL-18 stimulation, respectively (Dahl et al. 1992; Kim et al. 2005; Shoda et al. 2006). However, a systematic description of IL-32 expression in diverse lymphocyte subpopulations in response to different stimuli is missing to date. Here, the IL-32 response not only of iNKT cells but of an array of innate and adaptive lymphocytes was characterised. Furthermore, the potential of different stimuli on IL-32 expression in these lymphocyte subsets was investigated. Fig. 34A depicts a gating strategy for conventional T cells next to iNKT cells and other innate T cells like γδ T cells and MAIT cells. As the $CD161^{hi}CD8^+$ T cell population consists up to 95 % of mucosal associated invariant T (MAIT) cells, this marker combination was used as a surrogate for bona fide $Va7.2^+CD8^+$ MAIT cells (Fergusson et al. 2014).

Interestingly, IL-32 expression was strongly biased towards cytokine mediated activation. A combination of IL-12, IL-15 and IL-18 showed the strongest increase in IL-32 positive

cells in all tested lymphocyte populations that was consistent in a more than 10-fold range of concentration (Fig. 34B and Supplementary Fig. 3A). In contrast, TCR mediated activation by anti-CD3 beads played only a minor role as IL-32 expression was only slightly elevated above background levels and ranged from a mean of 2.71 % in $CD8^+$ T cells to a mean of 13.82 % in NK cells (Fig. 34C). This IL-32 expression also in non-T cells probably was caused by anti-CD3 mediated cytokine secretion and downstream low level IL-32 induction. Besides, IL-2 evoked a moderate induction of IL-32 in all tested lymphocyte populations that was strongest in NK cells (Supplementary Fig. 3B).

The most IL-32 expression after IL-12, IL-15 and IL-18 stimulation was observed in NK cells (mean 52.07 %) and $CD161^{hi}CD8^+$ T cells (mean 69.07 %). Only a minority of 20 % or less of conventional $CD4^+$ and $CD8^+$ T cells were IL-32 positive whereas intermediate levels of double negative T cells (mean 30.43 %) – that are believed to contain cells with innate like characteristics (Yang et al. 2021) – and gamma delta T cells (mean 24.93 %) induced IL-32. An average of 37.07 % of invariant NKT cells showed IL-32 expression after IL-12, IL-15 and IL-18 stimulation, which was slightly lower than NK cells. Of note, this confirmed the RNA-sequencing results, showing IL-32 upregulation after stimulation with IL-12, IL-15 and IL-18 on the protein level. This indicates a TCR-independent activation mechanism of IL-32 mediated by proinflammatory cytokine stimuli that is most potent in NK cells and innate T cells (Fig. 34B). In summary, this suggests a role of IL-32 in the early antigen independent proinflammatory immune response that is mediated mostly by innate like lymphocytes.

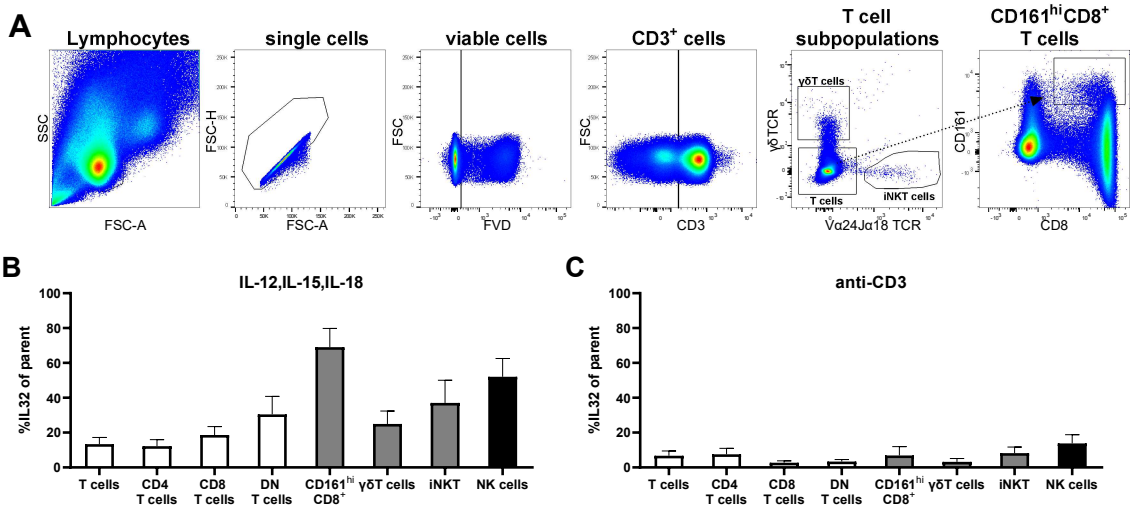

Figure 34. IL-32 expression by distinct lymphocyte subpopulations after different stimuli. Healthy donor buffy coat derived PBMCs were stimulated with (B) 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 or (C) anti-CD3/28 Dynabeads for 24 hours. BFA was added for the last 4 hours to every sample. (A) shows the gating strategy for the analysed lymphocyte populations. Bars represent the mean of 3 individual donors. Error bars show the standard deviation. At least 24 iNKT cells were analysed in each sample.

6.7.3. IL-32 is expressed by conventional and unconventional T cells in PWID

Invariant iNKT cells are activated during HCV infection in acute as well as chronic disease (Senff, Menne et al. in submission). In conjunction with the observation that IL-32 is upregulated in in vitro activated iNKT cells, this led to the assumption that IL-32 could also be differentially expressed in PWID with either chronic or resolved HCV infection. For this purpose, IL-32 expression by iNKT cells and various other lymphocyte populations was analysed by flow cytometry in five HCV-RNA positive and five HCV-RNA negative PWID after stimulation with IL-12, IL-15 and IL-18. Expression of IL-32 was highly variable in iNKT cells and reached from 3.95 % to 26.10 % in all samples. Thereby, IL-32 expression was comparable in HCV-RNA positive and negative patients with a median of 17.90 % and 15.25 %, respectively (Fig. 35A). Expectedly and like seen before, conventional T cells and γδ T cells showed lower frequencies of IL-32$^+$ cells than iNKT cells. CD161^{hi}CD8$^+$ T cells displayed the highest rate of IL-32$^+$ cells amongst innate like T cells (median 24.3 % and 18.6 % in HCV-RNA positive and negative, respectively). Opposing to the difference in IL-32 expression between T cell populations, expression levels of IL-32 between HCV groups were comparable.

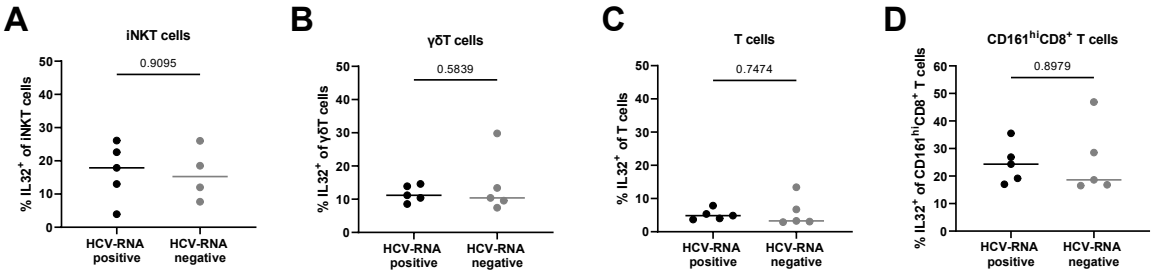

Figure 35. IL-32 expression in distinct lymphocyte subsets in HCV-RNA positive and HCV-RNA negative PWID. PBMCs from HCV-RNA positive and HCV-RNA negative PWID were stimulated with 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 for 24 hours and BFA was added for the last 4 hours. IL-32 expression was analysed in various lymphocyte subpopulations. (A) Invariant NKT cells, (B) gamma delta T cells, (C) conventional T cells and CD161^{hi}CD8^{+} T cells were gated as described above. Each dot represents one individual donor. Only sample with more than 10 iNKT cells were included in the analysis.

6.7.4. IL-32 expressing iNKT cells display a unique phenotype

IL-32 is expressed upon stimulation with other proinflammatory mediators primarily in innate immune cells like iNKT cells. However, it is not known if IL-32 producing cells display a specialised subset within the heterogeneous pool of iNKT cells. Hence, IL-32 expressing iNKT cells were comprehensively characterised in order to determine if these cells differ from non-IL-32 producers in terms of functionality and phenotype. Therefore, IL-32 production in iNKT cells from healthy donor PBMCs were analysed after stimulation with αGalCer and IL-12, IL-15 and IL-18 for 8h. Invariant NKT cells were gated into an IL-32^{+} and IL-32^{-} population and for the expression of surface markers associated with activation, differentiation and exhaustion as well as release of functional cytokines was analysed. The IL-32 positive population was significantly diminished in CD38 (Fig. 36A, mean 14.29 %, p=0.0212) and PD-1 (Fig. 36B, mean 23.12 %, p=0.0156) iNKT cells which were preferentially found in the IL-32^{-} population (mean 34.90 % CD38^{+} and mean 36.63 % PD-1^{+}). Interestingly, the frequency of CD4^{+} iNKT cells was slightly, but significantly diminished in IL-32^{+} iNKT cells (Fig. 36C, mean 36.10 % vs. 48.88 % in IL-32^{+} vs IL-32^{-}, p=0.0154). While CD8^{+} iNKT cells were unaltered, DN iNKT cells were reciprocally elevated in IL-32 positive iNKT cells (Fig. 36C, mean 48.28 % vs 37.98 % in IL-32^{+} vs IL-32^{-}, p=0.0394). This might reflect a bias towards a stronger proinflammatory response by CD4^{-} iNKT cells as previously reported (Gumperz et al. 2002; Krovi and Gapin 2018; Liu et al. 2019) indicated by IL-32 production in this subset.

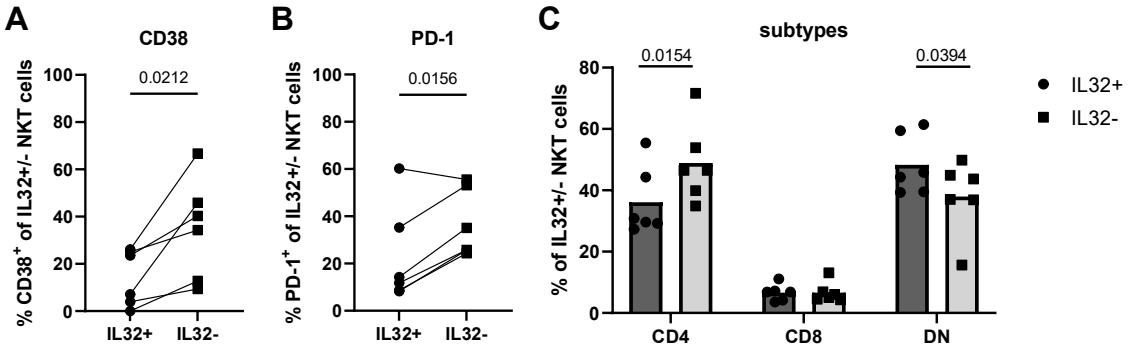

Figure 36. Phenotype of IL-32 expressing iNKT cells. Healthy donor buffy coat derived PBMCs were stimulated with 1 µg/ml αGalCer and 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 for 8 hours and BFA added for the last 4 hours. Invariant NKT cells were gated into a IL-32$^+$ and IL-32$^-$ population and the frequency of (A) CD38$^+$, (B) PD-1$^+$ and (C) CD4$^+$, CD8$^+$ and DN subsets was analysed. Each dot represent an individual of 6 donors. Bars show the mean of 6 donors. Paired t-test was used to compare groups and p-value is depicted. Only samples with at least 10 cells in the IL-32$^+$ and IL-32$^-$ iNKT population were included in the analysis

6.7.5. IL-32 expression occurs independently from other proinflammatory cytokines

Next, the expression of the effector molecules IFN-γ, TNFα and Granzmye B in dependence of IL-32 was analysed. Notably, while high levels of these molecules were expressed next to IL-32 in iNKT cells, secretion of all three was not significantly altered between IL-32$^+$ and IL-32$^-$ iNKT (Fig. 37). This indicates that IL-32 is expressed independently from distinct cytokine secretion profiles and universally distributed among effector iNKT cells.

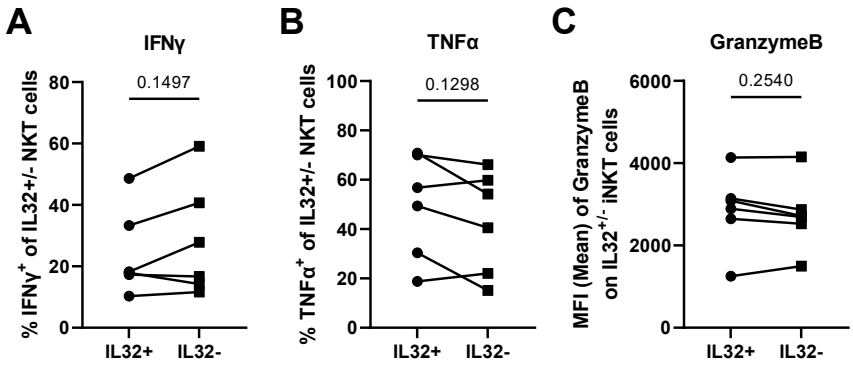

Figure 37. Cytokine expression in IL-32 secreting iNKT cells. Healthy donor buffy coat derived PBMCs were stimulated with 1 µg/ml αGalCer and 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 for 8 hours and BFA added for the last 4 hours. Invariant NKT cells were gated into an IL-32$^+$ and IL-32$^-$ population and the frequency of (A) IFN-γ$^+$ cells, (B) TNFα$^+$ cells and (C) the mean fluorescence intensity of Granzyme B was analysed. Each dot represent an individual of 6 donors. Paired t-test was used to compare groups and p-value is depicted. Only samples with at least 20 cells in the IL-32$^+$ and IL-32$^-$ iNKT population were included in the analysis.

7. Discussion

Despite the discovery and recent success of DAAs, Hepatitis C virus infection still poses a major public health burden highlighting the necessity of a prophylactic vaccination to achieve the WHO goal of virus elimination(McConnell and Lim 2018; Thimme 2021). Since up to one third of all acutely infected patients spontaneously clear the infection without therapeutic intervention (Westbrook and Dusheiko 2014), the immunological determinants of viral clearance in these patients can be used as a blueprint for vaccination induced immune responses (Thimme 2021). Even though acute HCV infection evokes innate immune signalling, HCV has evolved different mechanisms to dampen the innate immune response. In turn, adaptive responses against HCV are markedly delayed due to impaired induction of T and B cell responses (Cashman, Marsden, and Dustin 2014; Heim and Thimme 2014). In order to mount a successful adaptive response that achieves viral clearance, crosstalk between innate and adaptive immune cells is essential. The best-characterised example for an innate immune cell subset that induces adaptive responses are dendritic cells, which are crucial for the priming of virus specific T cells. Another important cell type that bridges the innate and the adaptive immune system are iNKT cells that can modulate the downstream immune response by the release of a multitude of pro- and anti-inflammatory chemokines and Th1, Th2 and Th17 cytokines (Godfrey, Stankovic, and Baxter 2010; Taniguchi, Seino, and Nakayama 2003). While the responses from individual branches of the immune system are well characterised, e. g. the innate interferon or NK cell response as well as the adaptive response from conventional $CD8^+$ T cells, iNKT cells as bridging cells between these two branches are not well studied in acute HCV infection. Therefore, in this work, not only a detailed analysis of iNKT cells during acute HCV infection was conducted but also the general features of activated human iNKT cells were closely investigated.

7.1. Invariant NKT cells in acute HCV infection

In this study, a comprehensive, longitudinal characterization of the iNKT cell frequency and phenotype in acute HCV infection during the first year post infection was conducted in patients with different disease outcome and correlated with clinical markers for liver inflammation. Most studies that analysed the frequency of iNKT cells in HCV infected

patients focused on the analysis of patients within the chronic phase of hepatitis C and compared those to patients with resolved HCV infection or healthy controls (Deignan et al. 2002; Inoue et al. 2006; Lucas et al. 2003; van der Vliet et al. 2005). One study that characterised the cellular innate immune response in 12 healthcare workers exposed to HCV by needlestick injury also analysed iNKT cells during the early phase of infection. They found activation of iNKT cells marked by increased FasL expression and NKG2D expression at two to six weeks post exposure compared to baseline levels in the majority of all individuals (Werner et al. 2013). Previous studies that focused on the analysis of iNKT cells during chronic hepatitis C are highly inconsistent. Some report significantly less circulating or intrahepatic iNKT cells in chronically infected patients compared to resolvers or controls (Deignan et al. 2002; Lucas et al. 2003), while others report no difference between patient groups (Inoue et al. 2006; van der Vliet et al. 2005). In line with this, in this work it was shown that peripheral iNKT cell frequencies remain stable after HCV exposure for at least one year post infection and furthermore are unaffected by infection outcome. Invariant NKT cells are known to have distinct effector functions dependent on their expression of CD4 and CD8. $CD4^+$ iNKT cells are believed to display Th1 and Th2 effector functions while the $CD4^-$ subpopulations are more limited towards a Th1 phenotype and cytotoxicity (Gumperz et al. 2002; Krovi and Gapin 2018). Here, the occurrence of the three described iNKT cell subgroups was neither affected by disease outcome nor did it change during the first year post infection. This indicates that HCV does not cause major perturbations in iNKT cell subset composition and that all iNKT cell subsets are equally implicated during HCV infection.

Analysis of the T cell activation markers CD38 and CD69 revealed that iNKT cell activation declined during the first year post infection in resolvers but not in chronic progressors. This hints towards an association between HCV outcome and iNKT cell activation. Notably, increased levels of CD38 on iNKT cells during chronic HCV infection compared to resolved HCV infection was confirmed in cross-sectional analysis in previous work in our lab (Senff 2018). The similar levels of iNKT cell activation during the early phase of acute infection in combination with the sustained high iNKT activation levels in chronic progressors – in the presence of detectable HCV RNA – suggests that iNKT cell activation is not directly predictive of HCV infection outcome.

The pattern of CD38 expression on iNKT cells in resolving patients resembles what was initially reported for HCV specific $CD8^+$ T cells in a patient that spontaneously resolved acute infection after needlestick injury in an early study about the immune response in acute HCV infection with different outcomes (Thimme et al. 2001). There, the initially present $CD38^+$ HCV-specific $CD8^+$ T cells disappeared and were replaced by $CD38^-$ HCV specific $CD8^+$ T cells that potently produced IFN-γ which preceded a 10^5 fold drop in viral load. Similar to this, the disappearance of $CD38^+$ iNKT cells – that were in independent experiments in this work shown to constitutively produce less IFN-γ – decreased in patients that resolved acute infection.

Importantly, CD38 but not CD69 expression patterns of iNKT cells and bulk conventional $CD4^+$ and $CD8^+$ T cells appeared to be similar in most of the patients with acute HCV infection (data not shown). This would suggest a non-specific activation of iNKT cells, in accordance to a mechanism proposed for HCV-nonspecific T cells that are abundantly present in the inflamed liver in chronic hepatitis C patients (Larrubia et al. 2014; Rehermann and Bertoletti 2015; Shin, Sung, and Park 2016). Invariant NKT cells showed higher CD69 expression upon alterations in ALT levels than conventional T cells indicating a stronger antigen nonspecific response by these cells, which hints towards an involvement in the inflammatory response during acute HCV infection (data not shown). Invariant NKT cell activation during early HCV infection has been shown to strongly correlate with the HCV-specific downstream T cell response (Werner et al. 2013). This involvement of activated iNKT cells in the modulation of the anti-HCV immune response is further corroborated by the findings in this work.

The observation that iNKT cells become activated during acute hepatitis led to the hypothesis that these cells contribute to the immune response against HCV. Correlation of activation markers CD38 and CD69 as well as the liver homing receptor CXCR6 and markers that are associated with T and iNKT cell cytotoxicity like CD56 and CD8 (Van Acker et al. 2017) with serum ALT levels indicate that they might play a direct role in the immune response against HCV. Intriguingly, in chronic progressors, this correlation was absent, which might be caused by sustained iNKT cell stimulation during the chronic phase of HCV infection and thus persistently elevated ALT levels . Since this study was correlative, it requires further evaluation if iNKT cells directly contribute to hepatocyte killing or if the inflammatory processes of the immune response against HCV secondarily

activate them. Invariant natural killer T cells have been shown to mediate direct perforin dependent cytotoxicity (Gumperz et al. 2002; Metelitsa et al. 2001). Furthermore, in autoimmune hepatitis mouse models, ConA activated iNKT cells directly mediated hepatocyte killing and αGalCer activated iNKT cells acted as central immunomodulators regulating a diverse set of other immune cells mediating liver damage (Takeda et al. 2000; Kaneko et al. 2000; Santodomingo-Garzon and Swain 2011; Mattner 2013; Osman et al. 2000; Biburger and Tiegs 2005). Together with the results from this work, this supports an active role for human iNKT cells in in liver inflammation for iNKT cells.

A hallmark of HCV infection is the delayed adaptive immune response, which kicks in about 6-8 weeks after HCV infection (Heim and Thimme 2014). Future analysis of iNKT cells as bridging cells between the rapidly induced innate and the delayed adaptive immune response thus requires samples from patients within the first days to weeks of infection. However, as symptoms in acutely infected patients develop only after several weeks of incubation, if at all, a major obstacle is the identification of very recently infected patients without a known exposure event, such as needlestick injuries in health care workers (Werner et al. 2013; Thimme et al. 2001; Cox et al. 2009; Blackard et al. 2008). Additional analysis of iNKT cell functionality could link the observed phenotype to a profile of effector functions and shed light onto the exact role iNKT cells play during acute hepatitis C. Zeissig et al. could show that iNKT cells but also type 2 NKT cells directly recognize HBV infected hepatocytes in mouse models of transgenic HBV expression. Thereby, iNKT cells modulated the downstream immune response as early as 2 days post infection before adaptive immune cells were first detected after 5 days (Zeissig et al. 2012). Unfortunately, an endogenous ligand for invariant NKT cells that is implicated in viral hepatitis could not be detected in this, or other, studies. Detailed knowledge of CD1d restricted, endogenous glycolipids that are recognized by iNKT cells would greatly add to the understanding of the early immune response by iNKT cells, e.g. by the use of tetramers loaded with this ligand or by functional in vitro studies. So far, some endogenous iNKT ligands have been identified (Mattner et al. 2005; Kain et al. 2015; Kain et al. 2014), however, most iNKT cell studies still utilize αGalCer multimers as a gold standard. A putative endogenous iNKT ligand induced by HCV infection could either be presented on the surface of hepatocytes due to perturbations in lipid metabolism evoked by the virus or by hepatic antigen presenting cells that upregulate CD1d after PRR mediated viral sensing

(Raftery et al. 2008). It was previously shown, that CD1d expression is upregulated in HCV infected and cirrhotic human livers, mainly on the surface of infiltrating professional APCs (de Lalla et al. 2004). Independently, it was shown that increased CD1d signalling stimulates a variety of non-invariant, CD1d restricted T cells that are abundant in the human liver (Yanagisawa et al. 2013; Bandyopadhyay, Marrero, and Kumar 2016). In conclusion, this could also be a possible mechanism of iNKT stimulation during HCV infection.

Collectively, this data demonstrates that human peripheral iNKT cells are activated during acute HCV infection and this activation is sustained upon viral persistence. This association with infection outcome was corroborated by the correlation of an activated and cytotoxic-like phenotype of iNKT cells with serum levels of ALT in resolving patients. In line with the previous observation that iNKT cells can directly mediate liver damage, this strongly indicates an important role for these cells in the anti-HCV immune response and subsequent liver inflammation.

7.2. Intrahepatic iNKT cells

Understanding the immune response against Hepatitis C virus requires detailed knowledge about the complex interactions between a diverse range of immune cells within the immunologically specialized intrahepatic environment. The liver contains a unique composition of immune cell types with an enrichment of innate immune cells (Heymann and Tacke 2016; Racanelli and Rehermann 2006; Gao, Jeong, and Tian 2008; Crispe 2009) like iNKT cells that are believed to be crucial for liver immunology (Kumar 2013; Bandyopadhyay, Marrero, and Kumar 2016). In order to obtain insights into the phenotype of intrahepatic iNKT cells, perfusates of liver specimens derived from tumour resection surgery were used in this study. This facilitated a generalized insight into the iNKT cell population in the human liver, even in the absence of viral hepatitis, to define a baseline phenotype of intrahepatic iNKT cells.

Murine iNKT cells are highly enriched in the liver (Eberl et al. 1999; Bandyopadhyay, Marrero, and Kumar 2016) in stark contrast to human intrahepatic iNKT cell frequencies which are reported to fall into the same range as in the peripheral blood or are only moderately increased (Kita et al. 2002; Kenna et al. 2003). On a side note, type 2 non-

invariant NKT cells are abundantly present in the human liver (Yanagisawa et al. 2013). In this study, iNKT cell numbers from healthy liver tissue and the circulation were indeed comparable. Interestingly, the phenotype of intrahepatic iNKT cells was substantially different from their peripheral blood counterparts. This was shown by multidimensional analysis, which revealed a unique intrahepatic iNKT cell phenotype distinct from PBMC derived iNKT cells. This intrahepatic iNKT cell phenotype was enriched for markers associated with lymphocyte activation (CD38, CD69) but also exhaustion and differentiation (PD-1), similar to what has been shown for conventional T cells even in the absence of viral hepatitis (Grabowska et al. 2001). The observed unique phenotype of intrahepatic iNKT cells could reflect a previous encounter of iNKT cells with an endogenous ligand in the liver as suggested before (Kenna et al. 2003). Overall, this indicates a highly active subset of iNKT cells present in the human liver, confirming and extending previous knowledge of the iNKT cell phenotype in HCV infected as well as – for $Va24^+$ cells – nondiseased livers (Lucas et al. 2003; Kenna et al. 2003).

Given the fact that in this study only healthy liver tissue could be used that was not affected by viral hepatitis, it remains elusive how activated iNKT cells contribute to liver damage during hepatitis C. The occurrence of $CD38^+$ iNKT cells – that are enriched in the liver even under steady-state conditions – in the circulation might reflect a spillover from the liver to the periphery caused by liver inflammation. Due to the descriptive nature of this study, this question cannot be decisively answered and needs further investigation, ideally by comparison of matched liver and blood samples from HCV infected as well as healthy donors.

If liver-derived iNKT cells behave differently from conventional intrahepatic T cells or from circulatory lymphocytes upon HCV infection, needs further evaluation to shed light on the early inflammatory processes after infection. To date, a detailed analysis of liver derived iNKT cells for instance by transcriptome analysis is still lacking due to limited sample availability and low cell numbers.

7.3. Functional characterization of iNKT cells

In order to characterise the functionality of human iNKT cells, an in vitro stimulation protocol was established that relied on more physiological activation with cognate antigen

instead of an unspecific PMA and ionomycin stimulus. Moreover, the proinflammatory cytokines IL-12, IL-15 and IL-18 were used to enhance iNKT cell activation. While iNKT cells can be activated by either a strong TCR stimulus – like αGalCer – or cytokines alone (Reilly, Wands, and Brossay 2010), it is believed that in vivo they are activated by a weak TCR signal provided by an endogenous ligand combined with a cytokine stimulus (Brigl et al. 2003). Well in line with a previous study that observed higher production of the Th2 cytokine IL-13 in chronically HCV infected patients, the frequency of IFN-γ positive iNKT cells was not associated with infection outcome (Inoue et al. 2006), even when stimulated physiologically. Invariant NKT cells can modify their cytokine expression based on the prevailing cytokine milieu, as demonstrated for IL-17 producing iNKT cells (Moreira-Teixeira et al. 2011). Therefore, it is reasonable to hypothesize that the inflammatory environment in the chronically inflamed liver skews the iNKT cell cytokine response into a certain direction, even though it is initially similar in the periphery in all patients. This mechanism has previously been described by de Lalla et al. who reported a switch towards a Th2 cytokine profile of iNKT cells recruited to the cirrhotic liver of patients with chronic HCV infection mediating liver fibrosis (de Lalla et al. 2004) and could explain the similar cytokine profile of peripheral iNKT cells.

Since iNKT cells are believed to connect the innate with the adaptive immune system, it was tested if iNKT cell activation can modulate unrelated downstream immune responses. Addition of αGalCer to a culture of CMV specific T cells that were stimulated with their cognate antigen had no obvious effect on their expansion.

It was shown in mouse models that αGalCer mediated stimulation of iNKT cells can influence the $CD8^+$ T cell response (Valente et al. 2019; Reilly et al. 2012; Guillonneau et al. 2009; Qin et al. 2019). In humans, there are numerous clinical trials that utilized αGalCer activated iNKT cells for the treatment of different tumours (Waldowska, Bojarska-Junak, and Rolinski 2017). One study demonstrated that αGalCer injection is well tolerated and save, however it did not induce a profound anti-tumour response (Giaccone et al. 2002). In another trial, αGalCer pulsed dendritic cells were administered which resulted in activation of NK and T cells followed by increased IFN-γ levels. Even though the tumour did not subside, it was proven that αGalCer mediated iNKT activation can in vivo activate downstream immune cells in humans (Nieda et al. 2004).

In a recent work, it was further shown that αGalCer can promote human CD8$^+$ T cell responses against viral peptides in vitro. Thereby, αGalCer has to be covalently linked to a peptide antigen facilitating simultaneous presentation of both epitopes on the same professional APC to iNKT as well as CD8$^+$ T cells. This substantially augmented the CD8$^+$ T cell response in vitro (Speir et al. 2017). The authors also critically state that functional in vitro studies with human PBMCs are challenging to conduct due to missing three-dimensional co-localisation of interacting cells as it is present in the secondary lymphoid organs as well as low frequencies of antigen presenting cells and iNKT cells in human peripheral blood. In conclusion, even though adjuvanting CD8$^+$ T cells with activated iNKT cells is possible, recent discoveries underline the importance of well-suited cell culture systems for in vitro studies of human PBMC derived iNKT cells.

7.4. Functionality of CD38$^+$ iNKT cells

One striking finding in this work was the dysfunctionality of iNKT cells that are CD38$^+$ ex vivo. Due to the association of CD38$^+$ iNKT cells with outcome of HCV infection and liver damage, it was sought to conduct a detailed analysis of CD38$^+$ iNKT cells in order to determine the mechanisms responsible for this functionally deficient phenotype. CD38$^+$ iNKT cells produced less IFN-γ, TNFα, Granzyme B as well as IL-32 and proliferated less. They were also less activated after in vitro stimulation in terms of CD69 expression. Even though all iNKT cells induced CD38 expression beginning at 24 hours after in vitro stimulation, the cells already positive for CD38 ex vivo seemed to be remarkably different from their CD38$^-$ counterparts.

The functional deficiency of CD38$^+$ iNKT cells could possibly be explained by iNKT cell exhaustion. Exhaustion is a condition that is defined by poor T cell functionality evoked by repeated antigenic stimulation during chronic viral infections or cancer. In line with this, CD38$^+$ iNKT cells were enriched during chronic progression of hepatitis C, which is well known for its association with T cell exhaustion. Exhaustion is marked by diminished CD8$^+$ T cell reactivity towards cognate antigen, less cytotoxicity and lower production of IFN-γ and other inflammatory cytokines. T cell exhaustion is defined by the simultaneous expression of multiple immune checkpoint inhibitors such as PD-1, CTLA-4, Tim-3, LAG-3, CD160 and others (Wherry 2011). Today it is known that T cell exhaustion is not just a

side effect of continuous antigen exposure but rather a mechanism that evolved to protect the host against detrimental immunopathologies when the infection cannot be fully cleared (Wherry and Kurachi 2015). Indeed, iNKT cells have been shown to mediate liver pathology in experimental mouse models of Concanavalin A induced autoimmune hepatitis (Takeda et al. 2000; Kaneko et al. 2000). Thus, dampening iNKT cell responses in chronic HCV infection would be feasible to protect the liver against excessive immune responses. There is one report of iNKT cells with an exhausted phenotype in sarcoidosis patients marked by decreased frequency of multifunctional IFN-γ and TNFα secreting iNKT cells and PD-1 elevation suggesting that iNKT cells, similar to conventional T cells, can become functionally exhausted (Snyder-Cappione et al. 2013). Another publication reports that $CD62L^-$ iNKT cells rapidly acquire an exhausted phenotype after repeated antigenic stimulation in vitro as they expressed multiple hallmark exhaustion markers like PD-1, 2B4, LAG-3 and Tim-3 (Tian et al. 2016). In this work, the unique phenotype of $CD38^+$ iNKT cells was primarily investigated in healthy donors independently from any pathologies. If iNKT cell exhaustion can also occur in the absence of chronic infection requires further research. As iNKT cells are autoreactive to endogenous ligands (Bendelac, Bonneville, and Kearney 2001), repeated cognate antigen encounters are possible even in the absence of chronic viral infection. However, the natural ligand for iNKT cells is still not unequivocally defined, yet. Even though some putative ligands have been discovered that bind to the human invariant iNKT cell TCR, their influence on iNKT cell activation and differentiation in vivo has still to be elucidated (Brennan, Brigl, and Brenner 2013). Thus, it cannot be conclusively determined if $CD38^+$ iNKT cells were exposed to chronic antigenic stimulation or not.

Along this line, the presence of high levels of $CD38^+$ iNKT cells in healthy liver tissue could provide an explanation for the observed diminished functionality of CD38+ iNKT cells.

The liver is a unique immunological site characterized by immunological tolerance. As the liver receives a constant supply of microbial products and food-derived antigens via the portal vein, induction of immunological tolerance is important to avoid hyperreactivity immune cells in the liver resulting in tissue damage and autoimmunity (Zheng and Tian 2019). T cells that are primed in the liver undergo tolerance induction rather than effector activation partly due to the coexpression of inhibitory ligands like PD-1L on the surface of APCs and checkpoint inhibitors such as PD-1 on T cells in the liver (Thomson and Knolle

2010) (Bowen et al. 2004) . Therefore, expression of CD38 on iNKT cells could reflect a tolerogenic state in iNKT cells induced by the hepatic environment in order to prevent the cells from activation by non-pathogenic and gut derived antigens.

Due to its use as an activation marker, CD38 can be used in conjunction with other surface receptors to stain effector T cells. However, those activated T cells are not always reliably distinguishable from exhausted T cells as a lot of exhaustion markers are initially upregulated in fully functional, recently activated effector T cells (Hong et al. 2013; Simon and Labarriere 2017; Ahn et al. 2018). Lately, a mass cytometry approach revealed that CD38 is not only induced in activated effector T cells but also strongly linked to a severely exhausted T cell phenotype and transcriptional signature (Bengsch et al. 2018; Winkler and Bengsch 2019). CD38 exerts important ectoenzymatically functions on the outside of the cell thereby regulating Nicotinamide adenine dinucleotide$^+$ (NAD^+) metabolism. Via this route, CD38 acts as a critical enzyme implicated in purinergic signalling. Another ectoenzyme involved in purinergic signalling is CD39, which is also induced on activated T cells and was shown to identify terminally exhausted $CD8^+$ T cells (Gupta et al. 2015). As $CD8^+$ T cell exhaustion is a hallmark of chronic hepatitis C (Holz and Rehermann 2015), the accumulation of $CD38^+$ iNKT cells during chronic progression could reflect iNKT cell exhaustion, too. Admittedly, however, the lack of effector functions in $CD38^+$ iNKT cells was also observed in healthy individuals and so far, coexpression of PD-1 could not unequivocally be linked to CD38 expression in iNKT cells while other markers are to be tested in the future.

CD38 was originally discovered to be expressed on early thymic T cells and predominantly on naïve T cells but not on memory T cells in the circulation. However, it is re-expressed upon T cell stimulation why it was considered to act as an activation marker (Mehta, Shahid, and Malavasi 1996; Malavasi et al. 1992). Even though iNKT cells exhibit an almost exclusive effector memory phenotype, some iNKT cells express markers associated with naïve or central memory cells, like CD62L and CCR7 (Liu et al. 2019; Montoya et al. 2007; Loh et al. 2014; Sandberg, Bhardwaj, and Nixon 2003; Kim, Johnston, and Butcher 2002; Eger et al. 2006; Kim, Butcher, and Johnston 2002). Given its expression pattern in conventional T cells, CD38 expression might define a population of iNKT cells with a naïve like phenotype. Whether these iNKT cells are also less responsive to in vitro stimulation, like naïve $CD45RA^+$ conventional T cells (Dianzani et al.

1994; Bell 1992; Beverley 1992; Mackay 1992), is not clear yet. Some early preliminary data that is not included in this work, hints at a divergent expression pattern of CD38 and CD161 in iNKT cells, the latter being expressed preferentially in $CCR7^-$ memory-like iNKT cells. NK1.1, the mouse homolog of CD161 is used to define matured iNKT cells in the periphery (Godfrey et al. 2004; Liu et al. 2019). This further corroborates a role for CD38 as a marker of early, undifferentiated or immature iNKT cells; however, the existence of such a population in humans remains elusive as most data addressing thymic iNKT cell development is derived from mouse models.

Analysis of transcription factor expression in $CD38^+$ and $CD38^-$ iNKT cells revealed that $CD38^+$ iNKT cells expressed lower levels of T-bet which is considered the master regulator of Th1 responses in conventional T cells (Luckheeram et al. 2012; Jenner et al. 2009). Only a minority of $CD38^+$ iNKT cells secreted the classical Th1 cytokines IFN-γ and TNFα (Zeng, Zhang, and Chen 2018). In addition, the cytotoxic potential of $CD38^+$ iNKT cells was also diminished, an effector function that is also associated with a $CD8^+$ T cell response. The occurrence of a large part of $CD38^+$ cells in the $CD4^+$ iNKT subset also hints towards a diminished Th1 response rather than a complete lack of effector function. $CD4^+$ iNKT cells are capable to elicit a Th2 response next to a Th1 response while the $CD8^+$ and double negative iNKT cells are believed to mediate mostly Th1 functions and cytotoxicity (Krovi and Gapin 2018). It is possible that CD38 marks a subpopulation of $CD4^+$ iNKT cells that are Th2 polarized and thus only mediate weak Th1 effector functions. This is supported by a report about conventional $CD4^+$ T cells, which produced high amounts of IL-13 when they expressed CD38 while the $CD38^-$ cells were Th1 biased (Scalzo-Inguanti and Plebanski 2011).

This would also be in line with a previous report from Inoue et al. that reported an increase in IL-13 secretion from iNKT cells in patients with chronic HCV infection. Even though CD38 was not investigated in that study, the here observed accumulation of $CD38^+$ iNKT cells as part of the profibrotic response would be an intriguing and reasonable explanation for the observed elevation of the Th2 cytokine IL-13 (de Lalla et al. 2004; Inoue et al. 2006).

While data about the detailed phenotype and function of $CD38^+$ iNKT cells is basically non-existing, some studies were conducted about $CD38^+$ $CD4^+$ T cells in mice and humans. In mice, it was observed that $CD38^+$ memory T cells proliferated less than their

CD38$^-$ counterparts and were almost completely devoid of cytokine production while even suppressing the immune response (Read et al. 1998). In another study, the decreased proliferative potential of CD38$^+$ CD4$^+$ T cells was also observed, however the authors state that these cells produced more IFN-γ and IL-2 (Sandoval-Montes and Santos-Argumedo 2005). In humans, a CD38$^+$ CD4$^+$ T cell population has been identified that showed reduced proliferation and after stimulation retained its naïve T cell phenotype expressing CD45RA, CD62L and CCR7. While producing marginal amounts of Th1 cytokines, this T cell subset was a potent producer of IL-13 but no other Th2 cytokines (Scalzo-Inguanti and Plebanski 2011). Impaired IFN-γ secretion by CD38$^+$ CD4$^+$ T cells was likewise observed in a controlled human malaria infection study. In contrast to the data from this work and to other studies, these cells proliferated more and exhibited higher cytotoxicity than their CD38$^-$ counterparts (Burel et al. 2016). Collectively, this strongly hints towards a role of CD38 that does not only mark activated T cells but also defines a unique subpopulation of not only T cells but based on the results from this work, also iNKT cells that might have so far undescribed effector functions.

Even though some past reports are contradictory to a certain extent, they all show that CD38$^+$ T cells exhibit a unique phenotype and functional properties. Hence, the results obtained in this work suggests that CD38 marks a distinct population of iNKT cells, too. Due its unique effector functions, this iNKT cell subpopulation might be a crucial immunomodulator in immune responses that are paralleled by this phenotype. The occurrence of this cell type during the progression to persistent HCV infection thus may reflect immune failure similar to what is known from conventional HCV specific T cells during persistent infection.

7.5. IL-32$^+$ iNKT cells

In this study and a former study (Senff 2018), it was shown that iNKT cells are highly activated during acute and chronic HCV infection and that this phenotype correlated with liver damage indicating an important mechanistic role for activated iNKT cells. In order to elucidate the most important factors leading to the observed phenotype during HCV infection, the transcriptome of activated iNKT cells was examined by RNA-sequencing.

This approach could reveal target genes essential for the iNKT cell-mediated immune response not only against HCV but also against other viral infections.

Invariant NKT cells can interact with a multitude of other immune cells. This interaction includes but is not limited to crosstalk with cDCs and pDCs (Keller, Freigang, and Lunemann 2017; Montoya et al. 2006; Shimizu et al. 2013), B cells (Zeng et al. 2013), T cells (Lin et al. 2006), NK cells (Riese et al. 2015) and MDSCs (Hongo et al. 2014). The integration of all this interactions strongly influences the downstream immune response, however the iNKT cells themselves can be substantially influenced by other immune cells as well. This would dramatically change the outcome of a transcriptome analysis depending on the costimulation of other, more abundant PBMCs during iNKT activation. Therefore, an isolated iNKT cell population was stimulated to reveal the activation pathways that are intrinsic to iNKT cells. Since mature T cells and thus iNKT cells do not express CD1d, TCR mediated stimulation is abrogated in a pure iNKT population (Chaudhry and Karadimitris 2014). However, iNKT cells are readily activated by proinflammatory cytokines, which is a hallmark of unconventional T cells (Reilly, Wands, and Brossay 2010; Holzapfel et al. 2014; Holoshitz et al. 1993; Hinks and Zhang 2020). Since it was proposed that iNKT activation during chronic HCV infection and other infections is mostly mediated by proinflammatory cytokines (Brennan, Brigl, and Brenner 2013)(Senff, Menne et al.), this experimental approach closely reflects the physiological activation of iNKT cells.

Transcriptome analysis of iNKT cells revealed a large set of differentially expressed genes after activation indicating profound alterations in global gene expression. GO Term enrichment analysis revealed an altered metabolomic state as well as the response to the activation stimulus. Surprisingly, most cytokines and interleukins that are described to be expressed by iNKT cells were not induced on a transcriptional level as seen by unaltered mRNA content in the activated iNKT cells. The developmental trajectory of iNKT cells in the thymus includes acquisition of a pre-activated memory-like phenotype that facilitates the rapid effector response of iNKT cells upon antigen encounters. This pre-activated phenotype is marked by the presence of pre-formed effector mRNA that enables rapid synthesis of effector cytokines (Stetson et al. 2003; Gapin 2008) hinting towards a translational rather than a transcriptional regulation of these molecules in iNKT cells. Intriguingly, out of the cytokines that were induced after activation on a transcriptional

level, IL-32 showed by far the strongest induction. Therefore, its expression and function in iNKT cells and other lymphocyte subpopulations was investigated in more detail.

IL-32, was originally described in IL-2 activated NK cells, and was thus called NK transcript 4 (NK4) (Dahl et al. 1992) before it was renamed to its current name in 2005 (Kim et al. 2005). NK4 was assigned to be an interleukin since it became clear that it can induce other proinflammatory cytokines like TNFα and IL-8 from various cell types (Joosten et al. 2013). In the last years, this relatively new interleukin has been shown to be involved in a multitude of different immunoregulatory processes mostly in a proinflammatory way.

IL-32 is expressed by a variety of immune cells like NK and T cells (Shoda et al. 2006; Dahl et al. 1992), monocytes, monocyte derived DCs (Shoda et al. 2006), but also non immune cells like endothelial cells and hepatocytes (Nold-Petry et al. 2009; Moschen et al. 2011). It is induced by different mechanisms dependent on the cell type and stimulus, for instance by cytokines like TNFα, which is closely associated with IL-32 signalling and reciprocally amplifies it as described for THP-1 cells (Heinhuis, Netea, et al. 2012; Ribeiro-Dias et al. 2017). In monocytes, activation can also occur via PRRs like TLRs and Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which leads to IL-32 transcription via the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. IL-32 production by human lymphocytes is mostly mediated by cytokine receptor stimulation by IL-12 and IL-18, by TNFα signalling or by mitogen activation (Ribeiro-Dias et al. 2017; Heinhuis, Netea, et al. 2012; Dahl et al. 1992). Here, analysis of various innate and adaptive lymphocytes revealed that IL-32 is predominantly expressed by innate cells like NK cells and also innate T cells while only few conventional T cells induce IL-32 after stimulation.

IL-32 is expressed in nine distinct isoforms produced by alternative splicing (Aass, Kastnes, and Standal 2021). Of these, IL-32α, IL-32β, IL32γ and IL-32δ are the most thoroughly investigated of which IL32γ – the initially described transcript (Dahl et al. 1992) – is the biologically most active isoform (Choi et al. 2009). However, to this date there are only pan-IL-32 antibodies commercially available for flow cytometry analysis hampering a more detailed description of the different isoforms in various infection settings. Even though T cells express predominantly the beta isoform (Goda et al. 2006), the isoforms that are expressed by iNKT cells – and other innate T cells – in health and disease have to be elucidated in the future. Nevertheless, IL-32 induction seemed to act individually

from other cytokines that were induced after iNKT cell activation as IFN-γ, TNFα or Granzyme B were unaltered between IL-32^{+} and IL-32^{-} iNKT cells. Moreover, it was enriched in CD4- iNKT cells showing the proinflammatory potential of this subset.

In many of the IL-32 mediated signalling pathways, it is believed to function intracellularly rather than from the outside of the cell. This and the fact that based on the three dimensional structure it does not fit into any existing cytokine family, IL-32 is considered a non-classical cytokine. Among many intracellular pathways that IL-32 influences, amplification of NOD-like signalling to induce proinflammatory IL-6 (Netea et al. 2005) and integrin binding are well described mechanisms IL-32 utilizes to modulate the immune response (Heinhuis, Koenders, et al. 2012).

A prominent role of intracellular IL-32 in regulating activation induced cell death was described in anti-CD3 activated T cells. This mechanism was also speculated to be responsible for the presence of IL-32 in the supernatant of activated T cell cultures, since it does not seem to be secreted in large amounts and due to the co-presence of cytosolic GAPDH in the supernatant (Goda et al. 2006). The isoforms IL32γ and IL-32β were linked to this phenomenon whereas the IL-32α isoform did not induce apoptosis. In this study, IL-32 was expressed in activated but not apoptotic iNKT cells after stimulation with IL-12, IL-15 and IL-18. If this is due to the different stimulus that was used or because iNKT cells express predominantly the alpha isoform of IL-32 needs further evaluation.

Despite the compelling data concerning the intracellular effects of IL-32, it is believed to act from the outside of the cell as well. Even though IL-32 is actively secreted in only minimal amounts and mostly released by necrotic cells, it was demonstrated that exogenous, recombinant human IL-32 can activate and induce cytokine production in THP-1 cells (Kim et al. 2005) and triggers IL-6 release in human PBMCs (Choi et al. 2009). Furthermore, it induced TNFα, IL-1b, IL-6. CXCL1, CCL2, IL-10 and IL-1ra from different myeloid cells (Ribeiro-Dias et al. 2017), providing further evidence that exogenous IL-32 is capable of stimulating human immune cells, albeit secreted in only marginal amounts, if at all. Furthermore, IL32γ isoform possesses a signalling peptide with no transmembrane domain indicating a possible secretory pathway. Even though this hints towards an exogenous role of IL-32, no specific IL-32 receptor has been found to this day, complicating research in this area. Nevertheless, in numerous studies IL-32 was shown to modulate the downstream immune response as a potent proinflammatory cytokine that

is also implicated in various diseases (Ribeiro-Dias et al. 2017). If iNKT cells are able to secrete IL-32 needs to be determined in future studies.

Next to its induction in various cell types after stimulation with different inflammatory stimuli, IL-32 is also upregulated during infection with bacterial and viral pathogens. Along this line, it was increased in various experimental mycobacterial infection models and was associated with protection from Mycobacterium tuberculosis infection. (Ribeiro-Dias et al. 2017; Koeken et al. 2020).

The influence of IL-32 on different viral infections is well described. Serum levels of IL-32 are increased in IAV as well as in HIV infected patients in which it is also a biomarker for loss of viral control in slow progressors (Li et al. 2008; El-Far et al. 2016). Both viruses induced IL-32 in infected cell cultures (Rasool et al. 2008; Li et al. 2009) and recombinant human IL32γ was moreover able to suppress influenza A virus replication in vitro (Li et al. 2010). Unfortunately, the cellular source of plasma IL-32 was not determined in these studies.

Besides its implications in the aforementioned viral infections, there is strong evidence of IL-32 involvement in the immune response and immunopathology during viral hepatitis. Moschen et al. showed that in vitro infection of the hepatoma cell line Huh-7.5 with HCV, as well as TNFα and IL-1β treatment, resulted in IL-32 induction. In contrast, IL-32 overexpression or silencing did not influence replication. Importantly, these in vitro results transferred well to patients with chronic HCV infection in which IL-32 expression correlated with clinical markers for liver inflammation like liver steatosis, smooth muscle actin area and serum ALT levels (Moschen et al. 2011). Interestingly, the correlation of intrahepatic IL-32 with serum ALT and liver fibrosis was also observed in chronic HBV infected patients in another study (Xu et al. 2012). Moreover, IL32γ was upregulated in HBV infected hepatocytes in vitro which – shown by a different group – intracellularly suppresses HBV replication (Kim et al. 2018; Li et al. 2013). While exogenous IL-32 had no direct effect on HBV replication in hepatocytes in vitro, it potently induced IFN-λ1 (IL-29) secretion from PBMCs. This IFN-λ1 strongly suppresses HBV and also other viruses like HCV and HIV (Kim et al. 2018; Li et al. 2013). From this perspective, iNKT cells that are activated by the proinflammatory cytokine milieu in the liver during active hepatitis could potentially modulate the immune response by fine-tuning the IL-32 response leading to secretion of highly potent anti-viral IFN-λ1 from other intrahepatic immune cells.

In this study, iNKT cells from chronic hepatitis C patients did not show enhanced IL-32 expression compared to resolved patients – albeit with a very low sample size. As this analysis was restricted to circulatory iNKT cells, IL-32 expression might be differentially regulated in hepatic iNKT cells evoked by the inflammatory environment in the HCV infected liver. As IL-32 correlated with liver inflammation in previous studies (Xu et al. 2012), it may also be involved in acute hepatitis C virus infection, where inflammation is more pronounced compared to the chronic phase (Shin, Sung, and Park 2016). Hence, IL-32 signalling by iNKT cells could be a player in the inflammatory network during early HCVinfection when the innate immune response targets the virus and the delayed adaptive response has not yet started.

Another finding in this work was the abundant expression of IL-32 in innate immune cells, like NK cells and innate T cells. While adaptive $CD4^+$ and $CD8^+$ T cells also expressed some IL-32, it was upregulated in only a minority of all cells. Recently, in an RNA-sequencing approach, Gutierrez-Arcelus et al. defined an innateness gradient in lymphocytes that spanned from adaptive conventional T cells to NK cells with innate T cells lining up in between. This innateness was defined by distinct effector functions whereby a hallmark of innate T cells was the propagation of the inflammatory response by directing other immune cells to the site of inflammation (Gutierrez-Arcelus et al. 2019). IL-32 production by iNKT cells would perfectly fit into this model in which innate-like lymphocytes modulate the inflammatory environment, e.g. by kick starting other inflammatory pathways (Ribeiro-Dias et al. 2017) via IL-32 signalling.

Overall, IL-32 acts as an early proinflammatory modulator and is expressed in large amounts in iNKT and other innate T cells upon TCR-independent stimulation. The well described role of IL-32 in viral hepatitis and in IFN-λ1 signalling in conjunction with the activated phenotype of iNKT cells during acute and progressing HCV infection might constitute a mechanism of early immune regulation by iNKT cells during viral hepatitis.

7.6. Outlook

In summary, iNKT cell activation correlates with outcome of acute HCV infection. These findings corroborate data from a former study which showed higher levels of $CD38^+$ iNKT cells in patients with chronic compared to resolved hepatitis C (Senff 2018).Furthermore,

in patients with acute resolving HCV infection, iNKT cell activation associates with the amount of liver inflammation suggesting that these cells actively contribute to the anti-HCV immune response. However, as most studies in the human system are descriptive and based on correlations, the exact impact of NKT cells onto these inflammatory processes remain unclarified. The lack of small animal models for HCV infection makes it extremely challenging to define any iNKT cell mediated mechanisms that are responsible for anti-HCV effects or the infection outcome. In order to reveal if activated iNKT cells directly mediate liver damage or if their activity is restrictedto regulating other effector anti-HCV effector cells, analysis of liver and matched blood samples from HCV infected patients is required. Furthermore, the use of in vitro cytotoxicity assays with HCV replicon cell culture system (Lohmann and Bartenschlager 2014) can be helpful to determine, if human iNKT cells can mediate direct cytotoxicity against HCV infected cells.

Investigation of the $CD38^{+}$ phenotype, which was observed during acute hepatitis C, revealed a strong functional impairment of these cells. This suggests that iNKT cells are not only activated during acute HCV but also that this activation comes along with a unique functional profile possibly reflecting iNKT cell exhaustion, differentiation or polarization towards a non-cytotoxic subset. This phenotype was found in healthy individuals suggesting that CD38 marks a unique population of functionally impaired iNKT cells. It is of particular interest, if the accumulation of these cells – as seen during acute HCV – also transfers to other viral infections, such as HBV infection.

Transcriptome analysis of activated iNKT cells unravelled the gene signature of activated human iNKT cells in a so far unprecedented detail. This gene signature will provide helpful in the future to identify target genes that are associated with iNKT cell activation not only during hepatitis C but also other diseases involving iNKT cell activation. Lastly, the identification of IL-32 as an innate effector molecule might contribute to a better understanding of the inflammatory network of iNKT and other innate-like cells during various infections. Better resolution of the IL-32 isotypes in immunological analyses will provide a greater insight into the complex regulatory network of this innate effector molecule and the contribution of iNKT cells to this network.

8. References

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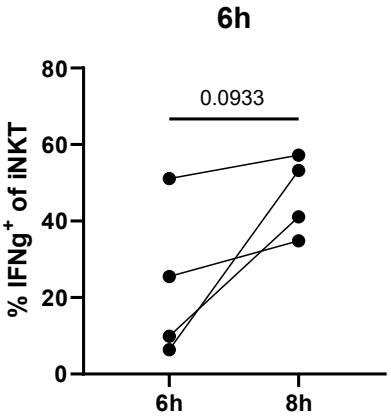
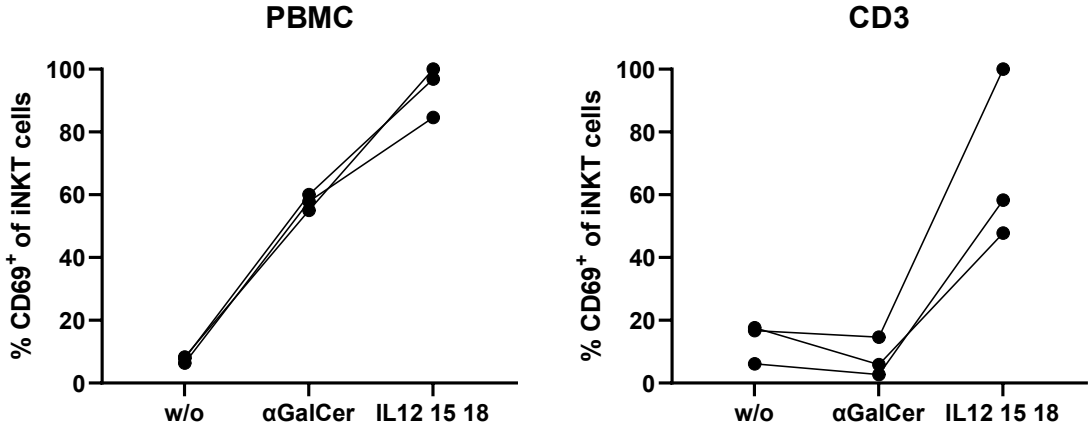
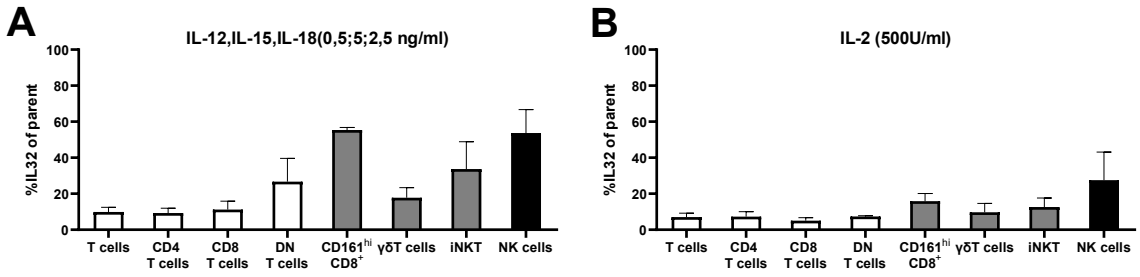
9. Supplements



Supplementary Fig. 1. Invariant NKT activation at 6 hours and 8 hours post stimulation. Healthy donor buffy coat derived PBMCs were stimulated for 6 hours or 8 hours with 1 µg/ml αGalCer, 10 ng/ml IL-12, 100 ng/ml IL-15 and 50 ng/ml IL-18 in the presence of BFA for the last 4 hours. IFN-γ secretion of iNKT cells was analysed by flow cytometry and ICS. Each dot represents one of four individual donors. Paired t-test was used to compare groups and the p-value is depicted.



Supplementary Fig. 2. Activation kinetics of in vitro stimluated iNKT cells. Buffy coat derived healthy donor PBMCs or $CD3^+$ cells negatively isolated by MACS were in vitro activated with the indicated stimuli and activation as marked by CD69 expression was analysed by flow cytometry at 24 hours post stimulation. Each dot represents an individual of three independent donors.



Supplementary Fig. 3. IL-32 expression by distinct lymphocyte subpopulations after different stimuli. Healthy donor buffy coat derived PMCs were stimulated with (A) 0.5 ng/ml IL-12, 5 ng/ml IL-15 and 2.5 ng/ml IL-18 or (B) 500 U/ml IL-2 for 24 hours. BFA was added for the last 4 hours to every sample. Bars represent the median mean of three donors and the bars indicate the standard deviation. Only sample with more than 17 iNKT cells were included in the analysis.

10. Abbreviations

Abbreviation	Meaning
ALT	Alanine transaminase
APC	Antigen-presenting cells
BCR	B cell receptor
BFA	Brefeldin A
BTB-ZF	BTB-zinc finger
CMV	Cytomegalovirus
DAA	Directly acting antivirals
DC	Dendritic cells
DEG	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DN	Double negative
EDTA	Ethylenediaminetetraacetic acid
EGR	Early growth response protein
ETI	Estimated time of infection
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FMO	Fluorescence minus one
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV-1	Herpes simplex virus type I
IAV	Influenza A virus
ICS	Intracellular cytokine staining
IFN	Interferon
IFNAR	Interferon alpha and beta receptor
IL-32	Interleukin -2
iNKT cells	Invariant natural killer T cells

ISG	Interferon-stimulated genes
KIR	Killer-cell immunoglobulin like receptors
KO	Knockout
KSHV	Kaposi sarcoma–associated herpesvirus
MACS	Magnetic activated cell sorting
MAIT	Mucosal associated invariant T
MDA5	Melanoma differentiation antigen 5
MDSC	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NAD	Nicotinamide adenine dinucleotide
NK	Natural killer cell
NK4	Natural killer transcript 4
NKT	Natural killer T cell
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
ODN	Oligodeoxynucleotides
PAMP	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PKR	Protein kinase R
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol-12-myristat-13-acetat
PRR	Pattern recognition receptors
PWID	People who inject drugs
RIG-I	Retinoic acid inducible gene-I

SLAM-SAP	Signaling lymphocytic activation molecule (SLAM)-associated protein
SNP	Single nucleotide polymorphisms
SVR	Sustained virological response
T-bet	T-box expressed in T cells
TCR	T cell receptor
Th	T helper cells
TLR	toll like receptors
TNFα	Tumor necrosis factor α
T_{reg}	Regulatory T cell
TZR	T-Zell Rezeptor
αGalCer	α-Galactosyceramide
αGluCer	α-glucosylceramides

11. List of figures

12. List of Tables

Danksagung

In der Online-Version nicht enthalten.

Curriculum Vitae

In der Online-Version nicht enthalten.

Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Düsseldorf, den _____