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The Role of Type 2 Innate Lymphoid Cells in the Pathogenesis of Liver Inflammation

Dissertation

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Content

1 Introduction

1.1 Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are a heterogeneous group of immune cells, which belong to the family of lymphoid cells but lack antigen-specific receptors or linage-specific markers (Cortez et al., 2015). Due to their relatively rare expression in most mammalian tissues, their diversity and the absence of unique surface markers, ILCs were not perceived as a distinct family and had first been identified as a population of important effector cells, which rapidly respond to inflammation-induced cytokines in 2011 (Spits and Di Santo, 2011, Walker et al., 2013). They are efficient producers of pro-inflammatory and immunoregulatory cytokines and in this way orchestrate immune responses in early stages of infection and throughout inflammation (Sonnenberg and Artis, 2015, Mjösberg and Spits, 2016).

1.1.1 Subsets of Innate Lymphoid Cells

The family of ILCs can be distinguished by their executive function into cytotoxic and non-cytotoxic ILCs. Non-cytotoxic ILCs can be further grouped into three different subsets by their cytokine production profile and dependence on transcription factors similar to the differentiated groups of cluster of differentiation (CD) 4^+ T helper (Th) cells (Spits and Di Santo, 2011). Naive $CD4^+$ T cells differentiate into a specific effector subset upon contact of their T-cell receptor (TCR) with an antigen presented via major-histocompatibility complex (MHC) class II of an antigen-presenting cells (APC) (Luckheeram et al., 2012). In this way $CD4^+$ T cells propel different immune responses: In type 1 immune responses Th1 cells contribute to the defence against intracellular pathogens by production of interferon γ (IFNγ), improvement of the phagocytic function of macrophages and production of class-switched immunoglobulin (Ig) type G antibodies. Type 2 immune responses are driven by Th2 cells, which are characterized by production of the cytokines interleukin (IL)-4, IL-5 and IL-13. In doing so, they support barrier immunity and the defence against extracellular parasites, such as helminths. Further, Th2 cells promote the production of Ig E antibodies and hereby they are linked with the pathogenesis of allergies. The third group of the T- helper subsets encompasses Th17 cells, facilitating type 3 immune responses by controlling

extracellular bacteria and fungi. Th17 cells produce IL-17 A,IL-17 F and IL-22, which improve neutrophil response and barrier function of epithelial cells by production of antimicrobial peptides (Stockinger and Veldhoen, 2007, Kolls et al., 2008). In analogy to the T helper subsets the subpopulations of ILCs were named group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3) ILCs based on the classification of immune responses (Artis and Spits, 2015). T-bet-dependent ILC1s predominantly produce the type 1 cytokine IFNγ upon activation (Artis and Spits, 2015, Klose et al., 2014). As representatives of type 2 immunity, GATA-binding protein 3 (GATA-3)-dependent ILC2s prevalently produce IL-5, IL-13, IL-4 and IL-9 (Hoyler et al., 2012, Mjösberg et al., 2012, Moro et al., 2010). Cells of the ILC3 subset, which require retinoic-acid-receptor-related- orphan nuclear receptor γ (RORγt) signaling for differentiation, are sources of IL-17 and/or IL-22 (Sawa et al., 2010). ILCs providing cytotoxic effects are natural killer (NK) cells, sharing phenotypic similarities with ILC1s as elaborated below (Fig.1).

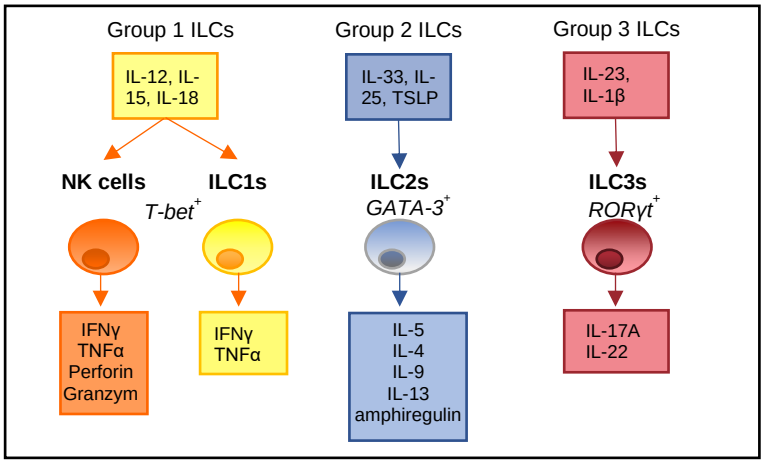

Figure 1: Subsets of ILCs.
ILCs respond to environmental mediators with cytokine production referring to their respective subset. IFNγ, interferon γ, IL, interleukin; NK cells, natural killer cells; TNFα, tumor necrosis factor α; TSLP, thymic stromal lymphopoietin.

Unlike T cells and B cells, ILCs do not express antigen-specific receptors that undergo somatic rearrangement and further lack expression of any known linage-specific marker. All ILCs share the expression of IL-7 receptor α (Rα) (CD127), IL-2Rα (CD25), the common gamma chain (γc) and thymocyte antigen 1 (Thy1, CD90) (Sonnenberg and Artis, 2015, Artis and Spits, 2015), with the exception of NK cells. In contrast to T and B cells, whose activation is facilitated by T- or B-cell receptor signaling, respectively (Pennock et al., 2013, Treanor, 2012), ILCs are dependent on the microenvironmental influences via cytokines due to their lack of an antigen-specific receptor (McKenzie et al., 2014, Diefenbach et al., 2014).

ILCs develop from the common lymphoid progenitor (CLP) (Artis and Spits, 2015), which gives rise to all lymphocytes of the adaptive immune system (Klose et al., 2014, Ichii et al., 2010, Yang et al., 2010), thus ILCs belong to the lymphoid cell lineage. CLP can be located in the fetal liver and the adult bone marrow (Klose et al., 2014, Rawlins et al., 2009). In contrast to T- and B-cell development all ILC subsets require the transcriptional regulator DNA-binding protein inhibitor ID-2 (ID2) (Yokota et al., 1999, Moro et al., 2010, Satoh-Takayama et al., 2010). This suggests the existence of another precursor cell line to give rise to ILCs. As there are different ILC-precursor populations described, it is current opinion that there are multipotent ILC precursors, which specify to unipotent ILC precursors, and give rise to NK cells and the respective ILC subsets (Klose et al., 2014, Constantinides et al., 2014). Once differentiated, ILCs maintain the capability to produce cytokines, which are not associated with their respective subset upon stimulation with certain environmental-derived substances (Lim et al., 2017). This phenomenon is called functional plasticity and is subject of ongoing research and currently not completely understood (Califano et al., 2015, Pikovskaya et al., 2016, Lim et al., 2016, Antignano et al., 2016, Lim et al., 2017).

ILCs are present throughout the body. In lymphoid tissue, they are relatively underrepresented, but they are enriched in mucosal and barrier surfaces such as the skin, the intestine and the lung (Klose and Artis, 2016, Sonnenberg et al., 2013). Parabiosis experiments showed that ILCs are tissue-resident and do not undergo continual replenishment from the bone marrow but maintain themselves by self-renewal and expand upon inflammatory stimuli (Gasteiger et al., 2015). Following an activating signal, tissue-resident ILCs proliferate and expand to facilitate various functions, including initiation and orchestration of immune defense, preservation of the epithelial integrity and regulation of metabolism and the microbiota (Artis and Spits, 2015).

Group 1 ILCs

Group 1 ILCs include IFNγ-producing lymphoid cells of the innate immune system: NK cells and ILC1s. Both cell populations have phenotypical similarities, like the expression of surface markers NKp46 and NK1.1. They can be activated by IL-12, IL-15 and IL-18 (Klose et al., 2014, Fuchs et al., 2013, Artis and Spits, 2015, Bernink et al., 2015), but are distinguishable by their expression of transcription factors. In contrast to T-bet$^+$ and Eomes$^+$ NK cells, ILC1 development and function only requires T-bet signaling (Daussy

et al., 2014). Further, there are differences in the localization and phenotypical appearance between NK cells and ILC1s. Distinct from NK cells, which circulate in the peripheral blood (Sojka et al., 2014, Daussy et al., 2014), ILC1s are tissue-resident and had been found in the liver, spleen, intestine, uterus and the retroperitoneal cavity. Furthermore, ILC1s and NK cells differ in their expression of the surface markers CD49a, CD49b, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), CD127, CD183 and CD186 (Gonzaga et al., 2011, Klose et al., 2014, Daussy et al., 2014). NK cells are well known to kill virus-infected cells by degranulation of granzymes and perforin (Biron et al., 1999) and via binding of TRAIL (Mandal and Viswanathan, 2015). In a similar manner, ILC1s from the liver are able to lyse target cells via TRAIL. Therefore, hepatic ILC1s and NK cells are both capable of cytolysis but differ in the expression of this mediators of cytotoxicity (Daussy et al., 2014, Klose et al., 2014, Cortez et al., 2015). In addition to their capability to produce IFNγ, ILC1s are potential sources of Tumor necrosis factor α (TNFα) (Klose et al., 2014). Thus, ILC1s are described as important player in defense against intracellular pathogens such as *Salmonella enterica* and *Toxoplasma gondii* infection (Klose et al., 2014, Klose et al., 2013, Artis and Spits, 2015). Further, ILC1s were reported to contribute to the pathogenesis of inflammation in different models of colitis and to be involved in inflammatory bowel disease in humans (Bernink et al., 2013, Fuchs et al., 2013).

Group 3 ILCs

ILC3s encompass lymphoid cells that express the transcription factor RORγt, like lymphoid tissue inducer-like (LTi)-like cells and conventional ILC3s (Cortez et al., 2015). This heterogeneous population differs in their expression of cell surface markers and cytokines. LTi-like cells are variable in their expression of CD4 and lack expression of NKp46 and T-bet (Sawa et al., 2010), whereas conventional ILC3s are NKp46^{+} RORγt^{+} T-bet^{low} cells (Klose et al., 2013, Sciumé et al., 2012). The functions of RORγt^{+} ILC3s are diversified: fetal LTi-like cells play a crucial role in the formation of secondary lymphoid organs like Peyer´s patches in the embryonal state (Mebius et al., 1997, Eberl et al., 2004) and also contribute to the formation of isolated lymphoid follicles after birth, which play an important role in the immunological defense of the intestine (Kruglov et al., 2013). As all ILC3s are capable to produce IL-22 and/or IL-17A in response to IL-23 or IL-1β (Magri and Cerutti, 2015), they maintain the epithelial integrity of barrier

surfaces and mucosal regeneration after infection. In the intestine, they facilitate and coordinate tolerance to commensal bacteria (Goto et al., 2014, Hepworth et al., 2013), but also inhibit their peripheral dissemination (Sonnenberg et al., 2012). ILC3s are reported to contribute to mucosal regeneration after intestinal infection (Sawa et al., 2011), tissue remodeling of the thymus after radiation damage (Dudakov et al., 2012) and lymphatic tissue repair upon acute viral infection (Scandella et al., 2008). But there is evidence that ILC3s are also involved in the pathogenesis of carcinogenesis and tumor growth of the gastrointestinal tract (Chan et al., 2014).

1.1.2 Biology and Effector Function of ILC2s

The group of ILC2s encompasses innate lymphoid cells that are capable to produce type 2 cytokines and therefore, mediate type 2 immune responses (McKenzie et al., 2014). ILC2s are enriched at barrier surfaces such as the skin, the lung and the intestine (Huang and Paul, 2016), but can further be found in solid organs like the liver, spleen, kidney, uterus, heart, adipose tissue, brain of mice and the cornea (Liu et al., 2017, Nussbaum et al., 2013). Further, a few ILC2s can be found in lymphoid organs such as the thymus, spleen, mediastinal and mesenteric lymph nodes, Peyer's patches, bone marrow and the liver (Nussbaum et al., 2013).

ILC2s express the surface markers stem cell antigen-1 (Sca-1), tyrosine kinase KIT (c-Kit), and Thy1. They further express the γc, which is a subunit of the heteromeric receptors for IL-2, IL-4 (Russell et al., 1993) , IL-7 (Noguchi et al., 1993) and IL-9 (Takeshita et al., 1992). As they also express the other subunit of the IL-2 receptor (CD25) and the IL-7 receptor (CD127), ILC2s are responsive to IL-2 and IL-7 signaling (Moro et al., 2010, Duerr and Fritz, 2016). They further express cytokine receptors, which enable ILC2s to react on tissue damage and cell death on early stages of infection, such as IL-33-receptor subunit ST2, IL-17Rb, an IL-25-receptor subunit, and receptor for thymic stromal lymphopoietin (TSLPR) (McKenzie et al., 2014, Halim, 2016) (Fig. 2).

ILC2s develop from CLP in the bone marrow to immature ILC2-like precursors (Hoyler et al., 2012). As these cells are efficient to reconstitute ILC2 population in alymphoid mice after adoptive transfer experiments and fail to differentiate into other lymphoid linages, these cells are likely to constitute a ILC2 precursor population (Halim et al.,

2012b, Hoyler et al., 2012). These cells resemble mature ILC2s in the expression of the surface markers Sca-1, IL-7a, ST-2 and CD25, but differ in the expression of α4β7, killer cell lectin-like receptor subfamily G member 1 (KLRG1) and CD68 and CD122 and CD184 (Halim et al., 2012b, Hoyler et al., 2012). The differentiation from CLP to ILC2-like precursors are determined by the transcription factors ID2 and Notch, which suppress B- and T-cell differentiation (Moro et al., 2010, Wong et al., 2012). For their further differentiation, they depend on the transcription factors RORα (Wong et al., 2012), GATA-3 (Hoyler et al., 2012), growth factor independent 1 (Spooner et al., 2013), T-cell factor 1 (Yang et al., 2013) and B-cell lymphoma 11b (bcl-11b) (Walker et al., 2015). As mice with disrupted RORα receptor (Hamilton et al., 1996) fail to initiate appropriate Th2 immune responses upon challenge with the gastrointestinal roundworm *Nippostrongylus brasiliensis (N. brasiliensis)* or intranasal papain (Wong et al., 2012, Halim et al., 2012b) and deletion of GATA-3 leads to ablation of ILC2 *in vivo* and impaired survival *in vitro* (Hoyler et al., 2012, Walker and McKenzie, 2013) RORα and GATA-3 are not only important for ILC2 development, but also their functionality. Highly decreased numbers of ILC2s in ST2 and IL-17RB double deficient mice, but not in single knock-outs suggest the presence of either IL-33 or IL-25 to be important for the development of ILC2s (Huang and Paul, 2016, Voehringer et al., 2006, Huang et al., 2015). However, ILC2s only undergo only minor replenishment from the bone marrow, but proliferation of tissue resident ILC2 enable local expansion to initiate a sufficient immune responses (Gasteiger et al., 2015). Thus, the role of ILC2-like precursors in the bone marrow is not yet completely understood and the exact mechanisms of ILC2-differentiation, -maturation and -migration remain to be determined.

ILC2s can generally be grouped into two different subsets (McKenzie et al., 2014, Halim, 2016): tissue-resident, homeostatic ILC2s (ILC2s) and inflammatory ILC2s (iILC2s). Homeostatic ILC2s are present in steady state and respond to IL-33 and in low efficacy to IL-25. These cells are IL-7Rα^{+}, tyrosine kinase c-Kit^{+}, Thy1^{high}, KLRG1^{intermediate}, ST2^{+}, IL-17 receptor B (IL-17RB)^{low}. In contrast, iILC2s arise rapidly after IL-25 release in response to infection. This IL-7Rα^{+} c-Kit^{low}, Thy1^{low}, KLRG1^{high} ST2^{-} IL-17RB^{+}- population does not respond to IL-33 (Huang and Paul, 2016). iILC2 show a functional plasticity upon the environmental cytokines. When cultured under

Th2 conditions (IL-4, IL-33, anti-IFNγ, anti-IL-12) iILC2s produce IL-13, IL-4 and moreover IL-17, which is associated with ILC3 function. Culture under Th17 conditions (IL-1β, IL-23, IL-6, TGFβ, anti-IL-12, anti-IL-4) further increased their capacity to produce IL-17. Upon infection with *N. brasiliensis*, iILC2s develop into homeostatic ILC2 *in vivo* and support worm expulsion. Upon transfer of iILC2s to mice infected with *Candida albicans*, iILC2s lose their capability to produce IL-13, but sufficiently produce IL-17, and in this way improve anti-fungal immunity Thus, iILC2s can act as temporary progenitors of homeostatic ILC2s and show functional plasticity dependent on the environmental cytokines (Huang et al., 2015).

ILC2s have emerging roles in infection by cytokine production, but they are also able to interact with other immune cells directly. They are potent sources of type 2 cytokines upon activation. They produce predominantly IL-5 and IL-13 but further IL-4, IL-9 and the epithelial growth factor amphiregulin (Moro et al., 2010, Neill et al., 2010, Price et al., 2010, Turner et al., 2013). The expression of MHC II, CD80 and CD86, OX-40-ligand (OX-40L), KLRG1, inducible T-cell costimulator (ICOS) enable activation and modulation of other immune cells (see also chapter 1.1.4. and 1.1.5.) and interaction with epithelial cells (Oliphant et al., 2014, Mirchandani et al., 2014, Drake et al., 2014).

In early stages of infection, innate immune cells such as macrophages, natural killer T cells and mast cells produce IL-33 (Hsu et al., 2010, Gorski et al., 2013). IL-33 is also released as a result of danger associated molecular pattern (DAMPs)-signaling (Patel et al., 2014), during necrosis. Further DAMP-signaling activates ILC2s as well as mast cells directly (Cayrol and Girard, 2014, Lefrançais et al., 2014). Activation of mast cells leads to production of prostaglandin D2, another potent activator of ILC2s, and release of non-caspase protease chymase as well as tryptase, which fortify the effect of IL-33 on ILC2s (Xue et al., 2014). Further, basophil-derived IL-4 enhances ILC2 proliferation (Kim et al., 2014). In this way, ILC2 activation is facilitated in early stages of inflammation and ILC2s rapidly initiate and orchestrate immune responses in a direct and indirect manner. ILC2s directly promote the Th2 response in early stages of infection, by production of type 2 cytokines. Moreover, ILC2-derived IL-5 can act on eosinophils to promote their activation and recruitment (Nussbaum et al., 2013). ILC2s are also able to interact with $CD4^+$ T cells to mediate the Th2 immune responses (see also chapter 1.1.5), but also promotes Th2 immunity indirectly as IL-13 supports

alternative polarization (M2 polarization) of macrophages (Bouchery et al., 2015) as well as their accumulation and activation (Wu et al., 2011). Moreover, ILC2-derived IL-13 drives the migration of dendritic cells (DCs) to lymph nodes, which is essential of Th2-cell priming (Halim et al., 2014).Taken together, ILC2s have sentinel function and modulate the immune responses on early stages of infection (Cortez et al., 2015). Hereby, ILC2s have been shown to play an important role in the pathologies of different organs, especially when they are linked with barrier function as well as their tissue repair upon infection. In the intestine, ILC2s are reported to contribute to defense against extracellular pathogens and further a role in autoimmune disorders and allergic reactions of the gut are assumed. In the model of gastrointestinal infection with *N. brasiliensis*, ILC2s are critical sources of IL-13 and IL-9, which mediate clearance of worms, and amphiregulin, which provides epithelial repair (Fallon et al., 2006, Turner et al., 2013, Allen and Sutherland, 2014, Bouchery et al., 2015). By promoting mucus production of goblet cells and smooth muscle contraction, ILC2s are essential for worm expulsion (Moro et al., 2010, Neill et al., 2010, Price et al., 2010). Furthermore, ILC2s are likely to contribute to colitis as IL-13 drives intestinal inflammation (Heller et al., 2005) and patients with Crohn's disease show increased numbers of IL-13-producing ILC2s in the intestinal tissue (Bailey et al., 2012). IL-33, IL-25- and TSLP signaling have been reported to play a role in the pathogenesis of food allergies and eosinophilic esophagitis (Chu et al., 2013, Noti et al., 2013, Blázquez et al., 2010), suggesting a role of ILC2s in the pathogenesis of allergic disorders of the gut (Halim, 2016). In the lung, ILC2s can be found under homeostatic conditions but they dramatically increase upon allergic inflammation (Halim et al., 2012a). As Th2 immunity plays a central role in the pathogenesis of allergic lung diseases (Wills-Karp, 2004), ILC2s are critical for allergen- and virus-triggered airway hyperreactivity, goblet hyperplasia and mucus overproduction (Chang et al., 2011, Halim et al., 2012a). In a model of lung infection with *Schistosoma mansoni* eggs, ILC2-derived IL-13 leads to collagen deposition in the lungs, which suggests a profibrotic role of ILC2s (Hams et al., 2014). Further, ILC2s are enriched in nasal polys of patients with chronic rhinosinusitis (Mjösberg et al., 2011). In the skin, ILC2s contribute to inflammatory type 2 immune response in atopic dermatitis and are enriched in the atopic lesions in the skin of patients (Kim et al., 2013, Salimi et al., 2013).

ILC2s are also discussed to have protective effects in various diseases: ILC2 are described to have an attenuating effect on encephalitogenic T cells and hereby lower the susceptibility for experimental autoimmune encephalitis in mice (Russi et al., 2015). High frequencies of activated ILC2s decrease the susceptibility to graft-versus-host disease and induction of ILC2s by administration of IL-33 plays a protective role in a murine model of cerebral malaria by promoting the polarization of M2 macrophages (Besnard et al., 2015).There are also hints that ILC2s have protective effects against atherosclerosis in the aorta by providing atheroprotective IL-5 and IL-13 (Perry et al., 2013, Newland et al., 2017). In the adipose tissue, ILC2s, eosinophils, alternatively activated macrophages and regulatory T cells (T_{reg}) cooperate to maintain metabolic homeostasis. The loss of this interplay comes along with obesity and insulin resistance (Odegaard and Chawla, 2015). Further, ILC2s promote a process called “beiging”, which describes the recruitment of uncoupling protein 1^{+} adipocytes in white adipose tissue, thereby increasing the caloric expenditure by lipid oxidation and therefore heat production. Thus, beiging shifts the adipocyte function from fat storage to thermogenesis and is a mechanism to limit adiposity (Brestoff et al., 2015). Moreover, in models of influenza infection, ILC2-derived amphiregulin is critical for tissue repair following acute infection of the lung (Monticelli et al., 2011). ILC2s were also found to increase upon corneal damage and drive epithelia repair (Liu et al., 2017). Taken together, ILC2s accomplish various functions: they serve as sentinels in epithelial tissues, orchestrate type 2 immune response, maintain tissue homeostasis and contribute to epithelial repair upon damage.

1.1.3 Regulation of ILC2s

ILC2s are tissue-resident cells that can be found in barrier surfaces under physiological conditions and expand upon an inflammatory stimulus. Therefore, ILC2s are influenced by their microenvironment and its changes in infection (McKenzie et al., 2014, Duerr and Fritz, 2016). As they can have pro-inflammatory, immunoregulatory and tissue-repair functions, strict regulation mechanisms are needed. These are facilitated by cytokines and cell-to-cell-interaction via surface marker such as ICOS and KLRG1 (Fig. 2).

Positive Regulation

The cytokines IL-25, IL-33 and TSLP were the first substances, which were described to elicit ILC2s and induce activation, increased type 2 cytokine production and proliferation (Diefenbach et al., 2014). IL-33 is a member of the IL-1 family binding to its receptor ST2. IL-33 is widely expressed by stromal and epithelial cells upon damage (Liew et al., 2010). IL-25, also known as IL-17E, is a member of the IL-17 family and is produced by Th2 cells, basophils, eosinophils, mast cells and a variety of parenchymal cells (Valizadeh et al., 2015). TSPL is an epithelial cell-derived cytokine, which binds to its receptor containing a TSLP-binding chain (TSLP bc) and the IL-7Rα-subunit (He and Geha, 2010) and is able to induce cytokine production by ILC2s in humans. This effect was not seen in mice, but by reinforcing GATA-3 signaling, TSLP contributes to the maintenance of the activating effect of IL-25 and IL-33 in murine models (Mjösberg et al., 2012, Duerr and Fritz, 2016).

IL-2 and IL-7 are critical for development and survival of ILC2s (Moro et al., 2010). IL-7 facilitates its function via IL-7a (CD127) receptor in combination with the γc. It is produced by tissue-resident non-hematopoietic stromal cells and epithelial cells in lymphatic organs such as lymph nodes, bone marrow, thymus and spleen. Also DCs and macrophages produce IL-7 on lower extent (Capitini et al., 2009). IL-2 binds to the trimeric receptor consisting of CD25, CD122 and γc, the dimeric receptor of CD122 and γc or CD25 or the dimeric receptor involving CD25 and γc. Major sources are activated $CD4^+$ T cells and $CD8^+$ T cells. Further, DCs can produce IL-2 under inflammatory conditions (Boyman and Sprent, 2012). IL-33 can induce IL-2 production by DCs and in this way synergizes its activating effect on ILC2s (Matta et al., 2014). IL-2 and IL-7 both facilitate their effect via activation of the transcription factor signal transducer and activator of transcription (STAT) 5 in responding cell populations (Boyman and Sprent, 2012, Mazzucchelli and Durum, 2007).

IL-4 is part of a positive feedback loop of ILC2s, as they express the IL-4 receptor and are potential sources of IL-4 as well (Motomura et al., 2014, Noval Rivas et al., 2016). Therefore, ILC2s can increase type 2 cytokine production in an autocrine manner via IL-4, which is synergistically further increased in presence of IL-33. Additionally, IL-33 and IL-2 induced ILC2 proliferation was potentiated in presence of IL-4. Further on, in type 2 immunity basophils were shown to be a potent source of IL-4 and basophil-

derived IL-4 was shown to control ILC2-dependent eosinophilic inflammation in allergic airway response (Motomura et al., 2014). Likewise, IL-9 can be produced by ILC2s, T cells and mast cells (Noelle and Nowak, 2010). IL-9 facilitates its role via the dimeric receptor of IL-9R and the γc and facilitates its effect via janus kinases 1-3, STAT1 and STAT5 signaling (Noelle and Nowak, 2010). IL-9-deficient mice show deficits in ILC2 accumulation and type 2 cytokine production. ILC2-derived IL-9 further is critical for tissue repair in the lung (Turner et al., 2013), recruitment of eosinophils upon infection with *N. brasiliensis* and worm expulsion (Licona-Limón et al., 2013). ILC2 activation via IL-33 and TSLP induces the interferon regulating factor 4/IL-9 program that promotes autocrine IL-9 production and reinforces expression of IL-5 and IL-13 (Mohapatra et al., 2016). IL-9 receptor-deficient mice show reduced numbers of ILC2s in the lung upon infection with *N. brasiliensis*, which was associated with a reduced expression of the antiapoptotic marker bcl-3 on ILC2s. Thus, IL-9 has anti-apoptotic effect on ILC2s (Turner et al., 2013). In this way, IL-9 supports ILC2 survival and cytokine production (Wilhelm et al., 2011, Turner et al., 2013).

Another mechanism to regulate ILC2s is via ICOS/ICOS-ligand (ICOS-L) signaling. ICOS is expressed on T cells and activated memory T cells and acts as a co-stimulatory molecule. Its ligand is expressed on B cells and DCs and plays an important role in cell-cell signaling, proliferation and is critical for the development of an adequate immune response (Sharpe and Freeman, 2002). Human and murine ILC2s were described to express both ICOS and ICOS-L simultaneously (Kamachi et al., 2015, Maazi et al., 2015). As ICOS-deficient mice show reduced numbers of ILC2s and blocking of ICOS signaling leads to increased expression of proapoptotic marker on ILC2s, such as the protein bcl-2 (Paclik et al., 2015), decreased cytokine production and amelioration of lung inflammation and airway hyperreactivity in a mouse model of fungal allergen challenge (Maazi et al., 2015), ICOS shows to be a crucial factor of ILC2 function and development (Duerr and Fritz, 2016). ICOS/ICOS-L signaling has further been described to play an important role in IL-33-induced expansion of T_{regs}. IL-33-elicited ICOS-L^+ ILC2s interact through ICOS, which is expressed by T_{regs}. This ICOS/ICOSL interaction is critical for T_{reg} survival (Molofsky et al., 2015). Vice versa interaction of ICOS-L^+ ILC2s with $ICOS^+$ T_{regs} is shown to suppress ILC2 cytokine production and ILC2-driven airway hyperreactivity (Rigas et al., 2017). As both ILC2s

and T_{regs}, expand upon IL-33 signaling and ILC2 mediated co-stimulation via ICOS support T_{reg} survival, which facilitate ILC2 suppression, ICOS/ICOS-L signaling is an important mechanism to counter-regulate ILC2s especially in IL-33-driven diseases.

Negative Regulation

Interferons are important players in the defense against viruses, bacteria, fungi and parasites. They drive immune responses by enhancing action of monocytes and dendritic cells as well as promoting activation and function of T-, B- and NK cells (McNab et al., 2015, Cho and Kelsall, 2014). However, regarding ILC2s, especially IFNγ has been reported to have a direct inhibitory effect. IFNγ is produced by Th1 cells, group 1 ILCs and APCs and facilitates its effect via the IFNγ receptor, a heterodimer consisting of the two chains IFNγR-1 and IFNγR-2 (Schroder et al., 2004), which are both expressed on ILC2s (Molofsky et al., 2015). IFNγ was shown to suppress proliferation, activation and cytokine production of ILC2s *in vitro* (Moro et al., 2016). *In vivo*, treatment with IFNγ decreased numbers of ILC2s and type 2 cytokines in the bronchioalveolar lavage fluid and reduced IL-33-induced airway hyperreactivity (Moro et al., 2016). Also in the adipose tissue IFNγ was shown to suppress ILC2 activation (Molofsky et al., 2013). Constitutive IFNγ expression caused reduced cytokine and ICOS-L expression of ILC2s (Molofsky et al., 2015).

Expression of KLRG1 varies in ILC2s dependent on their maturation. Tissue-resident ILC2s from the intestine are reported to express KLRG1 on a high level, whereas ILC2 progenitors in the bone marrow do not show substantial expression of KLRG1 (Hoyler et al., 2012). KLRG1 functions as a co-inhibitory receptor on NK cells and T cells (Ito et al., 2006, Tang et al., 1993). It binds to E-cadherin of epithelial cells, Langerhans cells and keratinocytes (Grundemann et al., 2006). E-cadherin expression is downregulated in lesions of patients with atopic dermatitis (Trautmann et al., 2001), whereas ILC2s are enriched in this tissue (Kim et al., 2013, Salimi et al., 2013). *In vitro* it was shown that culture of human ILC2s with plate-bound E-cadherin causes reduced GATA3 expression as well as decreased cytokine production by ILC2s (Salimi et al., 2013). As ILC2s are closely associated with barrier surfaces and can be found in the epithelia of the intestine (Neill et al., 2010) and the skin (Roediger et al., 2013) and the respiratory system (Chang et al., 2011, Mjösberg et al., 2011), KLRG1-E-cadherin

signaling is discussed as a mechanism to restrain the activity of ILC2s in epithelial tissue (Duerr and Fritz, 2016).

Programmed cell death protein 1 (PD-1) is expressed on activated T cells and B cells as well as on myeloid cells. Its ligand PD-L1 is expressed also on T cells, B cells and APCs (Yamazaki et al., 2002). Engagement of PD-1 by PD-L1 conterregulates TCR signaling and therefore inhibits T cells activation, cytokine production and survival (Parry et al., 2005). Taylor et al. also found ILC2s to be regulated by PD-1. PD-1 deficiency does not affect the development of ILC2s but increases the frequency of ILC2s and improves anti-helminth immunity via modulation of STAT5 signaling of KLRG1^{+} ILC2s. Thus, PD-1 can negatively regulate ILC2s (Taylor et al., 2017) and PD-1/PD-L1 signaling is a mechanism to suppress ILC2 function and maintenance by other immune cells, such as T cells.

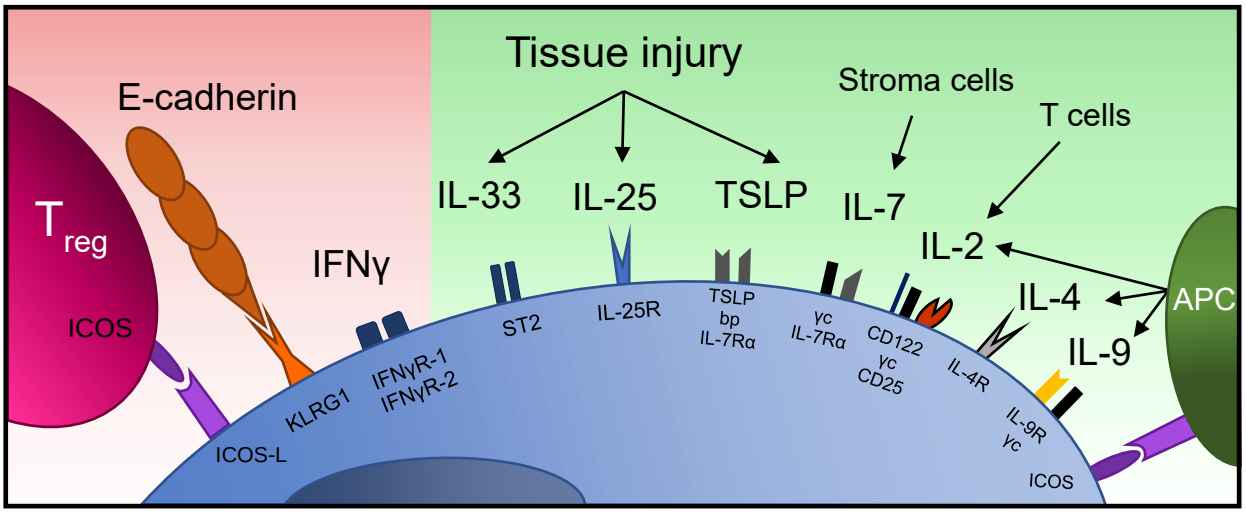

Figure 2: Regulation of ILC2s.
ILC2s are regulated by a variety of cytokines derived from the microenvironment or other immune cells and further are influenced by cell-cell contact. APC, antigen-presenting cell; bc, binding chain; CD, cluster of differentiation; ICOS, inducible T-cell costimulator; ICOS-L, ICOS ligand; IFNγ, interferon γ, IFNγR, IFNγ receptor; IL, interleukin; IL-4R, IL-4 receptor; IL-7Rα, IL-7 receptor α; IL-9R, IL-9 receptor; IL-25R, IL-25 receptor; KLRG1, killer cell lectin-like receptor subfamily G member 1; T_{reg}, regulatory T cell; TSLP, thymic stromal lymphopoietin; γc, γ chain.

1.1.4 Interactions of ILC2s and CD4$^+$ T Cells

A functional interplay between adaptive and innate immune cells is necessary to initiate an adequate immune response upon pathogen challenge, but also to maintain tissue homeostasis and prevent autoimmunity and hyperreactivity (de Visser and Coussens, 2005, Baecher-Allan and Hafler, 2004). To facilitate their function as sentinels and initiators of type 2 immune response, ILC2s cooperate with the adaptive counterpart CD4$^+$ T cells. Lack of ILC2s was shown to significantly impair Th2 response in different mouse models of Th2-driven inflammation (Halim et al., 2014, Oliphant et al., 2014, Gold et al., 2014).

The interaction of CD4$^+$ T cells and ILC2s occurs in direct and indirect manners. Ways of direct interactions are cell-to-cell contact influencing the maintenance or cytokine profile of both cell types. Direct effects of ILC2s/CD4$^+$ T cell-interaction can further be mediated by the paracrine effects of their cytokines on ILC2s and CD4$^+$ T cells, respectively. Furthermore, ILC2s can influence CD4$^+$ T cells by promoting their differentiation also indirectly by influencing other immune cells, such as DCs, to initiate Th2 immune responses. Production of type 2 cytokines by ILC2s have been shown to drive Th2 response in the initiation of allergic immune responses (Halim et al., 2012a). In a mouse model of allergic asthma, Halim et al. showed that ILC2-derived IL-13 promotes Th2 differentiation in the draining lymph node upon challenge with the antigen papain. As DCs, but not CD4$^+$ T cells, from the allergen-challenged mice express the IL-13 receptor, the Th2-promoting effect of ILC2-derived IL-13 is likely be facilitated via DCs. Indeed, IL-13 promotes the migration of DCs to the draining lymph node, a key event in Th2 priming (Halim et al., 2014). In this way, IL-13 secretion by ILC2s promotes Th2-cell differentiation indirectly (von Burg et al., 2015).

The direct effects of ILC2s on CD4$^+$ T cells are facilitated by their sufficient type 2 cytokine secretion: IL-4 plays a critical role in the development of an appropriate Th2 immune response. IL-4-deficient mice show great impairments in initiation of Th2-derived cytokine response (Kopf et al., 1993). Besides basophiles and mast cells (Perrigoue et al., 2009, Sokol et al., 2009, Yoshimoto et al., 2009), ILC2s are potent sources of IL-4 (Price et al., 2010). *In vitro*, addition of IL-33-elicited pulmonary ILC2s to cultured naive CD4$^+$ T cells promotes Th2 differentiation of CD4$^+$ T cells.

Th1 differentiation of naive $CD4^+$ T cells by IL-12 was inhibited in the presence of ILC2s (Mirchandani et al., 2014). As ILC2s from IL-4-deficient mice, did show significantly decreased levels of type 2 cytokines upon co-culture with wild-type $CD4^+$ T cells, ILC2-derived IL-4 plays a key role in upregulation of Th2 cytokine response in ILC2/$CD4^+$ T cell co-culture (Drake et al., 2014).

Additionally, interaction between ILC2s and $CD4^+$ T cells can be facilitated by cell-to-cell contact (Drake et al., 2014). Murine and human ILC2s are shown to function as APCs, process antigens and present them to T cells thereby inducing antigen-specific T-cell activation (Mirchandani et al., 2014, Oliphant et al., 2014). The presenting capacity of murine lung ILC2s is lower compared to DCs, however, MHC class II-mediated antigen presentation of ILC2s induces sufficient type 2 cytokine response *in vitro* (Oliphant et al., 2014). Oliphant et al. could show, that both cell types enter a MHC class II-dependent dialog that drives Th2 responses, displayed by type 2 cytokine production. Intracellular staining showed ILC2s to be the main source of type 2 cytokines, but also $CD4^+$ T cells contribute to type 2 cytokine response: MCH class II-mediated presentation of the antigen Ovalbumin (OVA) by ILC2s to transgenic OVA-specific $CD4^+$ T cells increased proliferation and production of IL-5, IL-13 and IL-9 of T cells. This effect is abrogated in presence of MHC class II-blocking antibodies. *In vivo* transfer of MHC class II-deficient ILC2s to *N. brasiliensis*-infected mice was associated with increased worm burdens. Only transfer of MHC class II-competent ILC2s induce sufficient worm expulsion (Oliphant et al., 2014). Similar results were achieved by Mirchandani et al.: in co-culture experiments, they show that OVA presentation via ILC2s induce proliferation and Th2 differentiation of $CD4^+$ T cells, whereas Th1 differentiation is inhibited. This effect is not seen upon separation of the cell types by transwells. As antigen recognition is the initial step of Th-cell differentiation (Luckheeram et al., 2012) and MHC II-mediated activation of $CD4^+$ T cells promotes type 2 cytokine production in both ILC2s and $CD4^+$ T cells, antigen presentation via MHC class II therefore is another mechanism of ILC2s to promote Th2 immune responses.

Moreover, antigen-specific activation in ILC2/T-cell interaction can be further supported by co-stimulation. As one molecule providing co-stimulatory capabilities, OX40

promotes Th2-cell differentiation (So et al., 2006). Drake et al. identified intracellular OX40L-expression in ILC2s and OX40 on the surface of $CD4^+$ T cells. Blocking of OX40/OX40L-signaling via polyclonal anti-OX40L antibodies partially inhibit type 2 cytokine production in ILC2/$CD4^+$ T-cell co-culture (Drake et al., 2014). Further, ICOS/ICOSL-signaling is crucial for both maintenance and cytokine production of Th cells (Hutloff et al., 1999) and ILC2s (Paclik et al., 2015, Kamachi et al., 2015). As ILC2 express ICOSL, signaling via this molecule is potential way to interact with $CD4^+$ T cells and to contribute to the initiation to Th2 immune responses. Moreover ILC2s also express the co-stimulatory molecules CD80 and CD86 (Oliphant et al., 2014), which potentially provides another mechanism of ILC2/T-cell interaction. CD80 and/or CD86 bind to the ligand CD28, which is expressed by all naive T cells, and hereby facilitate T-cell activation and proliferation (Luckheeram et al., 2012). In OVA-dependent ILC2/$CD4^+$ T cell-interaction, blocking antibodies of CD80 and CD86 markedly impaired ILC2 proliferation and IL-13 production (Oliphant et al., 2014). As blocking CD80 and CD86 primarily impairs activation of T cells, but not ILC2 directly, decreased ILC2 activation and cytokine production upon abrogated CD80/CD86 signaling underline the reciprocal effects of ILC2s and $CD4^+$ T cells upon antigen-specific interaction. Taken together, ILC2s have various molecular capabilities by providing co-stimulatory signals, therefore promoting T cell differentiation and activation.

As already mentioned the interaction of ILC2s and $CD4^+$ T cells is not unilateral. ILC2s in T cell and B cell- deficient $Rag1^{-/-}$ mice show reduced type 2 cytokine levels, suggesting ILC2s to require adaptive immune cells for efficient cytokine production (Wilhelm et al., 2011). Moreover, activated $CD4^+$ T cells induce proliferation of ILC2s (Mirchandani et al., 2014) as well as IL-5 and IL-13 production *in vitro* (Mirchandani et al., 2014, Drake et al., 2014). Depletion of $CD4^+$ T cells in mice, which were challenged with a systemic antigen, abrogates ILC2 proliferation and cytokine production upon re-challenge. Furthermore, the ability of ILC2s to produce IL-13 upon IL-33/IL-25 challenge correlates positively with the frequency of $CD4^+$ T cells in a model of allergic airway inflammation (Liu et al., 2015). In a model of infection with *N. brasiliensis*, depletion of $CD4^+$ T cells 7 days before infection impairs the ILC2-driven immune-mediated killing of the parasites. Depletion of $CD4^+$ T cells one day before infection did not influence the reduction of worm numbers, increased frequency of ILC2s and ILC2-

derived cytokine production. This indicates that $CD4^+$ T cells support long-term ILC2 maintenance and effector function, whereas their direct contribution to worm expulsion is minor (Bouchery et al., 2015).The ILC2-promoting effect of $CD4^+$ T cells is practically mediated by $CD4^+$ T cell-derived IL-2. IL-2 shows to be important for cytokine production (i.a. IL-9, IL-4 and IL-13) by ILC2s (Bouchery et al., 2015, Wilhelm et al., 2011). Furthermore, in ILC2/$CD4^+$ T-cell co-cultures presence of anti-IL-2 antibodies leaded to decreased numbers of $IL\text{-}13^+$ ILC2s. MHC II-dependent dialog of ILC2 and $CD4^+$ T cells was shown be necessary for IL-2 production as MHC II-blocking-antibodies abrogated IL-2 production by $CD4^+$ T cells (Oliphant et al., 2014) .

Taken together, ILC2s and $CD4^+$ T cells cooperate in different ways to drive Th2 immune response: besides contribution to type 2 cytokine responses ILC2 can improve Th2 immune responses by promoting DC migration and T-cell priming. Special focus in ILC2/T cell interaction comes to antigen-specific activation of $CD4^+$ T cells and co-stimulation, which drives not only T-cell-, but also ILC2 expansion and function and therefore shows mutual cross-talk of ILC2 and $CD4^+$ T cells to drive Th2 immune responses.

1.1.5 ILC2s in the Liver

Although ILC2s were first described in the liver in 2010 (Price et al., 2010), only little is known about ILC2s in particular from hepatic tissues and their exact function in liver inflammation. As the liver is an organ, which is constantly exposed to a variety of antigens, including dietary antigens by digested food, toxins and pathogens, it provides a variety of immunological functions including tolerance induction and immediate initiation of a sufficient immune response. For this purpose, a special role comes to innate immune cells, which accomplish both sides of this contrary roles (see also chapter 1.2) (Racanelli and Rehermann, 2006). In homeostatic conditions ILC2s are relatively underrepresented in the liver. Upon challenge with IL-33 or IL-25, hepatic ILC2s increase in number (Price et al., 2010, McHedlidze et al., 2013). In all studies addressing ILC2s as effector cells in hepatic inflammation, ILC2 function was closely related to IL-33 signaling. The alarmin IL-33 is released from stressed hepatocytes and necrotic cells (Schmitz et al., 2005) and has a critical role in different models of liver inflammation and hepatic tissue damage (Marvie et al., 2010, Arshad et al., 2012, Liang

et al., 2013). IL-33 levels positively correlated with levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels of patients with chronic hepatitis (Schmitz et al., 2005, Wang et al., 2012b). Elevated IL-33 levels were associated with increased numbers of ILC2s in the liver in murine modes of viral hepatitis (Liang et al., 2013), chemically induced liver fibrosis (McHedlidze et al., 2013) and immune-mediated hepatitis (Neumann et al., 2017).

However, ILC2s were also shown to have proliferative effects on damaged hepatic tissue. In a model of biliary atresia, in which liver damage is facilitated by rotavirus-type A, mice showed a greater liver damage and disruption of the epithelial lining of the hepatic bile ducts, when IL-33/ILC2-signaling was inhibited (Li et al., 2014). Li et al. showed further, that treatment with IL-33 induced ILC2-mediated cholangiocyte proliferation of the extrahepatic bile ducts *in vitro* and *in vivo*. As the proliferative effect of IL-33 on cholangiocytes was absent in ILC2-deficient recombination-activating gene (RAG) 2 $^{-/-}\gamma c^{-/-}$-and IL-13-deficient mice, but adoptive transfer of ILC2 or administration of IL-13, respectively, induced cholangiocyte proliferation, ILC2-derived IL-13 is likely to mediate the proliferative effect. In this way, ILC2s contribute to tissue repair of cholangiocytes upon damage. Moreover, ILC2s contribute to tumor development, as daily treatment with IL-33 for 10 weeks, induced murine extrahepatic bile ducts to increase in size and thickness and showed signs of glandular metaplasia. (Li et al., 2014). In a different model for cholangiocellular carcinoma, IL-33 was identified as a mediator in cancer development. As elevated IL-33 were associated with increased ILC2 numbers as well as amphiregulin and IL-13 levels, the IL-33-ILC2-circuit is hypothesized to contribute to biliary carcinogenesis (Nakagawa et al., 2016).

In immune-mediated hepatitis previous studies identified ILC2s to exert a pro-inflammatory role (Neumann et al., 2017). $CD4^+$ T cells drive concanavalin A (Con A)-triggered hepatitis (Fig. 3), which induced proliferation and substantial type 2 cytokine production by ILC2s. In this model, ILC2 levels correlated positively with hepatic expression and release of IL-33. Depletion of ILC2s ameliorated Con A-induced hepatitis whereas adoptive transfer of IL-33-elicited hepatic ILC2s reinforced liver inflammation, as well as hepatic expression of IL-5. These findings demonstrate that the IL-33/ILC2/Th2 cytokine-axis plays a crucial role in immune-mediated hepatitis.

ILC2-derived IL-5 drives activation and recruitment of eosinophils (Nussbaum et al., 2013, Roediger et al., 2013). Depletion of ILC2s impaired the recruitment of eosinophils in immune-mediated hepatitis, showing an immune-modulating effect of ILC2s (Neumann et al., 2017). Moreover, McHedilidze et al. identified ILC2s to contribute to liver fibrosis by promoting activation of hepatic stellate cells and collagen deposition, upon chronic liver damage. This pro-fibrotic effect was facilitated by ILC2-derived IL-13 signaling. IL-13-deficient mice showed a less severe phenotype of fibrosis. *In vitro*, ILC2-derived IL-13 stimulated hepatic stellate cells to proliferate and increase messenger ribonucleic acid (mRNA) expression of pro-fibrotic genes and also adoptive transfer experiments identified ILC2s to be critical effector cells to mediate liver fibrosis (McHedlidze et al., 2013).

Taken together, ILC2s have an important impact in the development of hepatic tissue damage, but also on subsequent repair mechanisms. At present state of knowledge, these effects are mediated by IL-33-induced production of type 2 cytokines. Nevertheless, their exact role and regulation in liver inflammation is not completely understood yet.

1.2 The Liver as an Immunological Organ

The liver is a solid organ located in the upper right quadrant of the abdomen, directly underneath the diaphragm. It is the largest gland in the human body and an essential metabolic organ. The liver can be macroscopically divided into 4 lobes: left lobe, right lobe, caudatus lobe and quadratus lobe. Functionally, the liver is divided into 8 segments according to the arterial supply areas. Special attention comes to the blood supply of the liver. Via the porta hepatis, three vessel called portal triad enter the liver: the Arteria hepatica propria representing the vasa private, which supplies the liver with oxygenated blood, the Vena porta, containing the nutrient-rich venous blood from the intestinal organs and the common bile duct, which drains the bile fluid (Abdel-Misih and Bloomston, 2010). Microscopically, the liver is made up of hexagonal hepatic lobules (Rappaport et al., 1954). The hepatic lobules include a plate of hepatocytes with a central vein, draining the blood into veins increasing in diameter, which finally coalesces into the Vena hepaticae and directly drain into the inferior Vena cava. In each corner of the hepatic lobules there is a portal triad composed of terminal vessels of the

elements of the porta hepatis: a branch of the hepatic artery, a branch of the portal vein and a branch of the bile duct (Burt et al., 2007). The plates of hepatocytes are traversed by sinusoids, enlarged capillaries with discontinuous liver sinusoidal endothelia cells (LSEC), with mixed blood from the artery and the portal veins that drains into the central vein. The presinusoidal space, also called space of Dissè, is located between the endothelia cells of the sinusoids and hepatocytes. Here, hepatocytes have contact to the plasma and discharge metabolites (Burt et al., 2007).

Due to its specific anatomy, the liver is a multifunctional organ with great metabolic, nutrition storage and detoxification function, but is also site of complex immunological activities (Robinson et al., 2016). Its functions vary through the development. The fetal liver is the location of hematopoiesis during gestation (Pahal et al., 2000). Postnatal hematopoiesis is relocated into the bone marrow (Fernández and de Alarcón, 2013). Further on, the liver plays an essential role in the metabolism of carbohydrates, fats and proteins, in the synthesis of various plasma proteins such as clotting factors and acute phase proteins, in the production of bile fluid, but also has storage functions and is essential in detoxification of the body (Burt et al., 2007). The liver is functionally connected with the intestine, as the venous blood from the intestine drains into the hepatic blood supply. Hereby, it is constantly exposed to dietary antigens and molecules from the microflora of the gut, which provide inflammatory potential (Mowat, 2003). Thus, besides hepatocytes accomplishing metabolic-, storage- and detoxifying-function, the liver is enriched with cells mediating immunological functions (Racanelli and Rehermann, 2006). The liver induces tolerance against harmless antigens and in this way prevents the organism from excessive immune reactions, but maintains immunosurveillance against pathogens and malignant cells (Robinson et al., 2016). On cellular levels two cell types can be differentiated the liver: parenchymal cells and non-parenchymal cells. Parenchymal cells, composed of hepatocytes, occupy about 60-80% of all hepatic cells (Racanelli and Rehermann, 2006). The biggest group of non-parenchymal cells are LSECs, which represent half of the non-parenchymal cells. They are involved in the immunological function of the liver, as they express molecules, that promote antigen uptake and antigen presentation, like MHC class I and II and co-stimulatory molecules (Lohse et al., 1996). They have enormous scavenger function (Smedsrød, 2004), act as a adherence platform for immune cells (Knolle and

Wohlleber, 2016), and respond sensitively to pathological molecules (Knolle et al., 1997). Moreover, LSECs can control T-cell activation and function (Tang et al., 2009) and support T_{reg} expansion (Wiegard et al., 2005). 25% of the non-parenchymal cells are lymphocytes, which can further be differentiated into T cells encompassing $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells (NKT cells), γδ T cells, as well as NK cells, and B cells (Racanelli and Rehermann, 2006). Another cell type with antigen-presenting capabilities in the liver are Kupffer cells (KC), which represent about 20% of the non-parenchymal cells (Racanelli and Rehermann, 2006). KCs are tissue-resident macrophages, which efficiently internalize endogenous and exogenous substances. They are a critical component of the mononuclear phagocytic system and are central in hepatic and systemic pathogen response, as these cells function as tolerogenic APCs, which promote tolerance induction (Thomson and Knolle, 2010). Nevertheless, they provide the ability to promote hepatic inflammatory responses by cross-presentation to $CD8^+$ T cells (Beattie et al., 2010) or NKT cells (Lee et al., 2010). They are typically located within the sinusoids of the periportal area (Dixon et al., 2013). Hepatic stellate cells, a small proportion (5%) of non-parenchymal cells, are also part of the intrahepatic mononuclear phagocytic system and are typically located in the space of Dissè (Racanelli and Rehermann, 2006). Within lipid droplets they store 50-80% of the bodies Vitamin A (Blomhoff and Blomhoff, 2006). Beside storage function, they have a key role in liver fibrosis, as they can produce extracellular matrix components and differentiate into myofibroblasts upon chronic liver injury (Weiskirchen and Tacke, 2014). Hepatic stellate cells have APC capability and can activate T cells and NKT cells *in vitro* and *in vivo* (Winau et al., 2007). Besides the non-parenchymal cells also hepatocytes itself are reported to contribute to the immunological function of the liver by providing APC function. Resting hepatocytes only express MHC class I (Senaldi et al., 1991), however, under inflammatory conditions hepatocytes additionally express MHC class II and in this way are able to activate $CD4^+$ T cells (Herkel et al., 2003, Tiegs and Lohse, 2010).

1.3 The Model of Concanavalin A-induced Hepatitis

The model of Con A-mediated hepatitis is an elaborated model of T cell-induced liver tissue damage in mice resembling human autoimmune hepatitis. Con A is a lectin isolated from the jack bean (*Canavalina brasilensis*) (Soares et al., 2011) with specific sugar binding sites, which bind to mannose residues of different glycoproteins expressed on the T-cell surface and therefore induces unspecific T-cell activation (Kanellopoulos et al., 1996). Upon injection, Con A accumulates specifically in the liver (Gantner et al., 1995) and induces a dose-dependent liver damage, displayed by increased levels of ALT and AST in the sera of Con A-challenged mice (Tiegs et al., 1992). In experiments with serve combined immunodeficiency (SCID) mice, which lack immunocompetent B cells and T cells, and also T-cell depletion experiments, Tiegs et al. further showed that $CD4^+$ but not $CD8^+$ T cells are essential for the development of Con A-induced hepatitis. Beside $CD4^+$ T cells, NKT cells play a critical role in the development of Con A-mediated hepatitis as CD1-deficient mice, which lack NKT cells, showed impaired development of Con A-induced hepatitis (Takeda et al., 2000). Moreover, Con A challenge after destruction of macrophages via silica particles resulted in significantly decreased serum ALT levels compared to controls, which identifies macrophages as an important effector cell population in this model of immune-mediated hepatitis (Tiegs et al., 1992).

Con A facilitates its T cell-mitogenic capabilities via binding to LSECs 15 minutes after intravenous injection (Knolle et al., 1996). This leads to disruption of the LSEC membrane, bleb formation and cytoplasm disappearance (Wang et al., 2012a, Tsui et al., 2007). Disruption and cell death of LSECs enables Con A to bind to macrophages 4 hours after injection (Knolle et al., 1996). $CD4^+$ T cells recognize Con A-modified macrophages via MHC class II-mediated interaction and become activated (Tsui et al., 2007, Wang et al., 2012a). Challenge with Con A, and thus activation of the different effector cells of Con A hepatitis, is linked to changes of the micromilieu of the liver via cytokine secretion (Fig. 3). T cell-derived IFNγ and KC-derived TNFα are the major cytokines in Con A-induced hepatitis and mediate necrotic cell death of hepatocytes and further recruitment of immune cells (Schümann et al., 2000, Kusters et al., 1996). Furthermore, T cell-derived IL-2 (Takahashi et al., 2011) and IL-4 (Miller et al., 2009)

are increased in the hepatic tissue upon Con A challenge. Likewise, IL-6 levels increase upon Con A-mediated activation of T cells (Miller et al., 2009) and KCs (Schümann et al., 2000). As anti-IL-12 antibodies prevent Con A-mediated hepatitis (Nicoletti et al., 2000) and anti-IL-18 antibody reduces ALT levels in the sera of Con A-challenged mice (Faggioni et al., 2000), macrophage-derived IL-12 and IL-18 are critical cytokines in the development of Con A-mediated hepatitis. Necrotic cell death of hepatocytes leads to the release of IL-33 in the liver and promotes hepatic inflammation by maintaining T-cell and NKT-cell activation (Chen et al., 2012). 24 hours after Con A injection, serum ALT and AST level decline and hepatic tissue starts to regenerate (Erhardt et al., 2007) by IL-6- and TNFα-driven proliferation of hepatocytes (Trautwein et al., 1998). After first challenge with Con A, mice develop a resistance against Con A-mediated liver damage when re-challenged with Con A 8 days to several weeks after the first administration. As tolerance induction was absent in IL-10-deficient mice, IL-10 signaling is crucial to mediate this effect. T_{regs} and KCs could be identified as main producers of IL-10 and the effector cells in tolerance induction (Erhardt et al., 2007). Hepatic injury can be prevented by immunosuppressive drugs such as dexamethasone, Cyclosporine A and FK 506 (Tiegs et al., 1992). In conclusion, the model of Con A-mediated hepatitis shows certain similarities to autoimmune hepatitis, such as the predominant role of $CD4^+$ T cells, the genetic disposition to autoimmune hepatitis resembled by the different susceptibly on Con A-mediated hepatitis of different mouse strains, the immunosuppressive state upon remission and the good responsiveness to immunosuppressive drugs (Tiegs et al., 1992). Although Con A-induced hepatitis is dominated by the Th1 cytokines IFNγ and TNFα ILC2s show to play an important role in this model of immune-mediated hepatitis. ILC2s increase in frequency upon Con A injection, most likely due to the release to IL-33, and have a proinflammatory function by expression of the type 2 cytokines IL-5 and IL-13, promoting recruitment of eosinophils. Depletion of ILC2s before challenge with Con A significantly ameliorated immune-mediated hepatitis (Neumann et al., 2017). However, the exact role of ILC2s in Con A-mediated hepatitis as well as their regulation during liver inflammation have not been revealed yet.

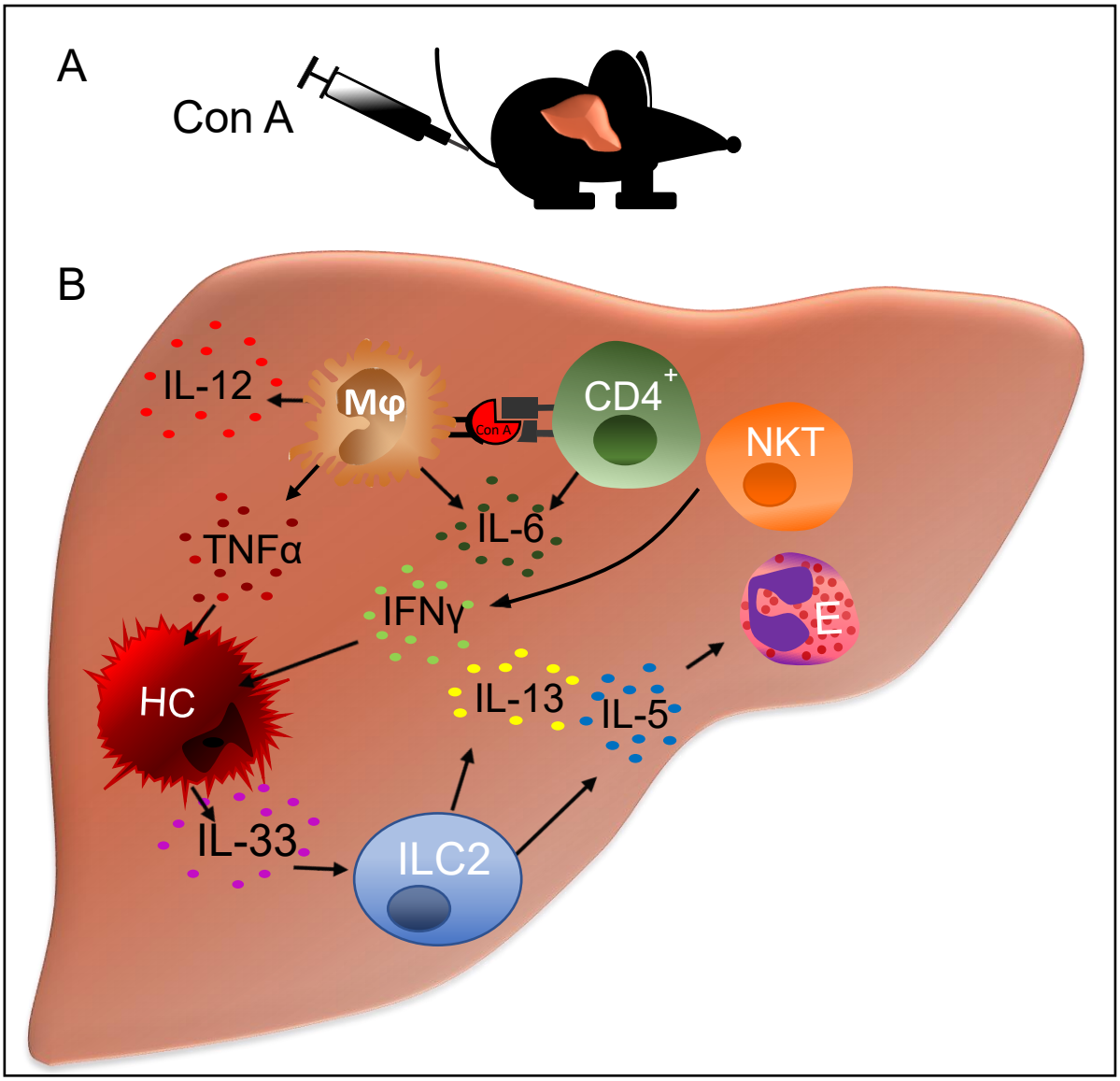

Figure 3: Model of Con A-mediated hepatitis.
(A)Injection of Con A into the tail vein leads to immune-mediated hepatitis. (B)Upon injection, Con A binds to macrophages and therefore activates $CD4^+$ T-cells. Activation of $CD4^+$ T cells, NKT cells and Kupffer cells leads to secretion of proinflammatory cytokines, cell infiltration and death of hepatocytes. IL-33 is released form necrotic hepatocytes, which further promotes hepatic inflammation by activation of $CD4^+$ T cells and NKT cells. ILC2s expand upon Con A-induced cell damage and subsequent release of IL-33. Hepatic ILC2s produce the type 2 cytokines IL-5 and IL-13, which promote activation and recruitment of inflammatory eosinophils. $CD4^+$, $CD4^+$ T cells; E, eosinophils; HC, hepatocytes; ILC2, type 2 innate lymphoid cells; IFNγ, interferon γ; IL, interleukin; Mϕ; Kupffer cells; NKT, natural killer T cells; TNFα, tumor necrosis factor α.

1.4 Aims of This Study

Hepatic ILC2s clearly facilitate a proinflammatory effect in Con A induced hepatitis, however the exact mechanisms are not completely understood. To gain insights into the role of ILC2s in the liver and upon liver inflammation, this study first addresses the functional differences of hepatic ILC2s in naive mice and their changes upon activation. Special focus is set to their expansion behavior, the expression of maturation markers as well as surface markers, which enable interaction and stimulation of other immune cells. In this context, hepatic ILC2s from naive C57BL/6 mice were isolated and analyzed by flow cytometry. As IL-33 is released upon liver damage and a potent activator of ILC2s, treatment with IL-33 resembles ILC2 activation upon liver inflammation *in vivo*. Mice were challenged with recombinant IL-33 via intraperitoneal injection for 4 days. Subsequently, hepatic ILC2s were isolated and characterized in the same way.

Given that ILC2 maintenance and function is highly influenced by cytokines, the second part of this study addresses the influence of cytokines, which dominate the hepatic microenvironment in Con A-induced hepatitis, on ILC2s *in vitro*. Therefore, hepatic ILC2s were isolated by fluorescence-activated cell sorting and cultured with liver inflammation-induced cytokines. The ILC2-derived cytokine production was analyzed in the supernatants and on cellular level. Hereby, the effect of liver microenvironmental cytokines on the phenotype of ILC2 are evaluated and ILC2-derived cytokines, which could reciprocally influence the hepatic microenvironment upon activation, are determined.

Since $CD4^+$ T-cell activation plays a crucial role in the development of Con A-mediated hepatitis this study further investigates the capability of hepatic ILC2s and $CD4^+$ T cells to crosstalk in presence of a certain antigen. The antigen-specific interaction of these cells was investigated by co-culture of hepatic ILC2s from C57BL/6 mice with OVA-specific $CD4^+$ T cells from OT II mice, which get activated upon presentation of OVA via MHC class II. To evaluate if ILC2-mediated antigen presentation induces $CD4^+$ T-cell activation, changes of phenotype, cell number and cytokine secretion of both cell types were evaluated in absence and presence of OVA and compared to an antigen-unspecific activation of T cells. Taken together, this study gives insights into the

phenotypically characteristics of hepatic ILC2s, their regulation by liver inflammation-induced cytokines as well as their ability of antigen-specific interaction with $CD4^+$ T cells. In this way, a better understanding of ILC2 biology, regulation and effector function will help to further reveal the exact role of ILC2s in liver inflammation.

2 Material and Methods

2.1 Materials

2.1.1 Technical Equipment

Table 1: Technical equipment.

TECHNICAL EQUIPMENT	SUPPLIER
Airflow-control EN 14175 cp	Airflow Lufttechnik GmbH, Rheinbach, Germany
ATILON ATL-423-I	Acculab Sartorius group, Göttingen, Germany
CANTO II	BD Bioscience, Franklin Lakes, USA
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Eppendorf Research Plus Pipettes	Eppendorf, Hamburg, Germany
FACS AriaFusion cell sorter	BD Bioscience, Franklin Lakes, USA
FACS AriaIIIu cell sorter	BD Bioscience, Franklin Lakes, USA
Flow cytometer tubes	Sarstedt, Nümbrecht, Germany
HandyStep® electronic	BRAND GmbH, Wertheim, Germany
Hera Cell 240 Incubator	Thermo Fisher Scientific,Waltham, USA
HERA Safe Clean Bench	Heraeus Instruments, Hanau, Germany
Infinite M200 Photometer	Tecan, Crailsheim, Germany
Innova CO-48 Incubator	New Brunswick Scientific, Nürtingen, Germany
LSRFortessa cell analyzer	BD Bioscience, Franklin Lakes, USA
MSC Advantag, Clean Bench	Thermo Fisher Scientific, Waltham, USA
MTS 2/4 digital, Rotating Shaker	IKA, Staufen, Germany
MyCycler thermal cycler	BioRad, München, Germany
MyCyclerTM Thermal Cycler	Bio-Rad, Hercules, USA
NanoDrop photometer ND-1000	PEQLAB Biotechnologie GmbH, Erlangen, Germany

TECHNICAL EQUIPMENT	SUPPLIER
Neubauer Improved Chamber	Roth, Karlsruhe, Germany
Pipetboy	Integra Bioscience, Huston, USA
RH basic 2 electric strirrer	IKA, Staufen, Germany
Schlauchpumpe TL/150	Medorex, Nörten-Hardenberg, Germany
Sevengo pH meter	Mettler Toledo AG, Schwerzenbach, Switzeland
Vortexer	Heidolph, Schwabach, Germany
VWR Mixer Mini Vortex 230V EU	VWR, Darmstadt, Germany

2.1.2 Reagents and Kits

Table 2: Reagents and kits.

REAGENTS AND KITS	SUPPLIER
2-Mecaptoethanol	GIBCO, Invitrogen, Darmstadt, Germany
Absolute qPCR SYBR Green Mixes	Thermo Fisher Scientific, Waltham, USA
Anti-allophycocyanin MicroBeads	Miltenyi Biotec,Bergisch Gladbach, Germany
Anti-CD3/CD28 Dynabeads	GIBCO, Invitrogen, Darmstadt, Germany
Anti-mouse linage Antibody-cocktail	BD Bioscience, San Jose, USA
Bovine serum albumin (BSA), protease free	PAA Laboratories, Pasching, A
Brefeldin A (BFA)	Sigma-Aldrich, München, Germany
$CD4^+$ T cell isolation Kit	Miltenyi Biotec,Bergisch Gladbach,Germany
Concanavalin A	Sigma-Aldrich, München, Germany
Dulbecco's Phosphate Buffered Saline (DPBS) (1x)	GIBCO, Invitrogen, Darmstadt, Germany
Heparin – Na (10000 I.E./ml)	Braun Melsungen AG, Melsungen, Germany
Ionomycin	Sigma-Aldrich, München, Germany

REAGENTS AND KITS	SUPPLIER
LEDGENDPlex ™ Mouse Th cytokine panel (13-plex)	BioLegend, San Diego, USA
NucleoSpin RNA Kit	Machery-Nagel, Duren, Germany
Ovalbumin peptide (OVA 323-339)	InvivoGen, San Diego, USA
Penicillin (10000 U/mL)/ streptomycin (10000 µg/mL)	GIBCO, Invitrogen, Darmstadt, Germany
Percoll	GE Healthcare, Uppsala, Sweden
Phorbol-12-myristate 13-acetate (PMA)	Sigma-Aldrich, München, Germany
recombinant IL-2	R&D Systems, Minneapolis, USA
recombinant IL-33	BioLegend, San Diego, USA
recombinant IL-7	Bio Legend, San Diego, USA
recombinant IFNγ	R&D Systems, Minneapolis, USA
recombinant IL-12	R&D Systems, Minneapolis, USA
RPMI-1640	GIBCO, Invitrogen, Darmstadt, Germany
Saponin	Sigma-Aldrich, München, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium pyruvate	GIBCO, Invitrogen, Darmstadt, Germany
Streptomycin	GIBCO, Invitrogen, Darmstadt, Germany
Tris/HCl	Carl Roth GmbH und Co. KG, Kahlsruhe, Germany
Verso cDNA Synthesis Kit	Life Technologies, Carlsbad, USA

2.1.3 Buffers and Solutions

Bi-distilled water was used for all buffers and solutions.

Table 3: Buffers and solutions.

SOLUTION	COMPOSITION
Ammoniochloride	19 mM Tris/HCl 140 mM NH_4Cl pH 7.2
FACS buffer	PBS (5x) 1% BSA 15.4 mM NaN_3 (0.1%) pH 7.1
Hank's balanced salt solution (HBSS)	5.4 mM KCl 0.3 mM Na_2HPO_4 x 7 H_2O 4.2 mM $NaHCO_3$ 1.3 mM $CaCl_2$ 0.5 mM $MgCl_2$ x 6 H_2O 0.6 mM $MgSO_4$ x 7 H_2O 137 mM NaCl 5.6 mM D-Glucose pH 7.4
MACS buffer	PBS (5x) 0.5% BSA 2 mM EDTA
PBS	137.9 mM NaCl 6.5 mM Na_2HPO_4 x 2 H_2O 1.5 mM KH_2PO_4 2.7 mM KCl pH 7.4

2.1.4 Antibodies

Table 4: Blocking antibodies.

NAME	ISOTYPE	CLONE	DILUTION	SUPPLIER
Anti-IL-4	rat	11B11	1:500	BioLegend, San Diego, USA
Fc-block (Anti-CD16/CD32)	rat	93	1:100	BioLegend, San Diego, USA

Table 5: Fluorescence-labeled antibodies for flowcytometry.

NAME	ISOTYPE	CLONE	CONJATED FLUORESCENCE	DILUTION	SUPPLIER
CD25	rat	PC61	PE/Cy7	1:300	BioLegend, San Diego, USA
CD4	rat	YTS	FITC	1:200	Immunotools, Friesoythe, Germany
eFlour 506 (Viability stain)			AmCyan	1:2000	eBioscience, San Diego, USA
ICOS (CD278)	rat	7E.17G9	FITC	1:200	eBioscience, San Diego, USA
ICOS-L (CD275)	rat	HK5.3	PE	1:200	BioLegend, San Diego, USA
IL-10	rat	JES5-16E3	apc	1:150	BioLegend, San Diego, USA
IL-10	rat	JES5-16E3	apc/Cy7	1:100	BioLegend, San Diego, USA
IL-13	rat	eBio13A	Alexa Fluor 488	1:100	eBioscience, San Diego, USA
IL-17A	rat	TC11-18H10	V 450	1:100	BD Pharmingen, San Jose, USA
IL-17A	rat	TC11-18H10.1	Alexa Fluor 700	1:100	BioLegend, San Diego, USA
IL-2	rat	JES6-5H4	Brilliant Violet 605	1:100	BioLegend, San Diego, USA
IL-4	rat	BVD4-1D11	PE	1:200	BD Bioscience, San Jose, USA

NAME	ISOTYPE	CLONE	CONJATED FLUORESCENCE	DILUTION	SUPPLIER
KLRG1/MAFA	Syrian hamster	2F1/KLRG1	Brilliant Violet 605	1:200	BioLegend, San Diego, USA
Lineage Ab Cocktail		145-2C11	apc	1:400	BD Bioscience, San Jose, USA
Ly-6A/E (Sca-1)	rat	D7	Pacific Blue	1:100	BioLegend, San Diego, USA
MHC II (I-A/I-E)	rat	M5/114.15.2	apc/Cy 7	1:200	BioLegend, San Diego, USA
OX-40 (CD134)	rat	OX-86	PE	1:200	BioLegend, San Diego, USA
OX-40-L (CD252)	rat	RM134L	BB515	1:200	BD Bioscience, San Jose, USA
ST2 (IL-33R)	rat	RMST2-2	PerCP-eFliuor 710	1:100	eBioscience, San Diego, USA
TCR (beta-chain)	Armenian hamster	H57-597	PE/Cy7	1:200	BioLegend, San Diego, USA
Zombie NIR (Viability Stain)			apc/Cy7	1:1000	BioLegend, San Diego, USA

2.1.5 Software

Table 6: Software.

SOFTWARE	SUPPLIER
FACSDiva Software	BD Bioscience, Franklin Lakes, USA
FlowJo	FlowJo LLC, Ashland, USA
GraphPad Prism V.5.02	GraphPad Software Inc., San Diego, USA
LEGENDplex Data Analysis Software	BioLegend, San Diego, USA
Microsoft Office 2016	Microsoft GmbH, Washington, USA

2.1.6 Consumables

Table 7: Consumables.

CONSUMABLES	SUPPLIER
Centrifuge tubes (15 ml and 50 ml)	Greiner BioOne, Frickenhausen, Germany
Falcon Polystyrene 5 ml FACS tubes	BD Bioscience, Franklin Lakes, USA
MicroWell™ 96-Well Optical-Bottom Plates	Nunc A/S, Roskilde, Denmark
Nylon meshes (100 µm)	BD Falcon, Heidelberg, Germany
Parafilm M	Brand, Wertheim, Germany
Pipette tips (10 µl, 100 µl and 1000 µl)	Eppendorf, Hamburg, Germany
Pipette tips with and without filter (10 µl, 100 µl and 1000 µl)	Sarstedt, Nümbrecht, Germany
Plastic pipettes (2 ml, 5 ml, 10 ml and 25 ml)	BD Bioscience, Franklin Lakes, USA
Reaction tubes (0.5 ml, 1.5 ml and 2 ml)	Eppendorf, Hamburg, Germany
Syringes	Braun Melsungen AG, Melsungen, Germany

2.2 Methods

2.2.1 Mice

2.2.1.1 Mice Strains

C57BL/6 and OT II mice were bred in the animal facilities of the University Medical Center Hamburg-Eppendorf. Male mice at the age of 6-12 weeks were used for all experiments. Experiments were approved by the institutional review board, Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz (Hamburg, Germany; approval code G44/15).

2.2.1.2 Housing

All mice mice received human care according to the guidelines of the National Institute of Health as well as to the legal requirements in Germany. They were held in individually

ventilated cages under controlled conditions of 20°C ±2°C, 50% ±5% humidity and 12-hour day/night rhythm. The mice were provided with autoclaved standard laboratory chow and water *ad libitum*.

2.2.1.3 Treatment

To induce immune-mediated hepatitis, C57BL/6- mice were injected with Con A (7 mg/kg) in the tail vein. After 8 h and 24 h, animals were sacrificed and the livers were harvested.

For *in vivo* expansion of hepatic ILC2s, C57BL/6- mice received recombinant murine IL-33 (0.3 µg/mouse) on 4 consecutive days via intraperitoneal (i.p.) injection. On day 5, mice were sacrificed and the livers were harvested.

2.2.1.4 Killing of Animals and Organ Removal

Mice were anesthetized by exposition to mixture of CO_2/O_2. Level of anesthesia was checked by loss of pedal withdrawal reflex of the hind limb, subsequently mice were killed by cervical dislocation. After opening the abdominal cavity, the gallbladder was extirpated. Livers of C57BL/6 mice were removed and transferred to Hank's balanced salt solution (HBSS) on room temperature. Spleens and mesenteric lymph nodes (LN) of OT II mice were excised and transferred to ice-cooled HBSS and transported on ice.

2.2.2 Isolation of Cell Populations

2.2.2.1 Mechanisms of Magnetic-activated Cell Sorting (MACS-Sort)

In magnetic-activated cell sorting, magnetic microbead-labeled cells are separated from a cell fraction by a magnet. The cell can either be labeled directly by direct binding of magnetic microbeads to an antigen on the cell surface or indirectly by labeling the cells with a primary antibody, which binds the surface antigen, and subsequently adding magnetic microbeads binding at the primary antibody. Magnetically labeled cells are passed through a magnetic column, which retains the labeled cells. The pass trough can be collected as unlabeled cell fraction. By removing the column from the magnet and washing of the columns, labeled cells can be removed from the column. In this way either a specific cell type can be isolated from a cell suspension (positive selection) or a sample can be cleared from certain cell types (negative selection).

2.2.2.2 Magnetic-activated Cell Sorting of CD4⁺ T cells from Spleens and LN of OTII Mice

Spleens and LN were passed through a 100 µm cell strainer, washed with 25 ml HBSS (4°C) and centrifugated for 5 minutes at 500 g (4°C). Erythrocyte removal was performed by resuspending the cell pallet with 10 ml ammonium chloride (140 mM) for 8 minutes. Erythrocyte lysis was stopped by washing with cold HBSS and centrifugation for 5 minutes at 500 g (4°C). To minimize unspecific binding of antibodies to the fragment crystallizable (Fc) region of an antibody, the cell suspension was incubated anti-mouse CD16/32 antibodies for 15 minutes at 4°C, washed with MACS-buffer and centrifugated at 500 g for 5 minutes. Subsequently. CD4⁺ T cells were isolated via MACS-Sort using CD4⁺ T Cell Isolation Kit according to the manufacturer´s instruction. Non-CD4⁺ T cells were depleted using a cocktail of biotin-conjugated antibodies against CD8a, CD11b, CD11c, CD19, CD45R, CD49b, CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ. Subsequently the primary antibodies were labeled with magnetic anti-biotin microbeads. Passing the cells suspension through a magnetic column retrained non-CD4⁺ T cells. Purity of CD4⁺ T cells was repeatedly checked by flow cytometry (Fig. 4).

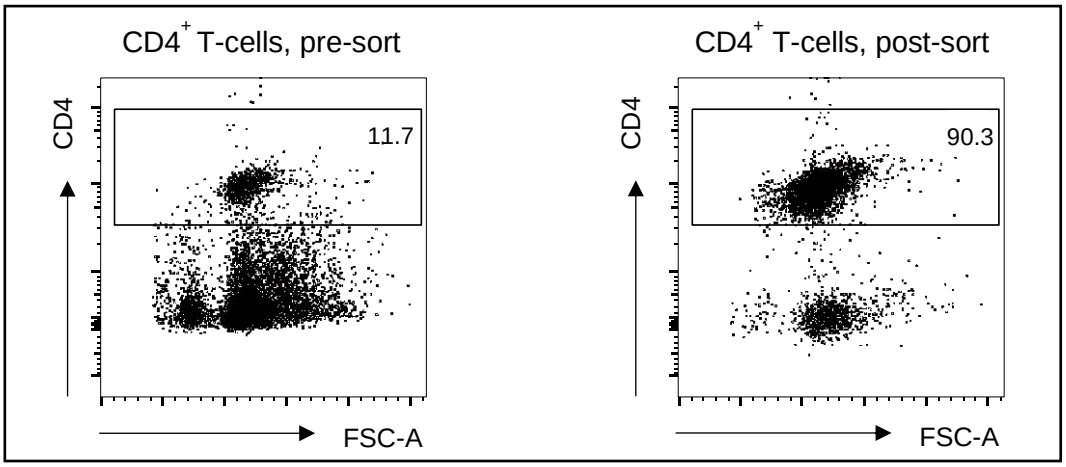

Figure 4: Purity of CD4+ T cells before and after MACS-Sort.
Lymphocytes from spleen and LN of OT II-mice were cleared from non-CD4⁺ T-cells by MACS- Sort. Frequency of CD4⁺ T cells was determined by flowcytometry. Cells were gated according to Figure 7 A-C.

2.2.2.3 Fluorescence-activated Cell Sorting (FACS-Sort)

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry (see below Chapter 2.2.4.1) in which a cell fraction can be separated from a cell suspension by their fluorescent characteristic. Cells are labeled with fluorochrome-linked antibodies, which binds to a specific surface antigen. A vibrating system separates the cell suspension into small droplets and enables analyzing each cell separately regarding their fluorescent characteristic. Thus, fluorochrome-labeled cells can be separated by electrostatic defection into a different container.

2.2.2.4 Fluorescence-activated Cell Sorting of ILC2s from Murine Livers

A single cell suspension was prepared from the harvested livers. Livers were mechanically disrupted by grinding them over narrow gaped petri dishes and passing through a 100 µm cell strainer. Subsequently, the cell suspension was washed with 25 ml HBSS (room temperature) and centrifugated at 500 g for 5 minutes. A density gradient centrifugation was performed after re-suspending the cells in 10 ml isotonic 36% Percoll/HBSS solution containing 100 U/L heparin solution to clear the cell suspension from hepatocytes. Percoll is a colloid solution containing silica particles, which fractionates the liver cell suspension according to the density of the different components. In this way hematogenous cells as leukocytes and erythrocytes can be separated from parenchymal cells. After centrifugation of the liver cell suspension in Percoll/HBSS for 20 minutes at 800 g hepatocytes and cell detritus, which were located on the top of the centrifuge supernatant, were removed. Subsequently, erythrocytes were lysed by resuspending the cell pallet with ammonium chloride (140 mM) for 8 minutes. Erythrocyte lysis was stopped by washing the cells with HBSS and centrifugation at 500 g for 5 minutes. The cell pallets were resuspended with MACS-buffer and pooled.

Single cell suspension was enriched for linage-negative (lin^-) cells by MACS-Sort using allophycocyanin (apc)–conjugated anti-mouse Linage Antibody Cocktail and anti-apc magnetic microbeads according to the manufacturer's instructions. To exclude the cell suspension form linage-positive (lin^+) cells, such as T and B lymphocytes, macrophages, NK cells, erythrocytes and granulocytes, cells were incubated with apc -conjugated antibody cocktail containing antibodies against surface proteins such as

CD3, CD11b, CD45-receptor, lymphocyte antigen (Ly-)76, Ly-6G, Ly-6C and mouse erythroid cells. After washing the cell suspension magnetic anti-apc microbeads were added to bind to the primary antibodies and to retain lin^+ cells from the cell suspension when being passed through a magnetic column. Subsequently, lin^- cells were stained for the ILC2-characteristic surface markers ST2 and Sca-1 for 20 minutes (4 °C), washed with phosphate buffered saline (PBS) and centrifugated at 500 g for 5 minutes. After resuspension in FACS-buffer lin^- $Sca\text{-}1^+$ $ST2^+$ ILC2s were sorted by FACS (BD Bioscience Aria Illu and Aria Fusion). ILC2 isolation via MACS-sort of lin^- cells and subsequent FACS-sorting reached purities of about 99% and were regularly checked by flow cytometry analysis (BD Bioscience CANTO II/ BD Bioscience FORTESSA) (Fig. 5).

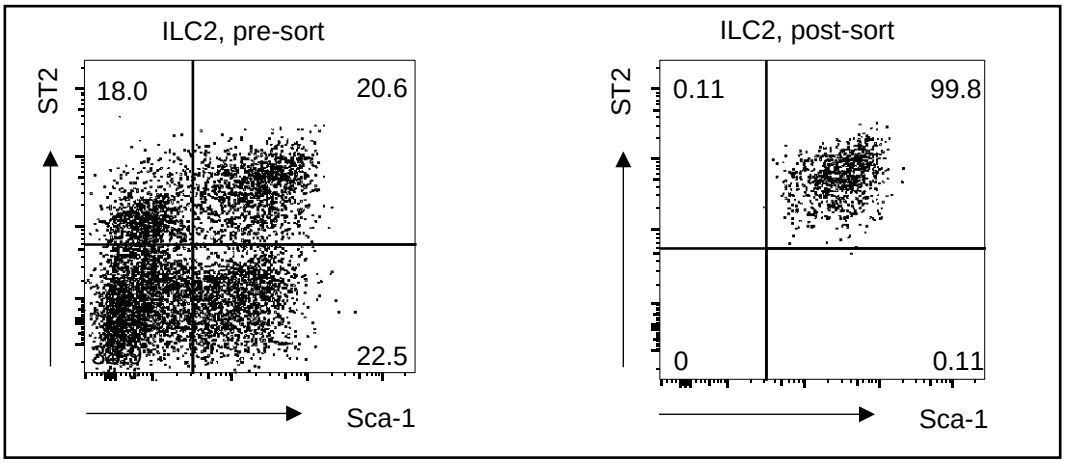

Figure 5: Purity of hepatic ILC2s before and after FACS-Sort.
Linage- cells were stained for their expression of ST2 and Sca-1 and sorted via FACS. Frequency was determined by flow cytometry. Cells were gated according to Figure 6 A-D.

2.2.3 *In vitro* Cell Culture

2.2.3.1 Mono-Culture

To investigate the phenotype of hepatic ILC2s and their modulation by liver-inflammation-derived cytokines, ILC2s were isolated from livers of IL-33-treated mice by MACS and FACS. Thereafter, $1x10^4$ ILC2s were cultivated in RPMI-1640 medium (supplemented with 1 mM sodium pyruvate, 0,05 mM 2-Mecaptoethanol, 100 U/ml Penicillin,100 µg/ml Streptomycin and 10 % fetal bovine serum) under supportive

cytokine milieu (IL-2 and IL-7) and in absence or presence of IL-12 and anti-IL-4 antibodies and/or IL-33 and/or IFNγ (according to Table 8) for 4 days.

Table 8: Systematic of ILC2 monocultures.

Cell culture	Supplements
1x10^4 HEPATIC ILC2s	IL-2 (10 U/ml) + IL-7 (10 U/ml)
	IL-2 (10 U/ml) + IL-7 (10 U/ml) + IL-33 (10 ng/ml)
	IL-2 (10 U/ml) + IL-7 (10 U/ml) + IL-12 (10 ng/ml) + anti- IL-4 (10µg/ml)
	IL-2 (10 U/ml) + IL-7 (10 U/ml) + IL-12 (10 ng/ml) + anti- IL-4 (10µg/ml) + IL-33 (10 ng/ml)
	IL-2 (10 U/ml) + IL-7 (10 U/ml) + IFNγ (10 ng/ml)
	IL-2 (10 U/ml) + IL-7 (10 U/ml) + IFNγ (10 ng/ml) + IL-33(10 ng/ml)

2.2.3.2 Co-Culture

To study the antigen-specific crosstalk of hepatic ILC2s and CD4$^+$ T cells, FACS-sorted hepatic ILC2s from IL-33-treated mice (2x10^4) and MACS-sorted, OVA-specific CD4$^+$ T cells from spleen and LN of OTII mice (1 x10^5) were cultivated in absence or presence of OVA peptide (2mg/ml; sequence: H-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-OH) for 4 days. To compare this antigen-specific interaction with an antigen-unspecific immune response, some ILC2/CD4$^+$ T cell co-cultures were stimulated by adding anti-CD3/CD28 Dynabeads in a 1:1 ratio of beads and CD4$^+$ T cells. The cell cultures were checked daily and RPMI-medium was stocked up to a total volume of 150 µl per well.

2.2.4 Phenotype Analysis

After culture, cells were harvested, counted and re-stimulated with phorbol-12-myristat-13-acetat (PMA) (20 ng/ml) and ionomycin (1 µg/ml) for 6 hours. PMA and ionomycin are substances known to synergize and stimulate cytokine response of T cells (Chatila et al., 1989, Hashimoto et al., 1991). After 1 hour, Brefeldin A (1µg/ml) and monensin (2 µM) were added to inhibit exocytosis and membrane transport in the Golgi membrane (Helms and Rotmann, 1992, Mollenhauer et al., 1990). Subsequently,

supernatants were removed and stored in the freezer at -20°C for later analysis via LEGENDPlex. Total cell number was counted before re-stimulation with PMA/ionomycin. Expansion of ILC2s was calculated according to the percentage of $TCR\beta^-$ cells after co-culture and the ILC2 input. Expansion of $CD4^+$ T cells were calculated simultaneously according to the percentage of $TCR\beta^+$ cells and the $CD4^+$ T-cell input. Phenotype of ILC2s and $CD4^+$ T cells was analyzed by flow cytometry.

2.2.4.1 Flow Cytometry

Protein Expression Analysis by Flow Cytometry

Flow cytometry enables multiparametric analysis of a single cell by detection of light scatter, intercept and emission. Lasers are used to determine cell number, cell complexion and fluorescent characteristics by passing the cells in a fluidic stream through a laser-based detection device. Cells are singularized from a sample by a fluidic system and pass the laser system. Thus, each cell can be analyzed separately.

Scattering of light occurs when a particle or cells deflect incident laser light while passing the laser. The extent of scattering depends on the physical properties of the cell such as size and internal complexity. The forward scattering light (FSC) is detected in the axis of the incident light by a photodiode. The extent of light diffraction is proportional to the size of the cell. Refraction of light is proportional to the internal complexity of a cell. This is measured by the sideward scattered light (SSC), which is collected in a 90° angle to the incident laser light.

A fluorescent compound absorbs light of a certain wavelength regarding its respective absorption spectrum. The energy of the light is used to raise the fluorescent compound to a higher energy level. The declination of the fluorescent compound to its ground state emits the energy as a photon. This transition of energy is called fluorescence and can be detected by photodetectors. When a particle enters the laser beam scattering light or fluorescence this light signal is converted into a voltage pulse and thereby gets digitalized. Each signal is displayed as a dot in a position on the data plot dependent on their respective intensity. Different lasers can be used to examine different fluorescent compounds at the same time. In this way, dyes with monoclonal fluorochrome-linked antibodies can be used for multiparametric cell analyzes and determine certain cell characteristics simultaneously to size and granularity. All

phenotype analyses were performed with BD LRSFortessa using a blue, red, violet and ultraviolet laser to detect up to 12 colors simultaneously.

Detection of Surface Proteins

To detect proteins expressed on the surface, cells were washed with PBS, centrifugated for 5 minutes at 300 g (4°C) and subsequently incubated with anti-CD16/CD32 antibodies for 10 minutes on 4 °C. Again, cells were washed with PBS and then stained for 20 minutes (4°C) with the antibody-cocktail for detection of specific surface proteins and the fixable viability dye Zombie NIR. Zombie NIR is not permeant to living cells, but get incorporated only by cells with compromised membranes. For direct FACS analysis cells were washed with PBS, centrifugated for 5 minutes at 300 g and transferred in FACS-buffer.

Detection of Intracellular Proteins

For detection of expressed intracellular proteins cells were stained for surface proteins and viability as described above and subsequently fixated with 2% paraformaldehyde solution for 10 minutes, washed with PBS and centrifugated for 5 minutes at 300 g (4°C). Cells were stored in PBS overnight at 4°C. Thereafter, cells were permeabilized by washing with saponin-buffer (0.5% solution in PBS) and centrifugation for 5 minutes at 300 g (4°C) twice. Subsequently intracellular staining with antibody-cocktail diluted in saponin was performed for 35 minutes (4°C). After washing the cells with saponin buffer and centrifugation for 5 minutes at 300 g (4°C) twice, cells were transferred into FACS-buffer and analyzed by flow cytometry.

Gating Strategies

Cells of medium size (FSC) and granularity (SSC) were gated as leukocytes (Fig. 6 A). To exclude unwanted confounding signals emanated by dead cells or debris, these cells were excluded by exclusively visualizing the cells of interest. Out of the population of leukocytes duplets were excluded on the basis of the FSC area and height (Fig.6 B). Dead cells were excluded from the remaining leukocytes by gating on cells, which were not marked by Zombie NIR fluorescence (Fig. 6 C). To determine ILC2s from hepatic leukocytes for phenotypical analysis or FACS-Sort, cells expressing a linage-specific marker were excluded (Fig. 6 D). Within linage negative cells, ILC2s were determined as ST2 and Sca-1 co-expressing cells (Fig. 6 E).

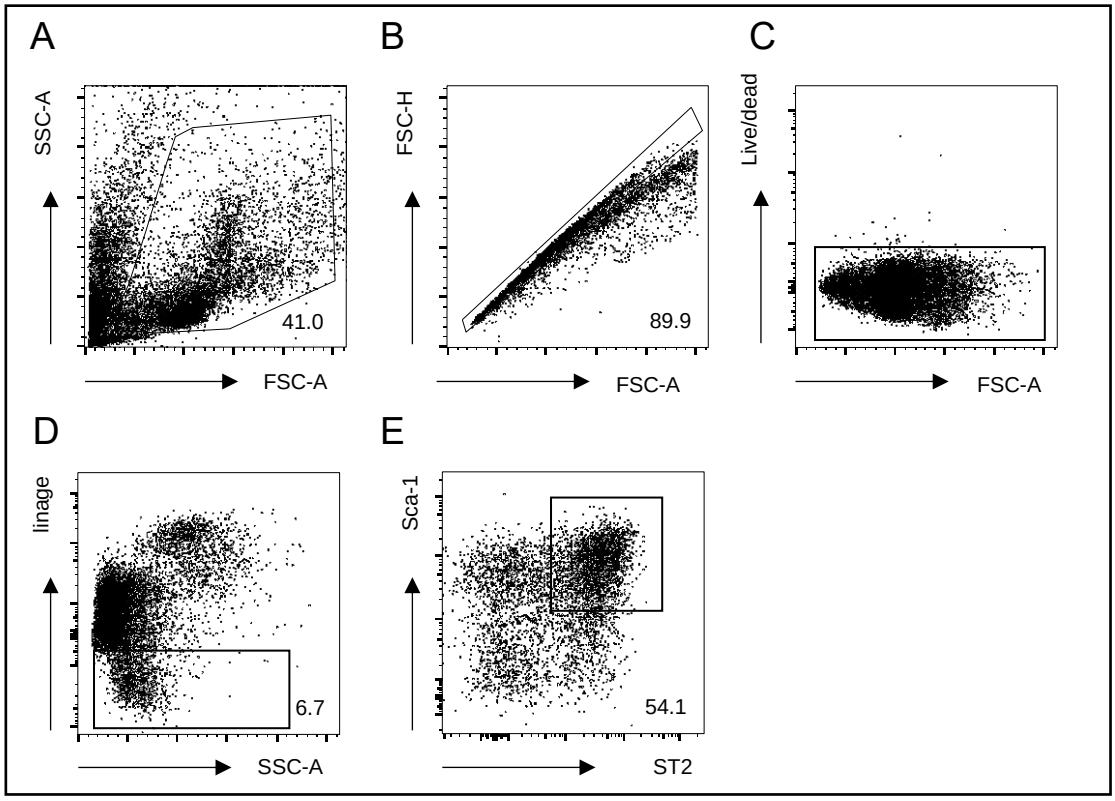

Figure 6: Gating strategy of ILC2s from hepatic leukocytes.
Hepatic leukocytes were analyzed for their physical characteristics as size and granularity (A) and FSC area and height (B). They were stained for viability (C) and the expression of a linage-specific markers (D). Hepatic leukocytes were stained for the expression of ST2 and Sca-1 (E). Frequency of hepatic ILC2 was determined by flow cytometry.

To distinguish ILC2s and $CD4^+$ T cells upon co-culture cells were gated similar to Fig. 6. Cells of medium size (FSC) and granularity (SSC) were gated as leukocytes (Fig. 7 A). To exclude unwanted confounding signals emanated by dead cells or debris, these cells were excluded by exclusively visualizing the cells of interest. Out of the population of leukocytes duplets were excluded on the basis of the FSC area and height (Fig. 7 B). Dead cells were excluded from the remaining leukocytes by gating on cells, which were not marked by Zombie NIR fluorescence (Fig. 7 C). The expression of TCRβ was used to distinguish $CD4^+$ T cells form ILC2s. $TCR\beta^+$ cells were determined as $CD4^+$ T cells, $TCR\beta^-$ cells were determined as ILC2s.

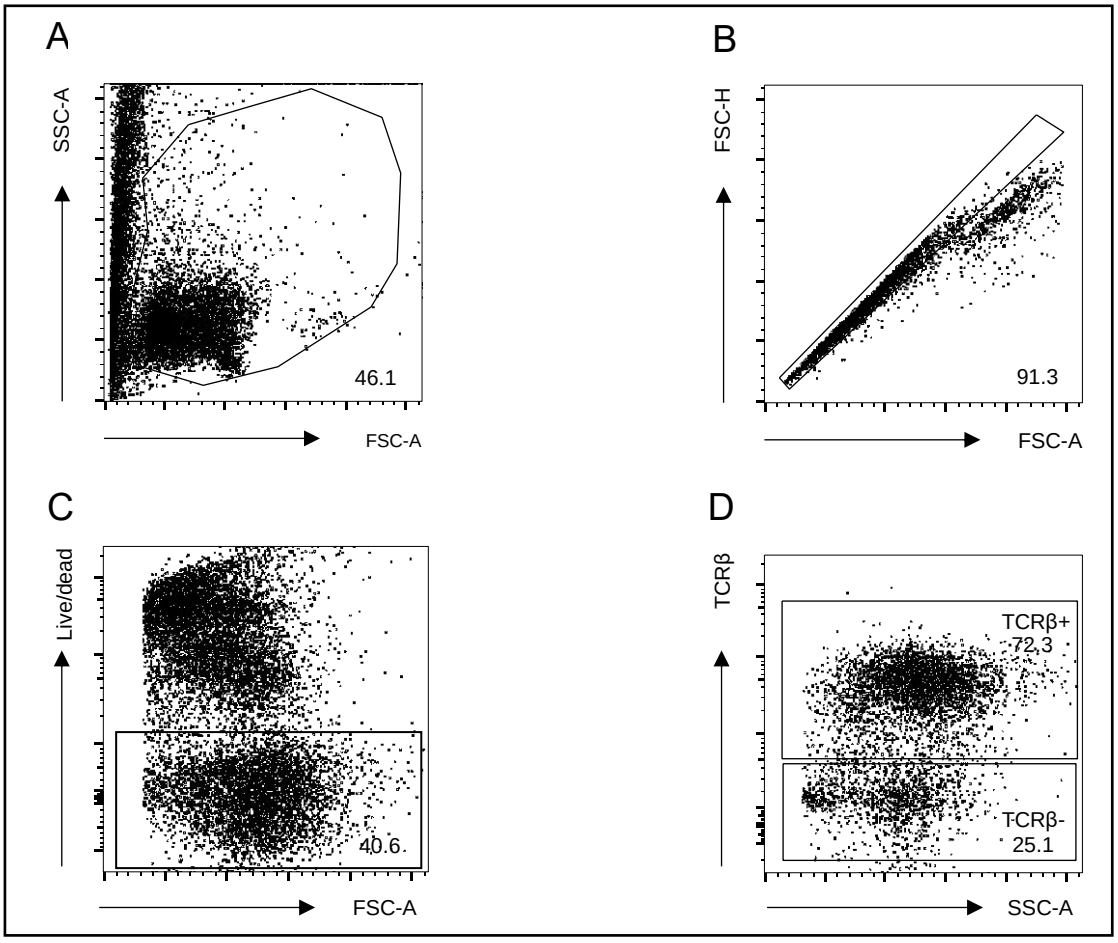

Figure 7: Gating-strategy of ILC2s and CD4⁺ T cells after culture.
After culture cells were analyzed for their physical characteristics as size and granularity (A) and FSC area and height (B). They were stained for viability (C) and the expression of TCRβ (D). Frequency of the cells was determined by flow cytometry.

2.2.4.2 Protein Analysis via LEGENGDPlex

This bead-based immunoassay uses the principle of sandwich-ELISA. According to every analyzed cytokine the beads have a specific antibody on their surface and serve as capture-beads. The beads have two sizes and every bead type differs in the expression of apc, which allows to distinguish between the different beads referring to different cytokines. Biotinylated detection antibodies bind to the specific analyte, which is bound by their respective capture antibodies. Streptavidin-phycoerythrin (SA-PE) is added, which binds to the biotinylated detection antibodies. By using flow cytometry, every cytokine can be distinguished by size of the bound capture beads as well as their expression intensity of apc. PE signal fluorescence intensity is used to quantify the level of cytokines in the sample compared to a standard containing the cytokines in a defined

concentration. The concentration of the cytokine is calculated by the LEGENDPlex software according to the standard curve.

2.2.4.3 Cell Proliferation Assay.

To verify the proliferation of $CD4^+$ T cells, T cells were labeled with the fluorescent dye eFluor670. Therefore, 5 x 10^6 T cells were incubated with 500 µl of 5 µM eFluor670 for 10 minutes in a water bath of 37°C under constant pivoting of the tube. Labeling with eFlour670 was stopped by adding RPMI and storing the incubated cells on ice for 5 minutes. After washing the labeled T cells with RPMI medium three times, cells were counted and co-cultured with ILC2s as described in chapter 2.2.3.2. The intensity of eFluor670 was determined by flowcytometry after co-culture. As eFluor670 binds to cellular proteins containing primary amines and is equally distributed to the daughter cells in every cell division, proliferation is displayed by the decrease of eFluor intensity.

2.2.4.4 mRNA Expression Analysis

RNA Isolation and Reverse Transcription

In the process of transcription, based on a specific DNA sequence, a gene is transcribed into an RNA equivalent by the enzyme RNA polymerase. As mRNA serves as a template for translation into the respective protein and thus, determine the protein expression profile of a cell, mRNA expression gives information about the functional activity of genes and their protein expression in the cells. To analyze mRNA expression, reverse transcription of mRNA into complementary deoxyribonucleic acid (cDNA) has to be done by using the enzyme reverse transcriptase, a RNA-dependent DNA polymerase that synthesizes first stranded cDNA. Levels of cDNA expression are analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to expression levels of a house keeping gene characterized by constant expression by all analyzed cell populations or tissues.

Quantitative Real-Time PCR Analysis (qRT-PCR)

In qRT-PCR analysis, messenger RNA (mRNA) expression levels are quantified. As a transcript of a certain gene, mRNA expression displays the activity of the respective gene. In SYBR Green-based qRT-PCR, complementary DNA (cDNA), which is synthesized from mRNA of the gene of interest is amplified into double-stranded DNA

by gene-specific primers and a DNA polymerase. SYBBR Green is a fluorescent dye which emits green flourecence (λ_{max}=497 nm) and binds to double-stranded DNA. Thus, DNA amount is monitored at each cycle of PCR as the DNA-SYBR Green-complex emitted light can be detected by the PCR machine and is proportional to the amount of PCR product. PCR follows three steps of denaturation (95°C), oligonucleotide specific annealing and elongation (72°C). The steps were repeated for at most 60 cycles. In qRT-PCR analysis, the cycle threshold (CT) describes the number of PCR cycles, which are needed to achieve a defined level of fluorescence crossing the level of background fluorescence signal and marking the exponential phase of the reaction. A low CT value means a high mRNA concentration and *vice versa*. To control the specificity of the PCR product melting curve analyses were performed. In this process the PCR product is constantly heated up from 50 to 95 °C with constant measurement of the florescence emission. For a PCR product there is a specific melting temperature, which induces denaturation of the DNA strands and is displayed by a strong increase of the florescent intensity.

Total RNA was isolated from shock-frozen liver tissue using the NucleoSpin RNA Kit according to the manufacturer's instruction. 1 µg RNA was transcribed into cDNA using the Verso cDNA Synthesis Kit on a thermal cycler (MyCycler Thermal Cycler). Quantitative RT-PCR was performed using the ABsolute™ QPCR SYBR Green. The relative mRNA levels were calculated using the ΔCT method after normalization to the housekeeping gene mitochondrial ATP synthase. Quantification was shown in x-fold changes to the corresponding control cDNA. Sequences of the primers are listed in Table 9.

Table 9: Sequences of the primers used for analysis of mRNA expression.

TARGET	FORWARD PRIMER REVERSE PRIMER	AMPLICON LENGTH	ANNEALING TEMPERATURE
mitochondrial ATP synthase	5´-ATTGCCATCTTGGGTATGGA-3´ 3´-AATGGGTCCCACCATGTAGA-5´	228 bp	60°C
IL-33	5´-ATGGGAAGAAGCTGATGGTG-3´ 3´-CCGAGGACTTTTTGTGAAGG-5´	150 bp	58°C
IL-12p40	5´-AGGTCACACTGGACCAAAGG-3´ 3´-TGGTTTGATGATGTCCCTGA5´	173 bp	60°C
IFNγ	5´- ACTGGCAAAAGGATGGTGAC-3´ 3´- GACCTGTGGGTTGTTGACCT-5´	212 bp	59°C

2.2.5 Statistical Analysis

Statistical significance was assessed with Mann-Whitney-Test using Graphpad 5 (Prism). The Mann-Whitney-Test is a non-parametric test to detect significant differences of not normally distributed data (Hart, 2001). *P*-value of <0.05 was considered as significant.

3 Results

3.1 Effects of IL-33 on Liver-resident ILC2s

3.1.1 IL-33 Induces Expansion of ILC2s in the Liver

ILC2s are tissue-resident cells that do not undergo constant replenishment from the bone marrow. They preserve the tissue-resident population by self-renewal from tissue-resident progenitors (Gasteiger et al., 2015). In parabiosis experiments, Gasteiger et al. showed that ILC2s facilitate their sentinel function mainly by local proliferation of tissue-resident cells upon an inflammatory stimulus and thus are highly influenced by the microenvironment of the respective organ.

IL-33 is released following hepatic tissue damage (Schmitz et al., 2005) and is involved in the pathogenesis of hepatic inflammation in the model on Con A-hepatitis (Chen et al., 2012). Hepatic IL-33 expression is upregulated in viral hepatitis, correlates with liver damage and is linked with hepatic fibrosis (Huan et al., 2016, Marvie et al., 2010, Wang et al., 2012b, McHedlidze et al., 2013). This suggests an immunomodulatory function of this cytokine in hepatic inflammation. As IL-33 is further known as a potent activator of ILC2s (Price et al., 2010), the influence of IL-33 on hepatic ILC2s was investigated. Therefore, the phenotype of ILC2s from livers of naive C57BL/6 mice was characterized and compared with hepatic ILC2s from IL-33-treated mice.

C57BL/6 mice received recombinant murine (rm) IL-33 intraperitoneally on 4 consecutive days. On day 5, livers were harvested and leukocytes were isolated by gradient centrifugation. Since ILC2s do not have a solitary linage-specific marker, cells expressing linage-specific markers were excluded. As the surface molecules ST2 and Sca-1 are constitutively expressed by ILC2s (Sonnenberg and Artis, 2015), the hepatic leucocytes were stained for these surface markers and $ST2^+$ $Sca\text{-}1^+$ cells were determined as ILC2s.

A small population of liver-resident lin^- $ST2^+$ $Sca\text{-}1^+$ ILC2s (0.45% ±0.12%) was found in naive mice that strongly expanded upon IL-33 treatment (24.4% ± 4.8%) (Fig. 8).

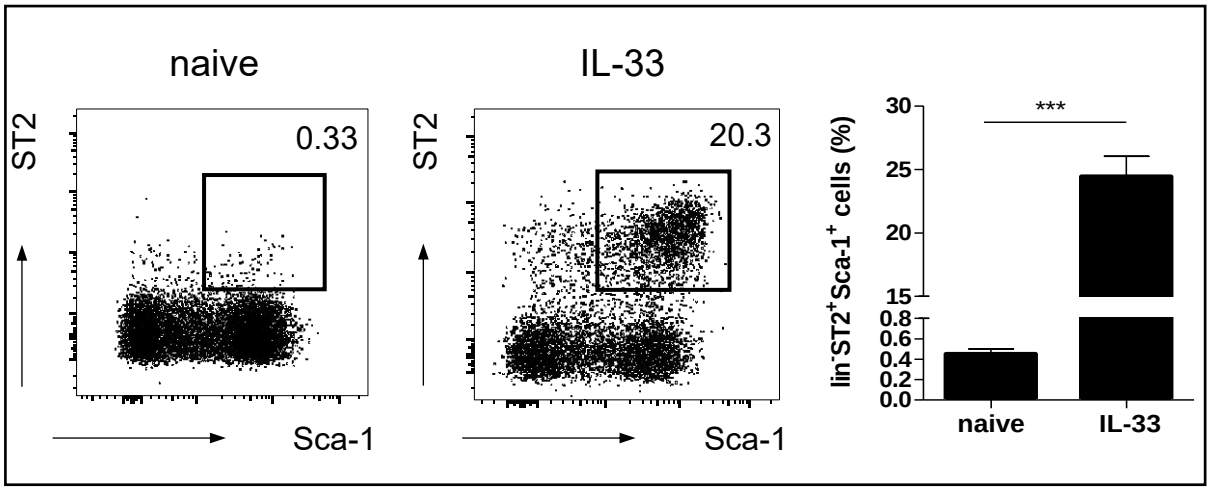

Figure 8: IL-33 treatment leads to expansion of hepatic ILC2s.
IL-33 was injected i.p. into C57BL/6 mice daily on four consecutive days. Frequency of ILC2s were determined by flow cytometry. Gated on lin- cells. Mean ± standard error of the mean (SEM) of three independent experiments are shown. ***p<0.001.

3.1.2 IL-33 Activates Hepatic ILC2s

CD25 and KLRG1 are surface markers linked with matureness and activation of NK cells and T cells (Henson and Akbar, 2009). Moreover, KLRG1 is described to be constitutively expressed by mature ILC2s from the lung (Huang et al., 2015) and the intestine (Hoyler et al., 2012). To analyze activation markers expressed by hepatic ILC2s in the liver under homeostatic conditions and upon challenge with IL-33, hepatic lin$^-$ ST2$^+$ Sca-1$^+$ cells were stained for KLRG1 and CD25 expression. ILC2s from healthy livers showed an inactive phenotype since only a small proportion of them expressed KLRG1 (26.1% ±3.2%) or CD25 (32.9% ±9.5%). Co-expression of both surface markers was seen in 15.1% (±2.7%) of ILC2s from naive mice. IL-33 treatment significantly increased expression of KLRG1 and CD25 by ILC2s in the liver: nearly all ILC2s expressed KLRG1 (96.3% ±1.8%) and a high percentage co-expressed CD25 (61.2% ±10.7%), demonstrating IL-33-induced hepatic ILC2 activation (Fig. 9).

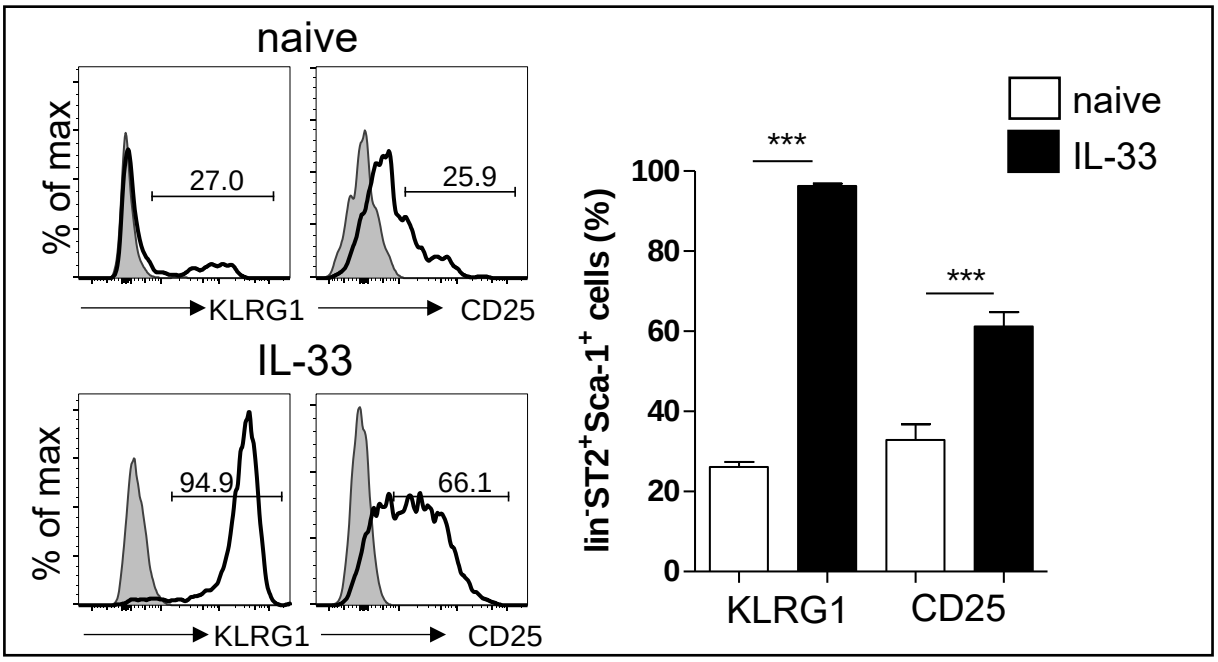

Figure 9: IL-33-elicited hepatic ILC2s upregulate expression of activation markers.
IL-33 was injected i.p. into C57BL/6 mice daily on four consecutive days. Expression of KLRG1 and CD25 by hepatic ILC2s was determined by flow cytometry. Histograms show frequency of protein-expressing ILC2s compared to fluorescence minus one (FMO) controls. Mean ± SEM of three independent experiments are shown. ***p<0.001.

3.1.3 IL-33 Predominantly Induces IL-5 Expression by Hepatic ILC2s

By performing qRT-PCR, hepatic mRNA expression of the type 2 cytokines IL-4, IL-5 and IL-13 and of the transcription factor GATA3, all of them linked with ILC2 effector function (Moro et al., 2010, Neill et al., 2010, Price et al., 2010), were analyzed. In liver tissue of IL-33-treated mice, mRNA expression of IL-4 (9.2-fold ±15.0-fold), IL-5 (1.8-fold ±1.3-fold) and IL-13 (3.9-fold ±0.6-fold) was induced, whereas PBS-treated animals did not show elevated hepatic expression of these cytokines. Furthermore, expression of the transcription factor GATA3, which is essential for ILC2 function (Hoyler et al., 2012, Mjösberg et al., 2012), was significantly increased in liver tissue of IL-33-treated mice compared to the PBS-treated controls. In contrast, mRNA expression of the type 1-associated pro-inflammatory cytokine IFNγ was not induced after IL-33 treatment (Fig. 10).

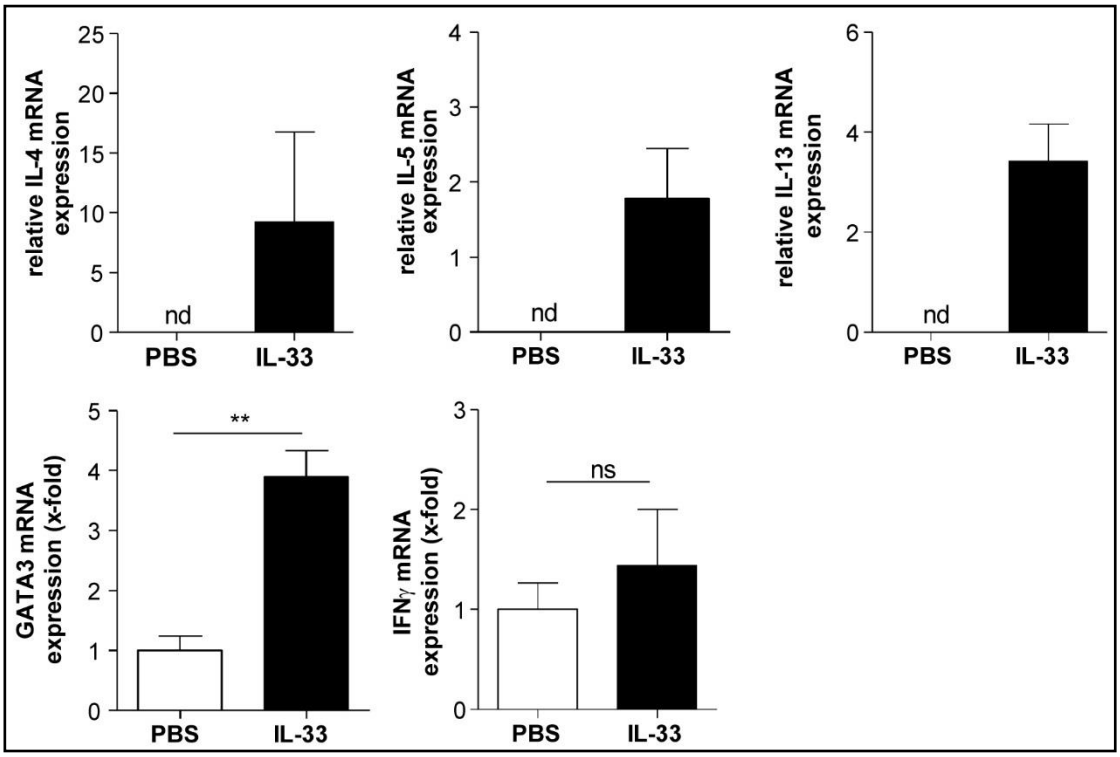

Figure 10: IL-33 treatment induces type 2 cytokine expression in liver tissue.
IL-33 was injected i.p. into C57BL/6 mice daily on four consecutive days. Hepatic mRNA expression was determined by qRT-PCR and normalized to the housekeeping gene mitochondrial ATP synthase. GATA3 and IFNγ mRNA expression in IL-33-treated mice was normalized to PBS-treated mice. Mean ± SEM of three independent experiments are shown. **p<0.01; ns, not significant; nd, not detectable.

Finding IL-33 to activate ILC2s in the liver and to induce type 2 cytokine mRNA expression in hepatic tissue, the question raised whether hepatic ILC2s contribute to the changes of the cytokine milieu following IL-33 treatment. To determine cytokine expression by ILC2s in the liver, hepatic leukocytes were isolated and restimulated with PMA and ionomycin in the presence of brefeldin A and monensin for 6 hours. Thereafter, intracellular cytokine expression of $lin^- ST2^+ Sca\text{-}1^+$ ILC2s was analyzed. Upon *in vivo* IL-33 challenge, hepatic ILC2s predominantly expressed type 2 cytokines. 31.5% (±12.8%) of hepatic ILC2s produced IL-5 following IL-33 injection. Moreover, a smaller percentage of IL-33-elicited hepatic ILC2s expressed IL-13 (8.4% ±4.2%), IL-4 (7.7% ±4.2%), IL-17A (6.8% ±2.9%) or TNFα (9.7% ±9.1%). In contrast, IL-10 (2.2% ±2.7%) was expressed on very low level whereas IFNγ (0.8% ±0.5%) was not induced (Fig. 11). In summary, this data shows that liver-resident ILC2s respond to IL-33-mediated activation by expansion and predominant IL-5 expression.

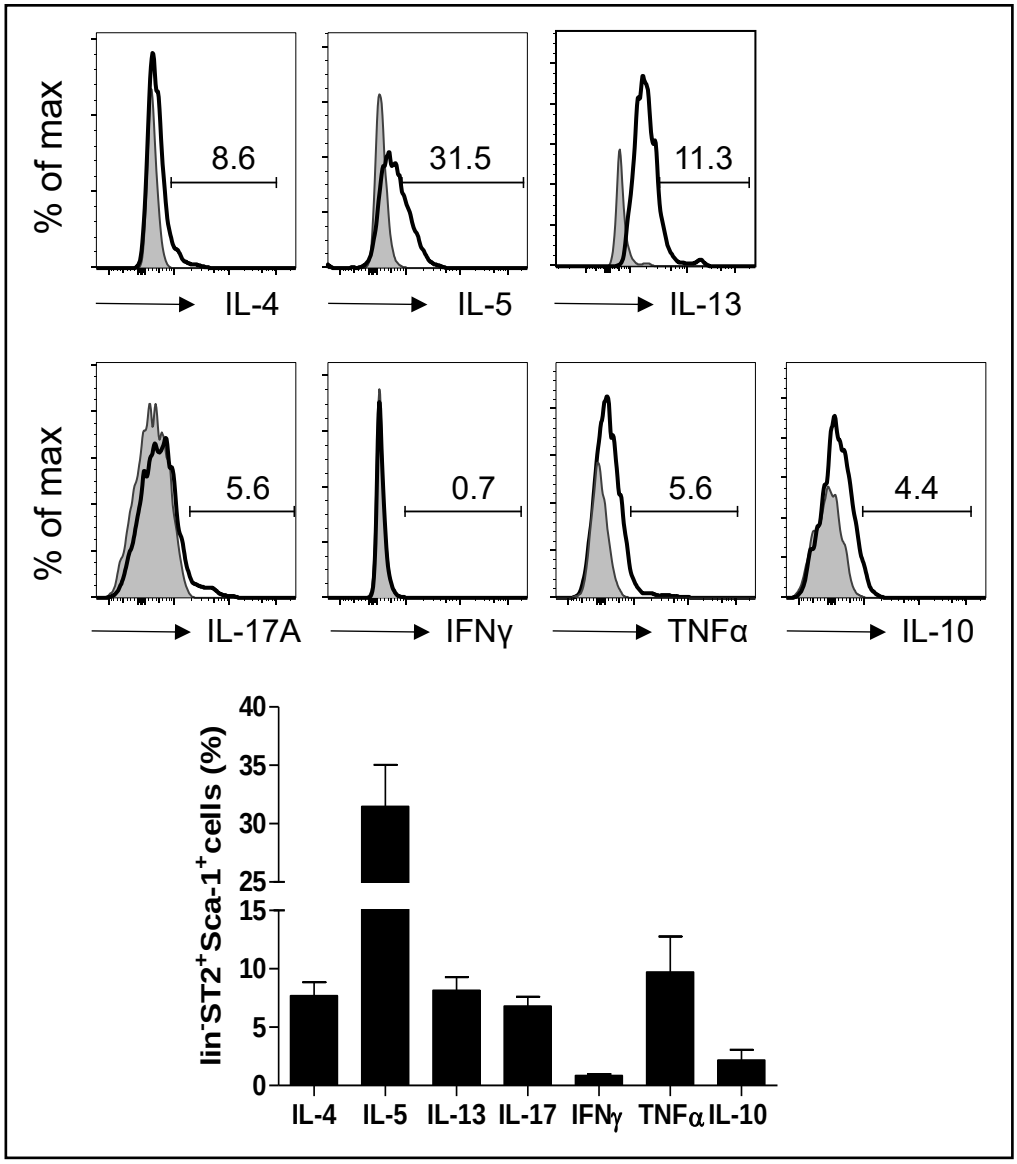

Figure 11: IL-33-elicited hepatic ILC2s predominantly express IL-5.
IL-33 was injected i.p. into C57BL/6 mice daily on four consecutive days. Hepatic ILC2s were analyzed for cytokine production by flow cytometry. Histograms show frequency of cytokine-producing ILC2s compared to FMO controls. Mean ± SEM of three independent experiments are shown.

3.2 Modulation of Hepatic ILC2s by Liver Inflammation-induced Cytokines

3.2.1 Induction of Hepatic IL-33, IFNγ and IL-12 Expression during Immune-mediated Hepatitis

ILC2s have been described to play a role in different models of liver inflammation such as viral hepatitis (Liang et al., 2013), biliary atresia (Li et al., 2014) and chronic hepatic fibrosis (McHedlidze et al., 2013). Previous findings of our working group showed that hepatic ILC2s expand during Con A-induced liver inflammation, most likely due to increased IL-33 levels in the liver tissue of Con A-treated mice (Neumann et al., 2017). Therefore, the hepatic cytokine expression profile during Con A-induced hepatitis was further investigated to determine inflammation-induced cytokines that potentially modulate ILC2 effector function in the inflamed liver.

Therefore, C57BL/6 mice were treated with Con A and mRNA expression levels in liver tissue of Con A-treated animals compared to PBS-treated controls were analyzed 8 h and 24 h after hepatitis induction. 8 h after injection of Con A, strongly increased hepatic mRNA expression of IL-33 (12.7-fold ±7.6-fold), IL-12 (4.9-fold ±2.6-fold) and IFNγ (68.5-fold ±15.4-fold) was detectable, which significantly decreased 24 h after hepatitis induction. While IL-12 mRNA levels normalized completely after 24 h (0.6-fold ±0.5-fold), IL-33 (3.5-fold ±1.2-fold) and IFNγ (13.3-fold ±2.7-fold) mRNA levels remained significantly increased compared to PBS-treated mice at this later time point of hepatic inflammation (Fig. 12).

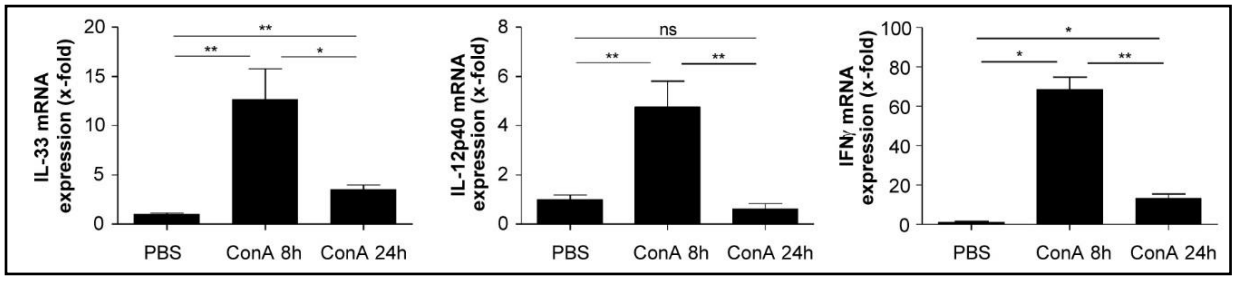

Figure 12: Induction of inflammatory cytokine expression in the liver during Con A-induced immune-mediated hepatitis.
Con A was intravenously injected into C57BL/6. Hepatic mRNA expression was determined by qRT-PCR and normalized to PBS-treated mice. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

3.2.2 IL-33 and IFNγ but not IL-12 Regulate Expansion and Activation of Hepatic ILC2s

IL-33 can act as an activation signal for ILC2s leading to expansion and type 2 cytokine production (Sonnenberg and Artis, 2015, Moro et al., 2010, Price et al., 2010, Saenz et al., 2008, Liew et al., 2010), whereas IFNγ was shown to suppress ILC2 function and proliferation (Molofsky et al., 2015, Moro et al., 2016). IL-12, in turn, is controversially reported to have modulating effects on human ILC2s (Lim et al., 2016, Bal et al., 2016, Ohne et al., 2016).

As IL-33, IFNγ and IL-12 are described to influence the phenotype of ILC2s in different ways, the question was raised how these cytokines modulate hepatic ILC2s expansion and activation when, similar as in the hepatic micro-milieu of Con A-mediated hepatitis, they are present simultaneously. Therefore, IL-33-expanded hepatic ILC2s were cultivated in the presence of IL-2 and IL-7, which are important for their maintenance and survival (Roediger et al., 2015, Diefenbach et al., 2014). In addition, IL-33, IL-12 or IFNγ or a combination of these cytokines were added.

Hepatic ILC2s cultured under the supportive conditions of IL-2 and IL-7 alone expanded to the 4.5-fold (±3.0-fold). In additional presence of IL-33, ILC2s expanded 2.0-fold (±0.7-fold) greater than ILC2s cultured only under supportive conditions. However, the presence of IL-12 alone did not significantly alter the expansion rate of ILC2s (1.0-fold ±0.7-fold), whereas the presence of IL-12 and IL-33 increased the expansion of hepatic ILC2s (1.7-fold ±0.9- fold) to similar levels, which are reached when ILC2s were cultured with IL-33 alone. The presence of IFNγ significantly inhibited the expansion of hepatic ILC2s (0.4-fold ±0.2-fold) compared to ILC2s cultured only under the supportive conditions of IL-2 and IL-7. However, in additional presence of IL-33, ILC2s expanded significantly more than ILC2s cultivated with IFNγ alone and reached expansion levels similar to ILC2s, which were cultured under supportive conditions (1.0-fold ±0.4-fold) (Fig. 13 A).

Similar effects of IL-33 and IL-12 were observed regarding the expression of the activation marker CD25. In the presence of IL-33, the frequency of hepatic ILC2s expressing CD25 significantly increased (70.9% ±22.0%) compared to ILC2s cultivated with supportive cytokines alone (43.3% ±23.5%). IL-12 did not affect expression of

CD25 (43.8% ±24.0%) and did also not significantly influence IL-33-induced CD25 expression by hepatic ILC2s (69.3% ±22.9%). Although the presence of IFNγ led to reduced hepatic ILC2 expansion, expression of CD25 was not affected. Indeed, in presence of IFNγ, the frequency of $CD25^+$ ILC2s was as high as in the group of IL-33-treated ILC2s (63.3% ±23.0%). The additional presence of IL-33 did not further increase CD25 expression (60.9% ±36.7%) (Fig. 13 B).

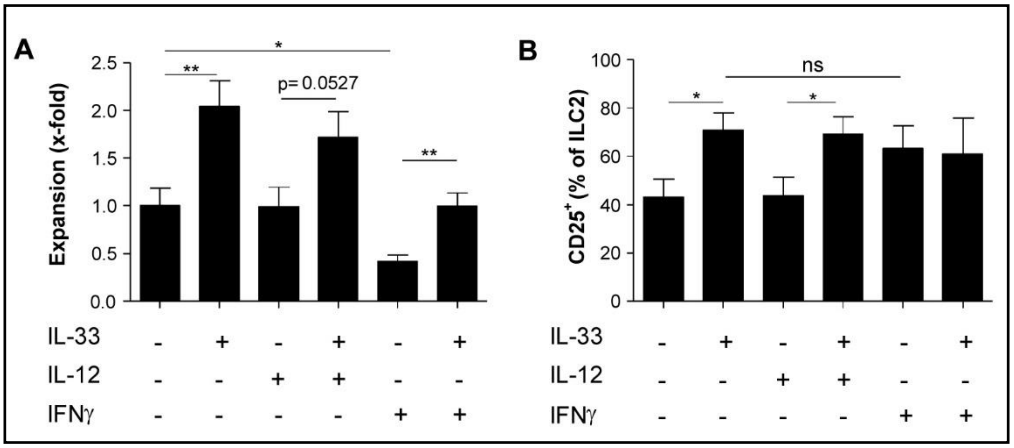

Figure 13: Effects of liver inflammation-induced cytokines on expansion and activation of hepatic ILC2s.
Hepatic ILC2s from IL-33-treated C57BL/6 mice were cultured with IL-2 and IL-7 in the absence or presence of IL-33 and/or IL-12 and/or IFNγ for 4 days. (**A**) Cells were counted and expansion was calculated based on cell number of the input and normalized to the cell number of ILC2s cultured with IL-2 and IL-7 alone. (**B**) Expression of CD25 was analyzed by flow cytometry. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ns, not significant.

3.2.3 IL-33 and IFNγ but Not IL-12 Modulate the Cytokine Profile of Hepatic ILC2s

After analyzing the effects of the inflammatory cytokines present in Con A-induced hepatitis on hepatic ILC2s in points of expansion and activation *in vitro*, the modulatory effects on the cytokine profile of hepatic ILC2s was further investigated. The supernatants of the ILC2 monocultures were analyzed for the typical type 2 cytokines IL-5 and IL-13, which are described to be produced by ILC2s after activation (Sonnenberg and Artis, 2015, Moro et al., 2010, Price et al., 2010) and confirmed via intracellular cytokine staining.

In the supernatants, IL-5 concentration of 126.3 pg/ml (±21.6 pg/ml) was detected when ILC2s were cultured under supportive conditions. In the presence of IL-33, the levels of IL-5 significantly increased to about 2728.5 pg/ml (±1238.4 pg/ml). In contrast, IL-12 alone did not alter IL-5 expression (96.0 pg/ml ±37.8 pg/ml). In additional presence of IL-33, significantly increased IL-5 levels were reached (3342.2 pg/ml ±1772.6 pg/ml). However, the presence of IFNγ significantly decreased IL-5 concentration in culture supernatants (36.3 pg/ml ±31.3 pg/ml) compared to ILC2s cultured under supportive conditions. Interestingly, IL-33 was able to antagonize the suppressive effect of IFNγ on IL-5 production of hepatic ILC2s. IL-5 concentrations in supernatants of ILC2s cultured with IFNγ and IL-33 were as high as IL-5 levels determined in the group treated with IL-33 alone (3893.0 pg/ml ±2516.0 pg/ml).

Concordant with the finding that presence of IL-33 increases the IL-5 levels in the supernatants of hepatic ILC2s, also higher frequencies of IL-5-producing ILC2s were detected on cellular levels. The frequency of IL-5$^+$ ILC2s increased in the presence of IL-33 (65.4% ±28.2%) compared to ILC2s cultured under supportive conditions (45.9% ±28.2%). This effect was not seen when ILC2s were treated with IL-12 (42.9% ±27.7%) or IFNγ (43.6% ±23.0%) alone. Here, no suppressive effect of IFNγ on the frequencies of IL-5-producing hepatic ILC2s was detect. Importantly, both cytokines did not suppress the IL-5-inducing effect of IL-33 on hepatic ILC2s (IL-12+IL-33: 68.2% ±26.3%, IFNγ+ IL-33:73.6% ±6.0%).

Further the median fluorescence intensity (MFI) was determined to identify differences in the cellular intensity of IL-5 expression by hepatic ILC2s. Similar to the increased frequency of IL-5-producing ILC2s after IL-33 exposition, a significant increase in the MFI of IL-5$^+$ ILC2s (4.4-fold ±1.2-fold) was shown compared to ILC2s cultivated under supportive conditions, whereas IL-12 had no effect (0.8-fold ±0.1-fold). In contrast IFNγ suppressed MFI of IL-5$^+$ ILC2s (0.5-fold ±0.3-fold). Also with regard to the MFI, the additional presence of IL-12 did not modulate IL-33-induced IL-5 expression of hepatic ILC2s (3.9-fold ±1.5-fold). The additional presence of IL-33 reversed the suppressive effect of IFNγ, thus, IL-5$^+$ ILC2s cultivated with IFNγ and IL-33 showed an increased MFI of 2.5-fold (±0.6-fold) (Fig. 14 A).

Likewise, IL-13 expression by hepatic ILC2s underwent similar changes following culture with one or a combination of different cytokines, respectively. In the supernatants, IL-13 concentration of 313.1 pg/ml (±66.3 pg/ml) was detected when ILC2s were cultured under supportive condition. In presence of IL-33, IL-13 concentration increased to 2949.9 pg/ml (±1260.3 pg/ml). IL-12 alone did not induce increased IL-13 production (230.6 pg/ml ±102.3 pg/ml), whereas the additional presence of IL-33 raised the IL-13 concentration to 4056.7 pg/ml (±1056.7 pg/ml). Similar to IL-5 expression, IFNγ also significantly decreased IL-13 production by hepatic ILC2s (102.3 pg/ml ±52.3 pg/ml). The additional presence of IL-33 restored IL-13 concentration to 2064.6 pg/ml (±995.3 pg/ml).

In line with the finding that IL-33 increases IL-13 levels in the supernatants of ILC2s, also an increase in the frequency of IL-13$^+$ cells following IL-33 treatment could be observed. 45.2% (± 36.0%) of ILC2 expressed IL-13 when cultured under supportive conditions. The presence of IL-33 increased the frequency of IL-13$^+$ cells to 71.7% (±26.0%). Neither did IL-12 alone influence the frequency of IL-13$^+$ cells (53.5% ±38.0%), nor did it effect the IL-13-inducing effect of IL-33 (74.5% ±22.1%). Furthermore, IFNγ alone did not decrease the frequency of IL-13-producing cells, neither when cultured alone (64.0% ±22.6%) nor in additional presence of IL-33 (77.6% ±11.0%).

Concordant with the finding that IL-33 increases the frequencies of IL-13-producing ILC2s, in the MFI of IL-13$^+$ cells increased following culture with IL-33 (4.3-fold ±0.9-fold) compared to ILC2s cultured under supportive conditions. The presence of IL-12 did not influence the MFI of IL-13$^+$ ILC2s, neither in absence (0.9-fold ±0.1-fold) nor in presence of IL-33 (4.2-fold ±1.2-fold). The presence of IFNγ did not decrease the MFI of IL-13$^+$ ILC2s (1.0-fold ±0.2-fold) and further did not modulate the MFI-increasing effect of IL-33 (3.3-fold ±0.9-fold) (Fig. 14 A).

Moreover, expression of other cytokines was detected in the supernatants of ILC2 culture. IL-10 was produced by ILC2s cultivated with IL-33 (163.8 pg/ml ±56.1 pg/ml), but not under supportive conditions or in the presence of IL-12 or IFNγ alone. The additional presence of IL-12 did not modulate the production on IL-33-induced IL-10 (130.8 pg/ml ±39.8 pg/ml), whereas IFNγ suppressed IL-33-mediated induction of IL-

10 expression in hepatic ILC2s (6.1 pg/ml ±8.1 pg/ml). Further induction of IL-9 (60.0 pg/ml ±31.2 pg/ml) and IL-22 (10.4 pg/ml ±1.4 pg/ml) production was detectable in the presence of IL-33, whereas IL-12 and IFNγ did not induce substantial amounts of both cytokines. IL-12 did not influence IL-33-induced expression of IL-9 (39.5 pg/ml ±3.2 pg/ml) or IL-22 (9.4 pg/ml ±2.6 pg/ml), but in the presence of IFNγ, IL-9 (12.7 pg/ml ±10.7 pg/ml) and IL-22 (4.2 pg/ml ±2.2 pg/ml) levels were significantly decreased compared to the group treated with IL-33 alone.

Interestingly, hepatic ILC2s produced high amounts of the pro-inflammatory cytokine IL-6, even when cultured under supportive conditions alone (138.2 pg/ml ±44.4 pg/ml). In presence of IL-33, IL-6 levels highly increased (6926.1 pg/ml ±1897.4 pg/ml). The additional presence of IL-12 did not modulate the IL-6-increasing effect of IL-33 (5801.8 pg/ml ±1710.6 pg/ml) and further IL-12 alone did not alter the IL-6 production of hepatic ILC2s (89.8 pg/ml ±29.2 pg/ml). IFNγ suppressed IL-6 production significantly (15.18 pg/ml ±4.7 pg/ml) compared to hepatic ILC2s, which were cultured solely under supportive conditions. The additional presence of IL-33 restored the suppressive effect of IFNγ (2349.0 pg/ml ±761.8 pg/ml); (Fig. 14 B).

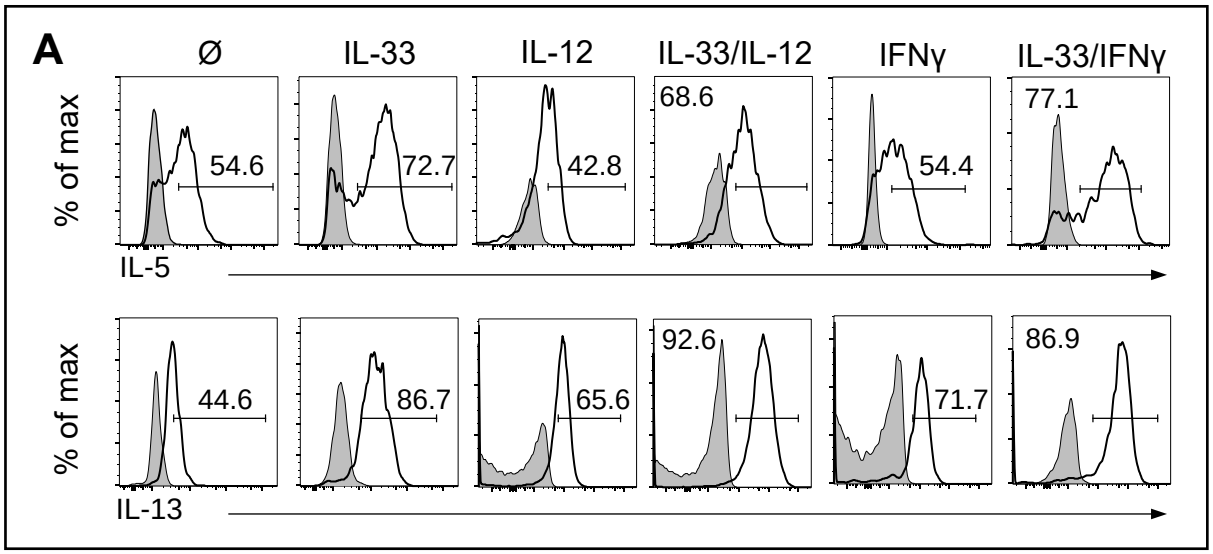

Figure 14A: Regulation of hepatic ILC2 cytokine response by liver inflammation-induced cytokines.
Hepatic ILC2s from IL-33-treated C57Bl/6 mice were cultured with IL-2 and IL-7 (Ø) in the absence or presence of IL-33 and/or IL-12 and/or IFNγ for 4 days. Frequency of cytokine-producing hepatic ILC2s were determined by flow cytometry. Histograms show frequency of cytokine-producing ILC2s compared to FMO control. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ***p<0.001; ns, not significant; nd, not detectable.

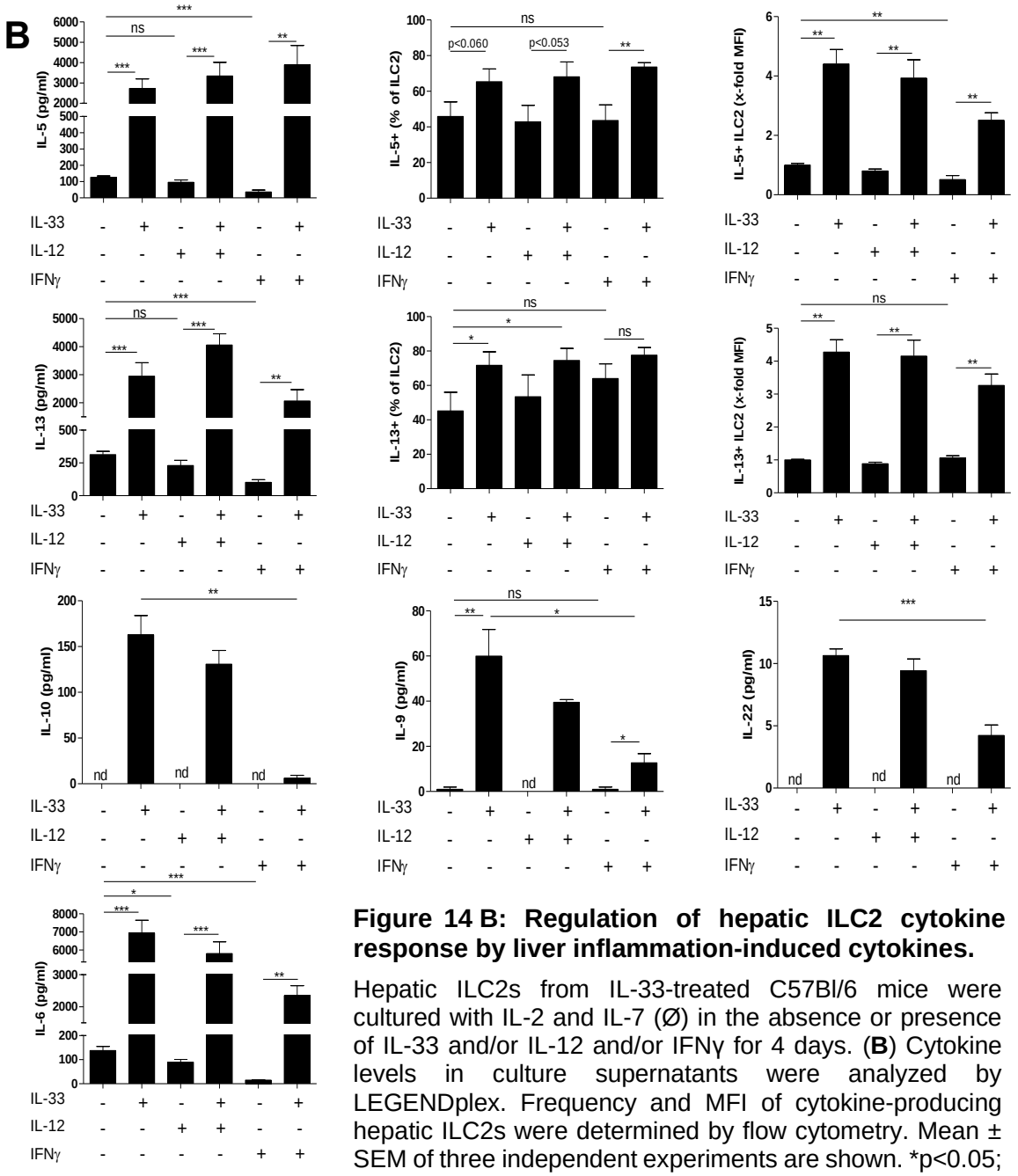

Figure 14 B: Regulation of hepatic ILC2 cytokine response by liver inflammation-induced cytokines.

Hepatic ILC2s from IL-33-treated C57Bl/6 mice were cultured with IL-2 and IL-7 (Ø) in the absence or presence of IL-33 and/or IL-12 and/or IFNγ for 4 days. (**B**) Cytokine levels in culture supernatants were analyzed by LEGENDplex. Frequency and MFI of cytokine-producing hepatic ILC2s were determined by flow cytometry. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ***p<0.001; ns, not significant; nd, not detectable.

3.2.4 Hepatic ILC2s Do Not Express IL-17A or IFNγ

To see if there is induction of plasticity by one of the liver inflammation-induced cytokines, production of IFNγ and IL-17A, which are not expressed by ILC2s, but are the dominant cytokines of group 1 and group 3 ILCs, respectively, were determined. None of the tested cytokines alone or in combination induced substantial production of IFNγ or IL- 17A by hepatic ILC2s (Fig. 15).

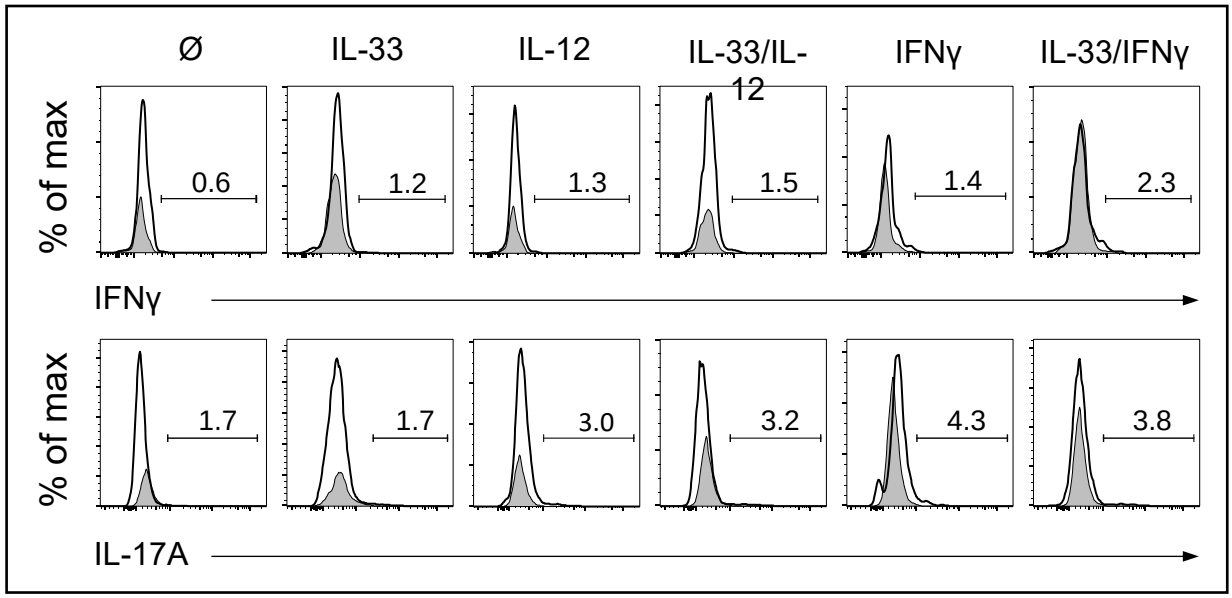

Figure 15: Hepatic ILC2s do not express IFNγ and IL-17A.
Hepatic ILC2s from IL-33-treated C57Bl/6 mice were cultured with IL-2 and IL-7 (Ø) in the absence or presence of IL-33 and/or IL-12 and/or IFNγ for 4 days. Frequency of cytokine-producing hepatic ILC2s was determined by flow cytometry. Histograms show frequency of cytokine-producing ILC2s compared to FMO controls. Representative data of three independent experiments are shown.

3.3 Mutual Influence of Antigen-specific Interaction of Hepatic ILC2s and CD4$^+$ T cells

3.3.1 IL-33-elicited ILC2s Upregulate Expression of Molecules Linked with APC-Function

As key effector cells of Con A-mediated hepatitis, CD4$^+$ T cells have major impact on pathogenesis and progression of immune-mediated liver injury (Tiegs et al., 1992, Wang et al., 2012a). ILC2s were described to have the ability to interact with CD4$^+$ T cells in indirect and direct manners (Drake et al., 2014, von Burg et al., 2015, Oliphant et al., 2014), which led us to investigate, if the interplay of hepatic ILC2s and CD4$^+$ T cells can modulate their respective immune responses and therefore effect the progression of immune-mediated hepatitis. The interplay of ILC2s and CD4$^+$ T cells might be accomplished by various molecules, which are reported to facilitate cell-to-cell interaction. Special focus comes to MCH II-mediated activation of CD4$^+$ T cells, as this mechanism is central for T-cell activation and differentiation and further MHC II/TCR-interaction is already described for pulmonary ILC2s and CD4$^+$ T cells (Oliphant et al., 2014).

To address if hepatic ILC2s can act as APCs and activate CD4$^+$ T cells by antigen presentation, MHC class II expression of hepatic ILC2s was investigated. As also co-stimulatory molecules are necessary for sufficient T-cell activation, expression of potent co-stimulatory molecules CD80 and CD86 was evaluated. Further, Drake et al. mentioned the involvement of the co-stimulatory molecule OX-40L in T-cell-ILC2 interaction in the lung (Drake et al., 2014), which prompted us to investigate expression of OX-40 and OX-40L on hepatic ILC2s. Given that the co-stimulatory molecule ICOS is known to control T-cell function and influence humoral immune response (Simpson et al., 2010) and ICOS$^+$ ILC2s were described to drive type 2 immune response in CD4$^+$ T cells, ICOS/ICOS-L signaling is an object of interest when it comes to T-cell interaction.

Another way to modulate T-cell response by T-cell-APC interaction is PD-1/PD-L1 signaling, which suppresses T-cell function by inhibiting TCR-mediated lymphocyte proliferation and cytokine production. PD-1 is expressed by activated T and B cells, whereas its ligand PD-L1 is also expressed on APCs (Freeman et al., 2000).

As PD-1 signaling was found to negatively regulate ILC2s (Taylor et al., 2017), PD-1/PD-L1 expression by hepatic ILC2s was also addressed in this study. To gain first insights about the ability of hepatic ILC2s to serve as APCs, the expression of this surface molecules on naive and/or IL-33-elicited ILC2s from hepatic tissue was analyzed.

In line with changes in number and CD25 expression, differences in the expression profile of MHC class II and co-stimulatory molecules of hepatic ILC2s were observed following exposure to IL-33. While only a small frequency of 9.0% (±6.6%) of ILC2s isolated from livers of naive mice expressed MHC class II molecules, IL-33 treatment strongly increased frequency of $MHCII^+$ hepatic ILC2s (31.5% ±10.8%). The expression profile of the surface molecule ICOS changed in a similar manner. About 14.3% (±4.9%) of naive hepatic ILC2s expressed ICOS, while 68.8% (±6.8%) of IL-33-elicited ILC2s were $ICOS^+$. The expression of its ligand (ICOS-L) on hepatic ILC2s did not undergo significant changes after IL-33 treatment and remained on low levels of 8.1% (±2.5%) in naive mice and 5.7% (±3.8%) in IL-33-treated animals (Fig. 16 A).

CD80 was expressed by about 8.0% (±2.5%) of IL-33-elicited hepatic ILC2s. CD86 was expressed by 2.0% (±1.7%) of IL-33-activated hepatic ILC2s. PD-1 expression was found in 63.2% (±4.3%) of the IL-33-elicited ILC2s and 75.9% (±14.3%) expressed PD-L1. There was no expression of OX-40 or its ligand detectable on the surface of ILC2s of IL-33-treated mice (data not shown). However, by performing intracellular protein staining 48.2 % (±37.4%) of hepatic ILC2s expressed OX-40 and 36.2% (±22.6%) expressed OX-40L intracellularly (Fig. 16 B).

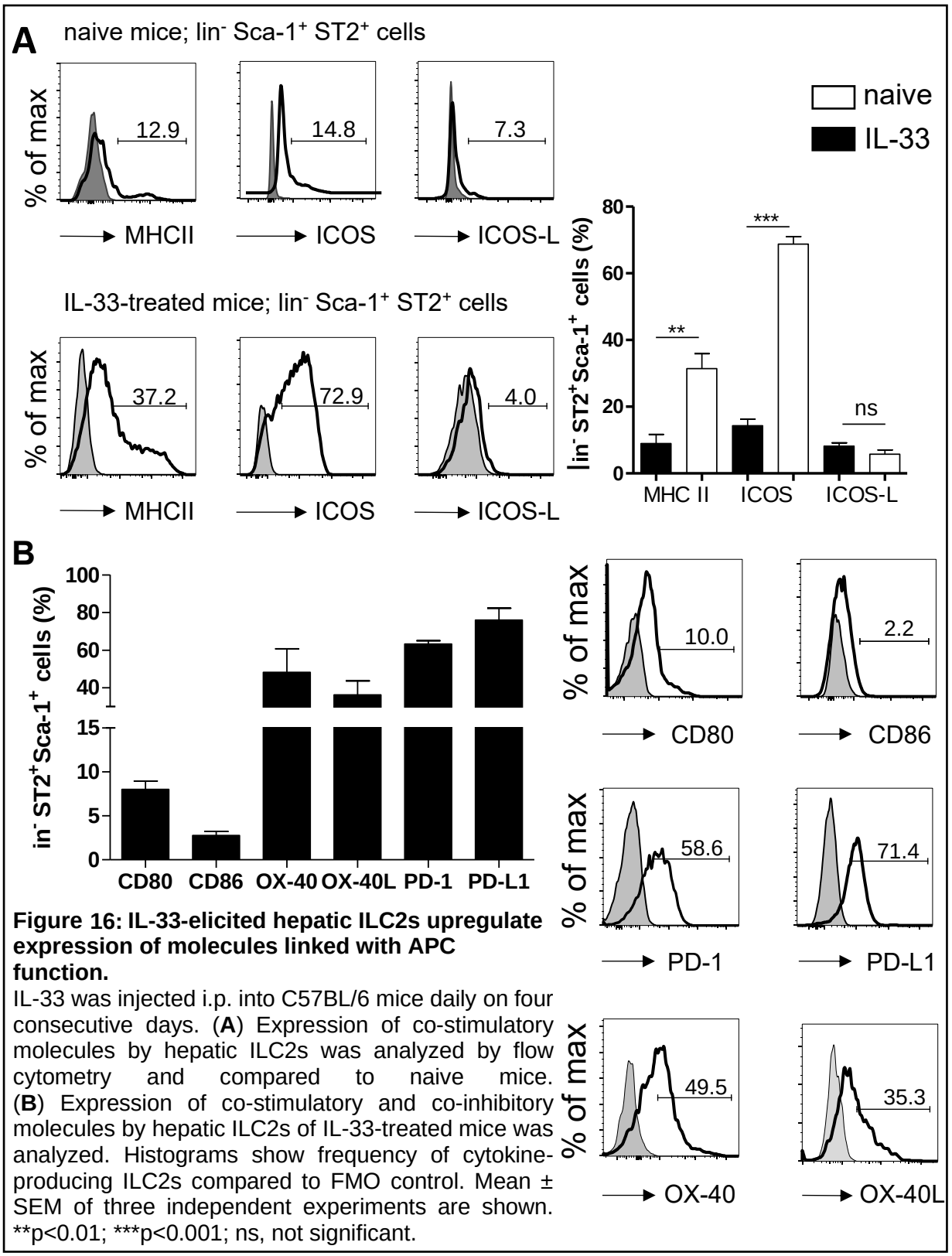

Figure 16: IL-33-elicited hepatic ILC2s upregulate expression of molecules linked with APC function.

IL-33 was injected i.p. into C57BL/6 mice daily on four consecutive days. (**A**) Expression of co-stimulatory molecules by hepatic ILC2s was analyzed by flow cytometry and compared to naive mice. (**B**) Expression of co-stimulatory and co-inhibitory molecules by hepatic ILC2s of IL-33-treated mice was analyzed. Histograms show frequency of cytokine-producing ILC2s compared to FMO control. Mean ± SEM of three independent experiments are shown. **p<0.01; ***p<0.001; ns, not significant.

3.3.2 Co-Culture of Hepatic ILC2s and CD4$^+$ T Cells Promotes Expansion and Activation of Both Cell Types

ILC2s are known to accomplish their function in type 2 immune responses by cytokine release (Bostick and Zhou, 2016, Halim, 2016, von Burg et al., 2015). Beyond this, ILC2s from the gut and the lung were described to interact with T cells via cell-to-cell contact (Drake et al., 2014, Mirchandani et al., 2014, Oliphant et al., 2014) and in this way further contribute to immune responses. As ILC2s were found to express surface markers, which enable T cell interaction (Fig. 16) it was investigated if hepatic ILC2s function as APCs, activate CD4$^+$ T cells by antigen presentation and in this way, modulate CD4$^+$ T cell immune response and enhance type 2 immunity. Therefore, hepatic ILC2s from C57Bl/6 mice were co-cultured with ovalbumin (OVA)-specific CD4$^+$ T cells from spleen and lymph nodes of OT2 mice *in vitro*. To see if these cells are capable to interact antigen-specifically, the OVA peptide was added to some of the cultures. CD4$^+$ T cells of OT2 mice recognize the OVA peptide when it is presented by APCs expressing MHC II resulting in TCR-mediated activation of CD4$^+$ T cells. As hepatic ILC2s express CD80/CD86 on low levels (Fig. 16 B), and therefore might not sufficiently provide a strong co-stimulatory signal, this antigen-specific interaction of hepatic ILC2s and CD4$^+$ T cells was compared with antigen-unspecific activation of the CD4$^+$ T cells. By adding anti-CD3/CD28 Dynabeads (aCD3/CD28) to some co-cultures it was analyzed if a strong co-stimulatory signal has influence on the phenotype of the cells. The anti-CD3 antibodies on the surface of the beads bind to the CD3-TCR-complex and induce TCR signaling, but further the anti-CD28 antibodies on the surface bind to CD28, the receptor of CD80 and CD86, and additionally provide co-stimulatory signals. Thus, by using aCD3/CD28, T-cell activation is induced in an antigen-unspecific manner (Trickett and Kwan, 2003). As a control, hepatic ILC2s and CD4$^+$ T cells were co-cultured in the absence of any stimulatory signal. After 4 days of co-culture, the cells were analyzed for their expansion and their cytokine profile. Both cell types were discriminated by the expression of the TCR, as CD4$^+$ T cells are TCR$^+$ and hepatic ILC2s TCR$^-$ (Fig. 17 A).

After 4 days of co-culture, both cell types, hepatic ILC2s and CD4$^+$ T cells, expanded significantly more in the presence of OVA and aCD3/CD28 than co-cultured without any stimulatory substance. While cell number of ILC2s remained on similar levels of

the ILC2 input in absence of a stimulatory signal (1.4-fold ±0.4-fold), ILC2s co-cultured with OVA expanded to the 2.7-fold (±1.0-fold). In presence of aCD3/CD28, ILC2s expanded even more greatly (4.2-fold ±0.6-fold). Antigen-specific interaction also led to expansion of $CD4^+$ T cells. $CD4^+$ T cells co-cultured in presence of OVA expanded to the 1.6-fold (±0.9-fold) whereas in absence of OVA, $CD4^+$ T-cell number decreased to the 0.5-fold (±0.3-fold). The stimulation via anti-CD3/CD28 did not lead to a further expansion of $CD4^+$ T cells compared to OVA-stimulated cells (1.3-fold ±0.26-fold).

There was no significant difference in the expansion of ILC2s and $CD4^+$ T cells between antigen-specific stimulation or antigen-unspecific stimulation (Fig. 17 C; E). We verified the enhanced expansion of $CD4^+$ T cells, which were cultured in presence of ILC2s and OVA, by performing a proliferation assay. Therefore, $CD4^+$ T cells were labeled with the proliferation dye eFlour670 before co-culture. eFlour670 binds to cellular proteins containing primary amines and is equally distributed to the daughter cells by every cell division. In this way, eFlour670 signal is bisected with every proliferation cycle and proliferation can be determined by flowcytometry by the decrease of eFlour670 signal. Thus, $CD4^+$ T cells co-cultured with hepatic ILC2s and OVA were confirmed to undergo multiple cell divisions, while $CD4^+$ T cells cultured with ILC2s in absence of OVA did not proliferate (Fig. 17 B).

Further, the expression of the activation marker CD25 by hepatic ILC2s and $CD4^+$ T cells was analyzed after 4 days of co-culture. In the absence of any stimulatory substance, a small frequency of both cell types expressed CD25. 5.5% (±2.2%) of $TCR\beta^-$ hepatic ILC2s were $CD25^+$ and 2.2% (±1.8%) of $TCR\beta^+$ $CD4^+$ T cells expressed CD25. Antigen-specific stimulation via OVA increased the frequencies of CD25-expressing cells in ILC2s (12.8% ±10.1%) and $CD4^+$ T cells (26.5% ±9.8%). Antigen-unspecific stimulation via aCD3/CD28 showed similar levels of activation in ILC2s (25.7% ±7.8%) and $CD4^+$ T cells (25.2% ±8.1%) but did not further increase the frequency of $CD25^+$ cells compared to the stimulation via OVA (Fig. 17 D, F).

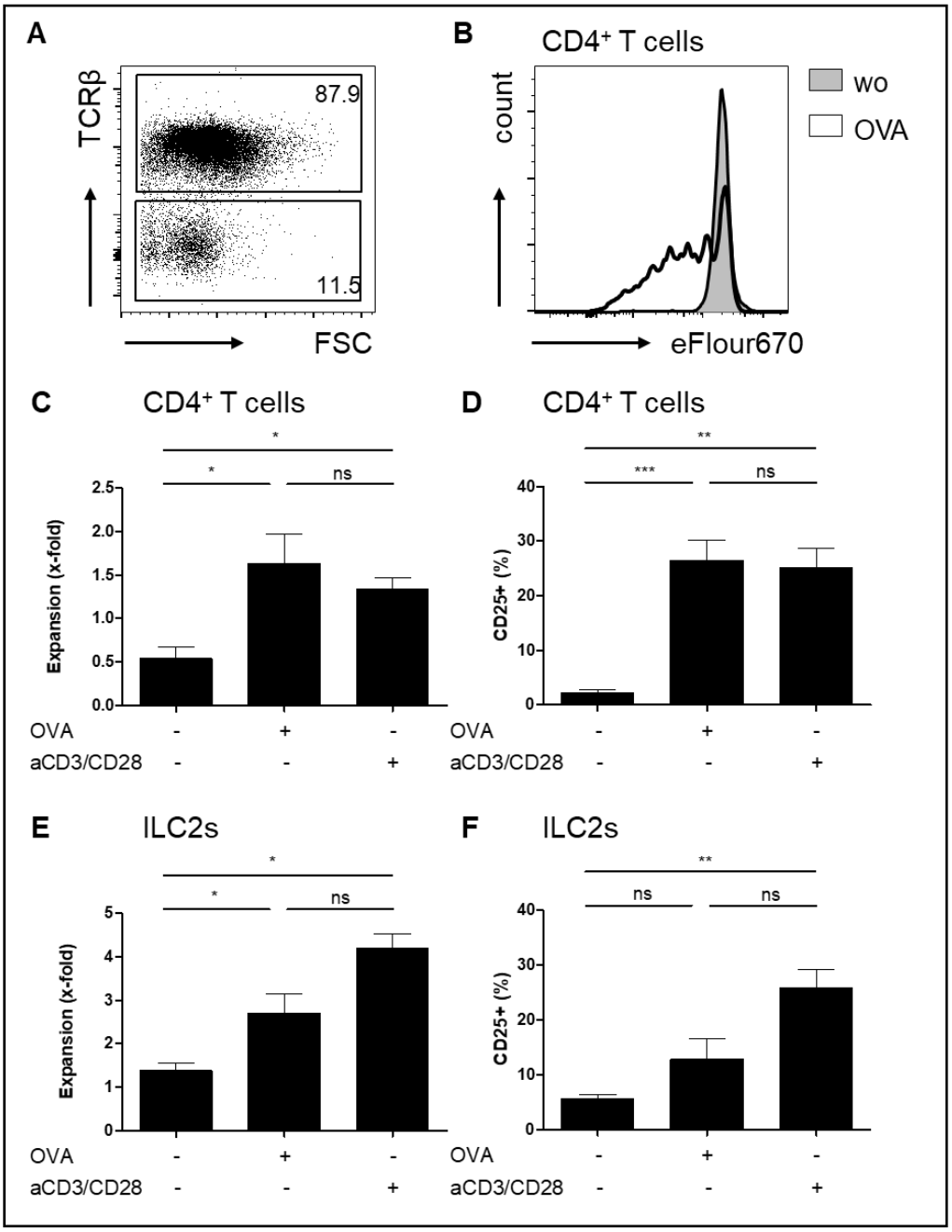

Figure 17: Co-culture of ILC2s and CD4+ T cells in the presence of OVA promotes activation and expansion of both cell types.
OVA-specific $CD4^+$ T cells were co-cultured with hepatic ILC2s from IL-33-treated C57Bl/6 mice in the presence of OVA or anti-CD3/CD28 Dynabeads for four days. (**A**) Total cells were harvested and stained for TCRβ to distinguish between $TCR\beta^+$ $CD4^+$ T cells and $TCR\beta^-$ ILC2s. (**B**) Proliferation of $CD4^+$ T cells was analyzed by flow cytometry. (**C**) Total cells were counted and $CD4^+$ T-cell expansion was calculated according to the percentage of $CD4^+$ T cells after co-culture and the input. (**D**) $CD4^+$T-cell activation was analyzed by flow cytometry. (**E**) Total cells were counted and ILC2 expansion was calculated according to the percentage of ILC2s after co-culture and the input. (**F**) CD25 expression by hepatic ILC2s was analyzed by flow cytometry. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

3.3.3 Increased Type 2 Cytokine Production in Co-Culture of Hepatic ILC2s and $CD4^+$ T cells

To investigate which cytokines are released during co-culture of hepatic ILC2s and $CD4^+$ T cells, the supernatants of the cultures were analyzed for their cytokine expression profile. In the absence of any stimulus low levels of type 2 cytokines were found in the supernatants. In presence of OVA, type 2 cytokine production increased significantly. IL-5 production raised from 7.8 pg/ml (±1.4 pg/ml) to 1121.4 pg/ml (±134.6 pg/ml). Likewise, IL-13 production was also increased from 5.2 pg/ml (±4.8 pg/ml) to 832.0 pg/ml (±109.5 pg/ml). In co-cultures stimulated with aCD3/CD28, similar concentrations, but no further increased levels compared to antigen-specific stimulation, were detected for IL-5 (1239.6 pg/ml ±243.5 pg/ml) and IL-13 (886.1 pg/ml ±329.9 pg/ml). IL-4 concentration in the supernatants was only moderately elevated when OVA was added to the cultures (7.4 pg/ml ±5.3 pg/ml) but significantly increased when the culture was done in the presence of aCD3/CD28 (47.2 pg/ml ±28.3 pg/ml) compared to co-cultures in the absence of any stimulus (1.3 pg/ml ±1.9 pg/ml). Beside increased type two cytokine production, increased IL-6 was observed in the presence of OVA (52.8 pg/ml ±11.7 pg/ml) and aCD3/CD28 (45.0 pg/ml ±17.9 pg/ml) compared to unstimulated co-cultures (2.1 pg/ml ±2.0 pg/ml). Moreover, in presence of OVA, levels of TNFα increased (190.5 pg/ml ±69.9 pg/ml) compared to unstimulated cultures (3.5 pg/ml ±3.0 pg/ml) and lFNγ (201.7 pg/ml ±78.3 pg/ml) and IL-10 production (29.0 pg/ml ±20.1 pg/ml) were induced. Antigen-unspecific stimulation did not induce a stronger cytokine production of the cells and reached a similar cytokine concentration in the supernatants of the co-cultures (TNFα: 248.8 pg/ml ±74.3 pg/ml; lNFγ: 144.5 pg/ml ±55.8 pg/ml; IL-10: 44.9 pg/ml 24.2 pg/ml) (Fig. 18).

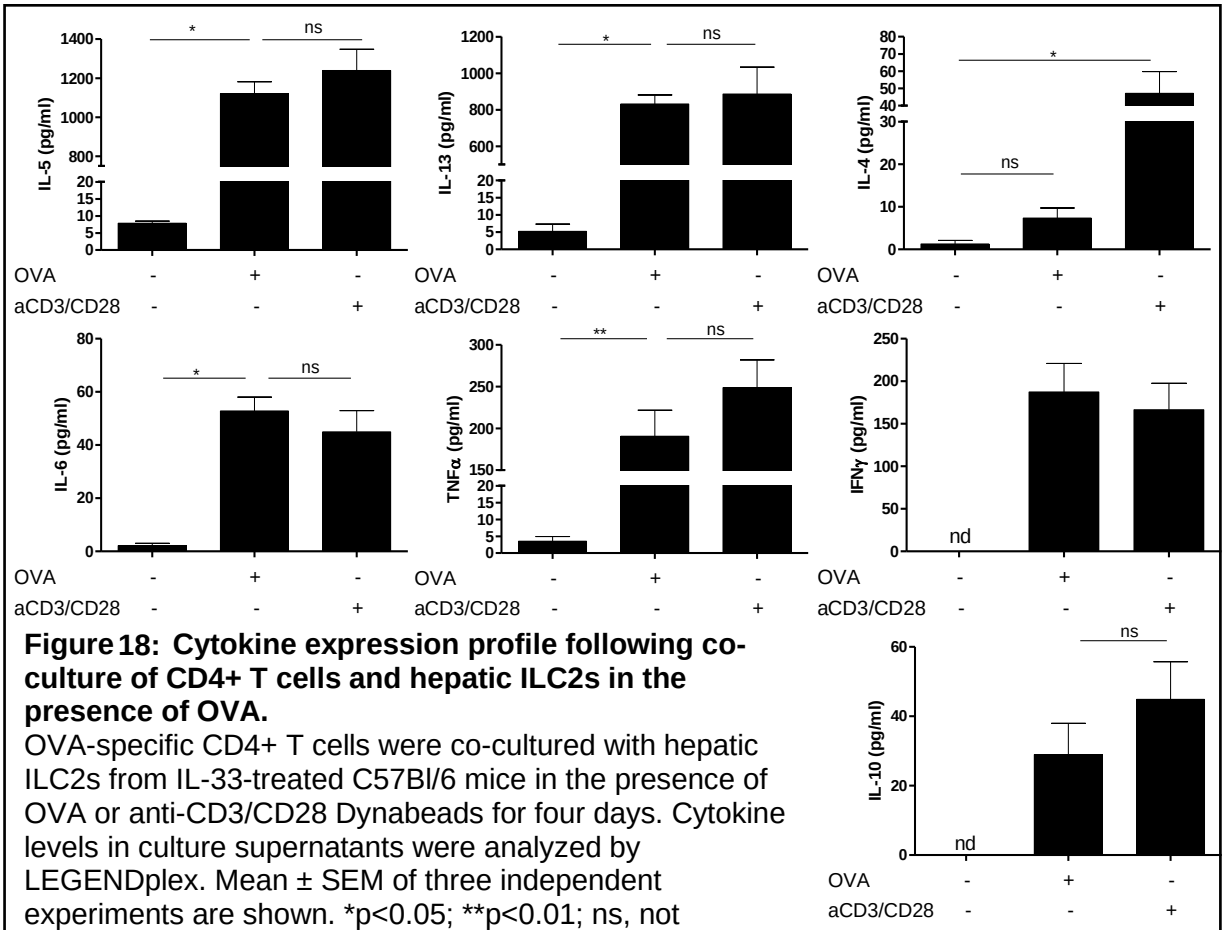

Figure 18: Cytokine expression profile following co-culture of CD4+ T cells and hepatic ILC2s in the presence of OVA.
OVA-specific CD4+ T cells were co-cultured with hepatic ILC2s from IL-33-treated C57Bl/6 mice in the presence of OVA or anti-CD3/CD28 Dynabeads for four days. Cytokine levels in culture supernatants were analyzed by LEGENDplex. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ns, not significant.

3.3.4 Hepatic ILC2s Increase Type 2 Cytokine Production after Co-Culture with $CD4^+$ T cells

To identify the cellular sources of these cytokines, intracellular cytokine expression of hepatic ILC2s and $CD4^+$ T cells was analyzed following co-culture via flow cytometry. Cells were discriminated by their expression of the T-cell receptor (Fig. 17 A).

After 4 days of co-culture with $CD4^+$ T cells and in the absence of any stimulus 8.7% (±8.1%) of the hepatic ILC2s produced IL-4. IL-5 production was detected in 12.6% (±5.8%) of the cells, while a total of 24.3% (±14.0%) of the ILC2s were IL-13^+. Following OVA stimulation, the frequency of hepatic ILC2s, which expressed IL-5, significantly increased to 25.5% (±13.3%). Antigen-unspecific stimulation via aCD3/CD28 did not further increase IL-5 expression and led to similar frequencies of IL-5^+ ILC2s (23.2% ±19.7%). Likewise, the frequency of $IL13^+$ ILC2s increased significantly to 54.9% (±12.0%) under stimulation with OVA, and, similar to IL-5 production, antigen-unspecific stimulation with aCD3/CD28 did not significantly alter the percentages of IL-13-producing ILC2s (49.9% ±31.3%).

In contrast to the type 2 cytokines IL-5 and IL-13, which were increased in the presence of OVA and aCD3/CD28 after 4 days, the increase of IL-4-producing cells was only slightly and not significant neither under OVA- (15.5% ±12.0%) nor aCD3/CD28-stimulation (18.2% ±14.3%) (data not shown). However, after 5 days of co-culture of hepatic ILC2s and $CD4^+$ T cells, stimulation via OVA significantly increased frequency of IL-4-expressing hepatic ILC2s (22.7% ±7.9%). Again, stimulation via aCD3/CD28 did not further increase IL-4 expression in hepatic ILC2s (28.9% ±7.9%).

Further, the frequency of IL-10-producing ILC2s was increased under stimulation with OVA and aCD3/CD28, but only aCD3/CD28-stimulation led to a significant increase in the frequency of IL-10-producing ILC2s from 4.9% (±7.4%) to 14.0% (±4.6%) after co-culture with $CD4^+$ T cells (Fig. 19).

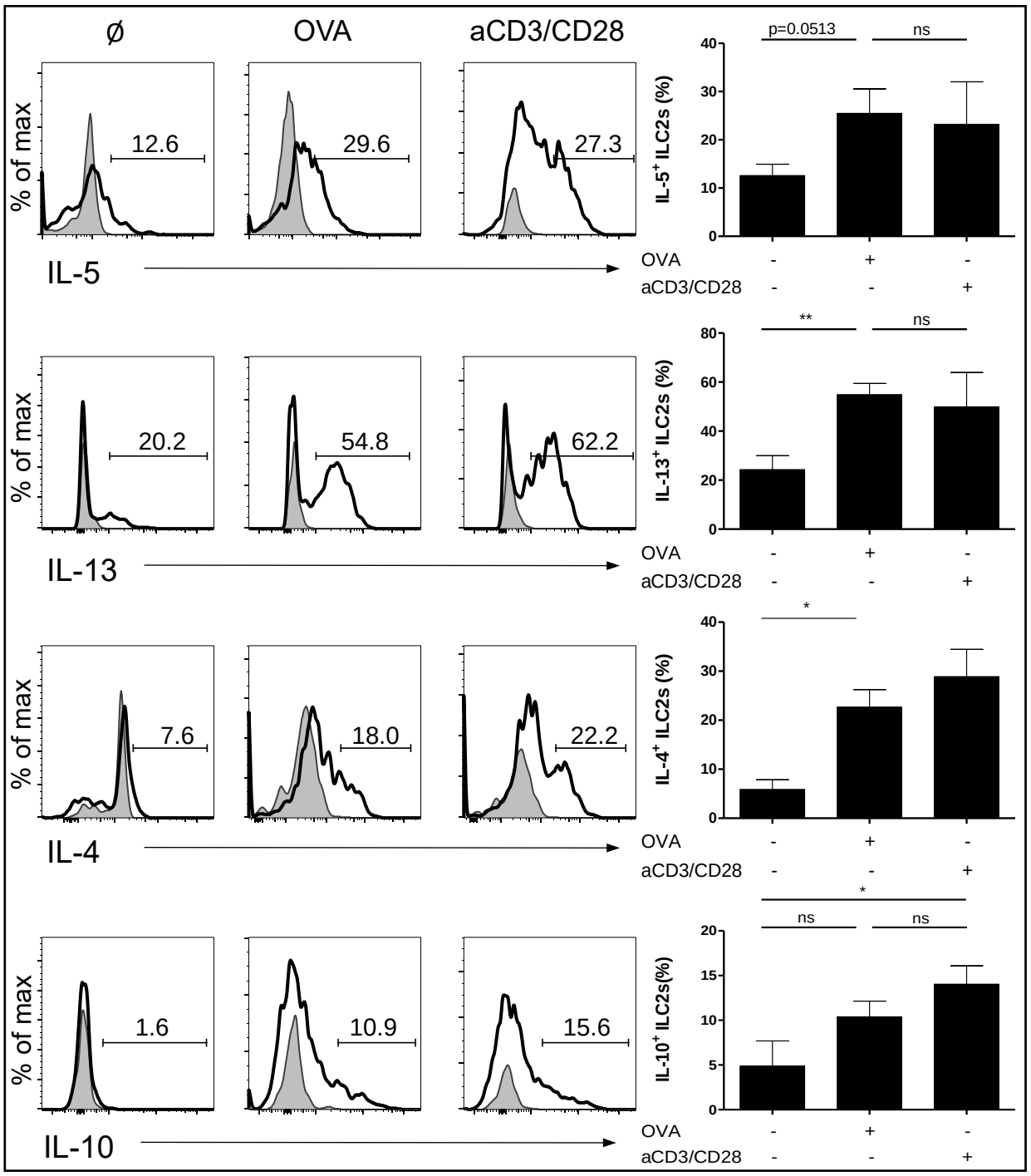

Figure 19: Co-culture of CD4+ T cells and hepatic ILC2s in the presence of OVA increases type 2 cytokine expression by ILC2s.

OVA-specific CD4+ T cells were co-cultured with hepatic ILC2s from IL-33-treated C57Bl/6 mice in the presence of OVA or anti-CD3/CD28 Dynabeads or in absence of any stiumulus (Ø) for four days. Frequencies of intracellular cytokine expression was determined by flowcytometry. Histograms show frequency of cytokine-producing ILC2s compared to FMO control. Mean ± SEM of three independent experiments are shown. *p<0.05; **p<0.01; ns, not significant.

3.3.5 Co-Culture of Hepatic ILC2s and CD4$^+$ T cells Do Not Induce IL-17A or IFNγ Production by Hepatic ILC2s

To see if the co-culture of ILC2s and CD4$^+$ T cells induces plasticity in hepatic ILC2s, the frequency of IL-17A- or IFNγ-expressing TCRβ$^-$ ILC2s was analyzed after 4 days of culture. In the presence of OVA, 1.1% (±1.0%) of ILC2s were IFNγ$^+$. This percentage did not differ significantly to ILC2s co-cultured in the absence of OVA (5.1% ±5.6%) or in the presence of aCD3/CD28 (2.5% ±2.5%).

The expression of IL-17A, which was seen in 6.0% (±4.4%) of the co-cultured ILC2s in absence of any stimulus, was even slightly but not significantly decreased upon stimulation with OVA (1.9% ±0.9%) or with aCD3/CD28 (3.4% ±2.1%) (Fig. 20).

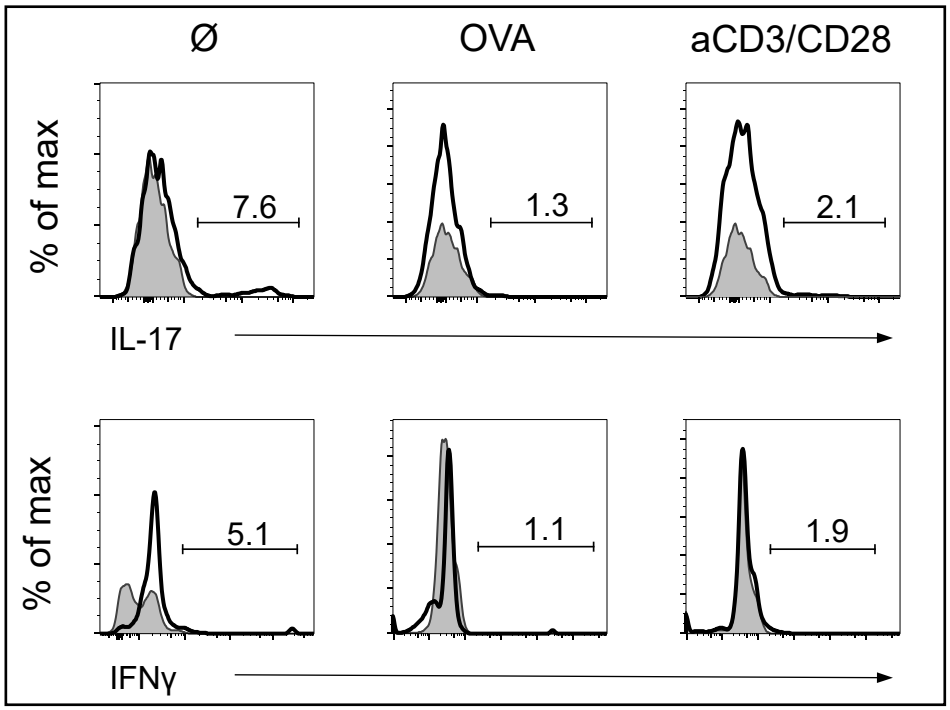

Figure 20: Co-culture of CD4+ T cells and hepatic ILC2s in the presence of OVA does not induce IL-17A or IFNγ production by ILC2s.

OVA-specific CD4+ T cells were co-cultured with hepatic ILC2s from IL-33-treated C57Bl/6 mice in the presence of OVA or anti-CD3/CD28 Dynabeads for four days. Intracellular cytokine expression by ILC2s was analyzed by flow cytometry. Histograms show frequency of cytokine-producing ILC2s compared to FMO control. Representative data of three independent experiments are shown.

3.3.6 Co-Culture of Hepatic ILC2s and CD4$^+$ T Cells Does Not Induce Substantial Cytokine Expression in CD4$^+$ T Cells

As co-culture of hepatic ILC2s and CD4$^+$ T cells led to antigen-specific expansion and activation of CD4$^+$ T cells, further the cytokine expression profile of ILC2-activated CD4$^+$ T cells was analysed. Increased levels of type 2 cytokines in co-culture supernatants raised the question whether CD4$^+$ T cells also contribute to type 2 cytokine production. The type 2 cytokines IL-5 and IL-13 were expressed by a small percentage of CD4$^+$ T cells in the absence of any stimulatory substance. 4.8% (±4.8%) of CD4$^+$ T cells expressed IL-5, while IL-13 was expressed by 2.2% (±2.2%). Stimulation via OVA or anti-CD3/CD28 did not increase the frequency of IL-5 (OVA: 2.1% ±2.7%; aCD3/CD28: 1.5% ±1.9%) and IL-13 (OVA: 1.9% ±2.5%; aCD3/CD28: 1.8% ±2.3%) producers under TCRβ$^+$ CD4$^+$ T cells. IL-4 was generally produced by a higher percentage of 14.2% (±22.6%) of CD4$^+$ T cells but also neither presence of OVA peptide (9.7% ±13.1%) nor aCD3/CD28 beads (11.4% ±15.7%) led to significant increase in the frequency of IL-4$^+$ CD4$^+$ T cells (Fig. 21).

The expression of typical pro- and anti-inflammatory cytokines of ILC2-activated CD4$^+$ T cells was analysed, but no significant increase in the frequency of IL-10-, IL-17A-, or IFNγ-expressing CD4$^+$ T cells was detected following antigen-specific (IL-10: 9.2% ±14.1%; IL-17A: 1.8% ±0.9%; IFNγ: 11.3% ±10.2%) or -unspecific stimulation (IL-10: 12.5% ±16.7%; IL-17A: 2.5% ±1.0%; IFNγ: 9.6% ±3.8%) (Fig. 21).

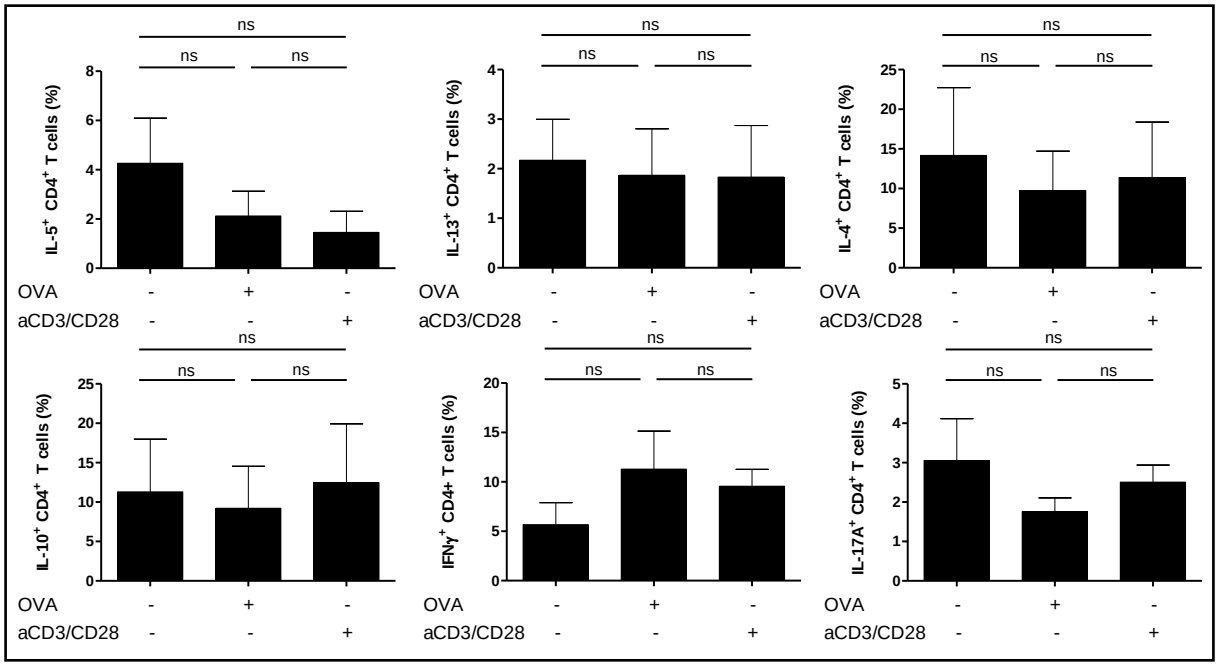

Figure 21: Co-culture of CD4+ T cells and hepatic ILC2s in the presence of OVA cells does not induce substantial cytokine response by CD4+ T cells.
OVA-specific CD4+ T cells were co-cultured with hepatic ILC2s from IL-33-treated C57Bl/6 mice in the presence of OVA or anti-CD3/CD28 Dynabeads for four days. Cytokine levels were analyzed by flow cytometry. Mean ± SEM of three independent experiments are shown. ns, not significant.

4 Discussion

Since ILC2s attracted the attention as important players in type 2 immunity, these cells have been studied intensively. Most of the knowledge about ILC2s was gained from research of ILC2s from the skin, the lung and the gut, as they are expressed in these organs in relatively high frequencies (Nussbaum et al., 2013). ILC2s do not undergo constant replenishment form the bone marrow but evolve from tissue-resident ILC2s resting in the respective organ. Thus, ILC2s are strongly influenced by microenvironmental changes. In the liver, ILC2s have first been described by Price et al. in 2010 (Price et al., 2010), but since then only a few studies addressed ILC2s in the pathogenesis of liver diseases, although there is solid evidence that ILC2s have a major impact on hepatic inflammation and tissue regeneration. Therefore, this study contributes to the knowledge about ILC2s in liver as well as their abilities to influence inflammatory processes in this organ.

4.1 Tissue-resident Hepatic ILC2s as Hepatic ILC2 Precursors?

This study shows ILC2s to be present in the liver under physiological conditions. From the entire pool of hepatic leukocytes, ILC2s constituted only an extremely small population in the steady state (Fig. 8). In the current opinion of ILC2 homeostasis, ILC2s maintain themselves by proliferation from tissue-resident pre-mature ILC2s and further expand in course of inflammation. Differentiation of ILC2s from haematogenous cells of the bone marrow only contributed to a minor degree to the maintenance of a matured ILC2 population (Gasteiger et al., 2015). As the surface marker KLRG1 is described as a marker of senescence and differentiation for T cells (Henson and Akbar, 2009) and also for ILC2s (Hoyler et al., 2012, Walker and McKenzie, 2013), KLRG1 expression of ILC2s from physiological livers was investigated. Furthermore, the activation marker CD25, which is a well-known to display T cell activation (M López-Cabrera, 1993) and moreover, is reported to be rapidly upregulated upon activation of ILC2s (Li et al., 2017), is of great interest, when it comes to characterization of tissue-resident hepatic ILC2s.

Concerning this, hepatic ILC2s of naive mice were shown to express the surface markers KLRG1 and CD25 on low levels (Fig. 9). The presence of cells, which phenotypically match ILC2s, but express markers of senescence and maturation only

on low levels indicates that these cells might act as tissue resident precursors to replenish the pool of mature ILC2s. Thus, the theory of ILC2 constitution by proliferation of tissue-resident premature ILC2s potentially also applies to the liver. Adaptive transfer experiments of tissue-resident ILC2s from physiological livers to $Rag2^{-/-}\gamma c^{-/-}$ mice, lacking T cells, B cells and ILC2s, should be performed to investigate if they give rise to a type 2 cytokine-producing ILC2 effector population *in vivo* and to confirm their role as ILC2 precursors in this way. ILC2 precursor cells are further described to lack type 2 cytokine production (Hoyler et al., 2012). Therefore, the cytokine profile of this $KLRG1^{low}$ $CD25^{low}$ ILC2s population from physiological livers should be checked to verify if they facilitate only the role of providing proliferative capacity for ILC2 maintenance and expansion or moreover, function as effector cells also under physiological conditions.

4.2 IL-33 as a Potent Activator of Hepatic ILC2s *in vivo*

To further identify the role of ILC2s in the liver, hepatic tissues were analyzed after daily injection of IL-33 for 4 days. Upon treatment with IL-33, the population of hepatic ILC2s expanded strongly (Fig. 8). IL-33-elicited ILC2s uniformly expressed KLRG1 and a majority co-expressed CD25 (Fig. 9). Given that these markers are linked with matureness and activation of T cells and ILC2s, the IL-33-dependent expansion of hepatic ILC2s identified IL-33 as a potent activator of hepatic ILC2s *in vivo*. As ILC2s are further described as potent producers of type 2 cytokines upon activation, the liver was analyzed for type 2 cytokine expression after IL-33 treatment. In hepatic tissue, mRNA levels of the type 2 cytokines IL-4, IL-5, IL-13, and the transcription factor GATA-3, which is characteristic for ILC2s and essential for their function and maintenance (Mjösberg et al., 2012) were increased (Fig. 10). Therefore, the activating effect of IL-33 on hepatic ILC2s was confirmed by flow cytometric analysis of hepatic leukocytes and verified the expression of mainly IL-5 but also IL-4 and IL-13 by ILC2s upon IL-33 treatment (Fig. 11). Thus, activation of hepatic ILC2s was shown to contribute to increased type 2 cytokine levels upon treatment with IL-33 *in vivo*.

Besides ILC2s, also other immune cells could provide type 2 cytokines upon IL-33 treatment. IL-33 is described to drive type 2 immune responses by cytokine induction of mast cells (Allakhverdi et al., 2007), basophils (Schneider et al., 2009), and

eosinophils (Cherry et al., 2008). Moreover, Th2 cells express the IL-33 receptor ST2 and IL-33 signaling was shown to promotes T-cell expansion, differentiation and cytokine production (Peine et al., 2016). Therefore, although the increase of type 2 cytokines in hepatic tissue upon injection with IL-33 is likely to be driven by expansion and activation of hepatic ILC2s, polarization of the hepatic milieu towards a Th2 phenotype cannot exclusively be drawn back to hepatic ILC2s. Nevertheless, IL-33-dependent expansion and type 2 cytokine production of hepatic ILC2s demonstrated that IL-33 can be potent activator of hepatic ILC2s *in vivo*. Increased GATA-3 expression in hepatic tissue along with increased type 2 cytokine levels indicate IL-33-driven influence of the hepatic microenvironment, most likely due to expansion and activation of hepatic ILC2s.

Furthermore, IL-17A expression by hepatic ILC2s isolated from IL-33-treated mice was shown (Fig. 11). As IL-17A expression is not described for tissue-resident ILC2s but for iILC2s, it is possible that these cells were included when analyzing lin^- $ST2^+$ $Sca\text{-}1^+$ cells. iILC2s are inflammation-induced ILC progenitors, which share phenotypical similarities with tissue resident ILC2s, but further have the ability to develop a tissue-resident ILC2-like phenotype or a ILC3-like phenotype. iILC2s are not detectable in the steady state but rise upon IL-25 signaling. Phenotypically they match tissue resident ILC2s. However, iILC2s are ST2- and only acquire ST2 expression upon stimulation with IL-25 (Huang et al., 2015). As hepatocytes constitutively express IL-25, the liver is a IL-25 rich organ (Sarra et al., 2013) enabling constant exposure of hepatic ILC2s to IL-25. IL-25 is also highly produced by Th2 polarized T cells (Fort et al., 2001) and IL-33 is known to reinforce Th2 differentiation and T cell proliferation (Peine et al., 2016). Besides this, IL-33 is described to activate mast cells (Saluja et al., 2015) and basophils (Suzukawa et al., 2008), which are potential sources of IL-25 (Saadoun et al., 2011). Thus, IL-33-induced activation of IL-25 producers might have led to induction of iILC2s, which were displayed by a small percentage of IL-17A-producing hepatic ILC2s. To verify this assumption, IL-25 levels should be evaluated upon IL-33 injection and IL-17A-producing ILC2s should be further characterized regarding co-expression of IL-13, responsiveness to IL-33 and surface markers, which can be used to differentiate iILC2s form tissue resident ILC2s, as IL-7Rα, c-Kit, Thy1.1, IL-17 RB (Huang and Paul, 2016).

Surprisingly, also TNFα production was detected by IL-33-elicited hepatic ILC2s (Fig. 11). TNFα is not described to be produced by ILC2s, but by ILC1s. However, IFNγ is characteristic for ILC1s and predominantly produced by these cells (Jiao et al., 2016). As no IFNγ production was observed in the investigated ILC population (Fig. 11), a contamination with ILC1s can be ruled out. The production of cytokines not commonly known for ILC2 might be an expression of their hepatic origin. Therefore, hepatic ILC2s might facilitate further functions differing for ILC2s of other organs. To target the roles of these cytokines in hepatic inflammation reconstitution of ILC2s from mice linages, unable to produce TNFα, to ILC2-free mice could provide insights about ILC2 derived TNFα upon hepatic inflammation.

However, the production of typical type 2 cytokines dominated the phenotype of hepatic ILC2s upon IL-33 challenge (Fig. 11). Taken together, this data shows the microenvironmental influence of IL-33 on hepatic ILC2s, resulting in proliferation and activation and vice versa hepatic ILC2s to contribute to microenvironmental changes upon this inflammatory stimulus.

4.3 Hepatic ILC2s in a Typical Type 1 Disease

IL-33 is further hypothesized to lead to ILC2 expansion and cytokine response in Con A-mediated hepatitis (Neumann et al., 2017). As the microenvironment is described to have a major impact on ILC2 maintenance and function (McKenzie et al., 2014, Diefenbach et al., 2014), it was investigated, whether other cytokines, which are prominent in Con A-induced hepatitis, influence ILC2s. After challenge with Con A, mRNA levels of IL-33, IL-12 and IFNγ were strongly increased 8 h after induction of hepatitis (Fig. 12), which are all described to influence ILC2s. To clearly identify how each cytokine affects hepatic ILC2 function and how these cytokines collude, ILC2s were cultured under the supportive conditions (IL-2 and IL-7) and in presence of IL-33, IL-12, IFNγ or in combination.

Similar to the results *in vivo*, induction of proliferation and activation of hepatic ILC2s by IL-33 was also observed *in vitro* (Fig. 13). Further IL-33 was shown to have a great impact on the type 2 cytokine expression. In the supernatants of IL-33-stimulated hepatic ILC2s, levels of IL-5 and IL-13 were strongly increased. This was also displayed by an increased percentage of IL-5^+ and IL-13^+ ILC2s. IL-33 not only led to an increase

in the frequency of type 2 cytokine-producing ILC2s but also to an enhanced relative mean fluorescence intensity of IL-5 and IL-13, indicating increased cytokine production of IL-5$^+$ or IL-13$^+$ ILC2s (Fig. 14). In line with the literature, IL-33 was confirmed as a potent inducer of hepatic ILC2 proliferation, activation, and type 2 cytokine production *in vivo* and *in vitro* (Moro et al., 2010, Neill et al., 2010, Price et al., 2010, Mjösberg et al., 2011, McHedlidze et al., 2013) .

IL-12 is controversy described to influence ILC2s. Lim et al. detected plasticity in human ILC2s from the blood upon stimulation with IL-12. In *in vitro* cultures of human ILC2s, they observed co-expression of IL-13 and IFNγ. IL-12 significantly increased IFNγ expression by clones of human ILC2s and furthermore, ILC2s from IL-12Rβ-deficient patients failed to express IFNγ, indicating IL-12 to induce plasticity in human ILC2s (Lim et al., 2016). However, in mice, Ohne et al. found IL-12 to diminish the viability of ILC2s. A IFNγ-inducing effect of IL-12 was dependent on previous treatment with IL-1β, whereas IL-12 alone had no effect on IFNγ mRNA expression of ILC2s (Ohne et al., 2016). In this study, no effect of IL-12 was observed on hepatic ILC2s. IL-12 itself did neither induce expansion, increased expression of activation markers, nor did it modulate the cytokine profile of hepatic ILC2s. Furthermore, it did not modulate the effect of IL-33 on hepatic ILC2s. This indicates that IL-12 does not influence the phenotype of hepatic ILC2s *in vitro* and supports the assumption that IL-12 plays a subordinate role in Con A hepatitis regarding the influence of ILC2s. However, since IL-1β is also elevated in Con A-mediated hepatitis (Mizuhara et al., 1994), the ability of IL-12 and IL-1β to induce plasticity in hepatic ILC2s should be further evaluated, when present contemporaneously.

IFNγ is described to be a suppressor of ILC2 function. IFNγ signaling via IFNγ-receptor 1 suppressed ILC2 proliferation and type 2 cytokine response *in vitro* (Moro et al., 2016). *In vivo*, IFNγ deficiency led to enhanced type 2 responses upon infection with *N. brasiliensis* displayed by increased numbers of ILC2s, eosinophils and levels of IL-5 and IL-13 in the bronchoalveolar lavage fluid (Moro et al., 2016). Yeti mice, which constitutively overexpress IFNγ, showed reduced clearance of *N. brasiliensis* as well as reduced numbers of ILC2s in the lung (Molofsky et al., 2015). In the model of Con A-induced hepatitis a strong Th1 cell-driven hepatic inflammation was observed (Wang

et al., 2012a). Given that IFNγ-deficient mice failed to develop hepatitis upon challenge with Con A (Kato et al., 2013), IFNγ plays a major role in the pathogenesis of Con A-induced hepatitis. Despite the suppressive effect described for IFNγ on ILC2s, previous work showed that ILC2s have a strong proinflammatory role in Con A-induced hepatitis (Neumann et al., 2017). To address this contradiction, this study investigated the effect of IFNγ on hepatic ILC2s *in vitro*. In line with the literature, suppression of ILC2 expansion was observed upon presence of IFNγ (Fig. 13 A). Although the expression of the activation marker CD25 did not significantly differ from hepatic ILC2 cultured in presence of IL-33, the presence of IFNγ was also associated with decreased overall cytokine levels in the supernatants (Fig. 14 B). Decreased IL-5 production was not displayed in the percentages of IL-5$^+$ ILC2s, but hepatic ILC2s cultured with IFNγ showed decreased MFI of IL-5. This suggests IFNγ to influence the functional ability to produce IL-5. Further decreased levels of IL-13 were detected in the supernatants of hepatic ILC2s cultured in presence of IFNγ, which was not seen on cellular levels. Neither the frequencies of IL-13$^+$ ILC2s nor the MFI was decreased in presence of IFNγ (Fig. 14 B). As the suppressive effect of IFNγ could exclusively be seen on cellular levels with regard to IL-5, it is likely that IFNγ decreased the functional ability to produce IL-5. Besides IL-5, IFNγ-mediated reduction of cytokine levels seems to be restricted to the ability of IFNγ to suppress ILC2 expansion. Hereby, IFNγ decreased the number of effector cells that contribute to cytokine production.

Importantly, IL-33 abrogated the suppressive effect of IFNγ and restored ILC2 expansion (Fig. 13). Further, hepatic ILC2s, which were cultured in presence of IL-33 and IFNγ, showed levels of IL-5 and IL-13, which did not significantly differ from ILC2s, which were cultured with IL-33 alone (Fig. 14). This shows that IL-33 overcame the suppressing effect of IFNγ *in vitro* and might serve as an explanation why hepatic ILC2s can act as important effector cells despite the presence of IFNγ in Con A-mediated hepatitis.

4.4 Hepatic ILC2s- More than a Source of Type 2 Cytokines

Besides the type 2 cytokines IL-5 and IL-13, several different cytokines were detectable in the supernatants of hepatic ILC2 cultures upon activation with IL-33. Interestingly, IL-6 was also detected in the supernatants of hepatic ILC2s on similar levels as IL-5 and IL-13. Already when cultured under supportive conditions, hepatic ILC2s produced IL-6, which dramatically increased in presence of IL-33 (Fig. 14 B).

Although IL-6 production by ILC2s was described since one of the very first descriptions of ILC2s (Moro et al., 2010), the role of IL-6 production was never pointed out, most likely due to the predominant effector function of ILC2-derived IL-5 and IL-13. IL-6 is secreted by innate immune cells such as neutrophils and macrophages upon toll-like receptor stimulation. In the liver, hepatocytes constitutively express the IL-6 receptor (IL-6R) and IL-6/IL-6R signaling induces the production of acute phase proteins in the liver (Schmidt-Arras and Rose-John, 2016). Acute phase proteins are a complex system of the early immune defense driving pathogen clearance, preventing dissimilation of infection and contributing to resolution by leukocytosis, complement activation, protease inhibition, opsonization and clotting (Cray et al., 2009). In this doing, IL-6 is a strong pro-inflammatory cytokine and a key factor of transition from innate to adaptive immune responses. In the liver, IL-6 is assigned to a special role as IL-6 is also shown to be essential for liver regeneration upon damage. Upon partial hepatectomy, non-parenchymal cells such as KCs and HSC produced TNFα and IL-6 thereby inducing hepatocytes to enter the cell cycle and start to proliferate (Taub, 2004). IL-6 knockout mice showed impaired liver regeneration (Cressman et al., 1996). Regarding Con A-induced hepatitis, IL-6 is described to be enriched in the sera of Con A-treated mice 4 hours after injection (Cao et al., 1998) and to have pro- and anti-inflammatory properties. IL-6-deficient mice and mice treated with anti-IL-6 antibodies showed less necrotic lesions in the liver 48 h after Con A injection and significantly decreased ALT levels in early phase of inflammation. IL-6 deficiency was also associated with decreased numbers of neutrophils in the liver of Con A-treated animals, which were also shown to be important for the development of Con A-hepatitis (Bonder et al., 2004, Malchow et al., 2011). Besides this pro-inflammatory role, IL-6 was also shown to have protective effects: IL-6 induces STAT3-dependent up-regulation of the

anti-apoptotic maker Bcl-X_L on hepatocytes (Hong et al., 2002, Tiegs, 2007). Given that injection of IL-6 before challenge with Con A prevented T cell-mediated hepatitis (Mizuhara et al., 1994, Klein et al., 2005) whereas injection of IL-6 after Con A treatment facilitated a hepato-destructive effect (Malchow et al., 2011) indicates differential roles for IL-6 along Con A-induced hepatitis. Taken together, IL-6 provides both pro- and anti-inflammatory capacities upon liver inflammation. Upon liver injury, macrophages, HSC and $CD4^+$ T cells were reported to be sources of IL-6 (Schmidt-Arras and Rose-John, 2016, Cao et al., 1998). In this study hepatic ILC2s were identified as potent IL-6 producers upon IL-33 stimulation, which suggests that hepatic ILC2s contribute to IL-6-mediated immunity in the liver.

Moreover, on markedly lower but still substantial level, IL-9 was detectable in the supernatants of hepatic ILC2s when cultured in presence of IL-33 (Fig. 14 B). This goes in line with previous reports about IL-9 production by ILC2s (Wilhelm et al., 2011, Turner et al., 2013, Mohapatra et al., 2016). IL-9 is described to promote ILC2 survival and accumulation as well as type 2 cytokine production by ILC2s (Wilhelm et al., 2011, Turner et al., 2013) also in an autocrine manner (Mohapatra et al., 2016). Further, IL-9 is known to be a pleiotropic cytokine that promotes growth of $CD4^+$ T cells, their type 2 cytokine production as well as Th17 and T_{reg} development and also to promote development and function of B cells and mast cells (Goswami and Kaplan, 2011). Moreover, IL-9 was shown to drive eosinophilia in the liver of parasite infection with *Schistosoma mansoni*, as transgenic mice with constitutive expression of IL-9 increased eosinophilia in the liver (Fallon et al., 2000). Here, hepatic ILC2s were identified as sources of IL-9 upon IL-33 challenge *in vitro* (Fig. 14 B). ILC2-derived IL-9 might contribute to Th2 response by autocrine promotion of ILC2 survival, accumulation and Th2 cytokine response or effecting other immune cells as T cells, B cells and eosinophils in the hepatic tissue.

Besides this, surprisingly IL-10 was also detectable in the supernatants of IL-33-activated hepatic ILC2s (Fig. 14 B). In line with this finding, a population of linage-negative, IL-10-producing ILCs was reported in a model of contact hypersensitivity (Kim et al., 2016) showing phenotypical similarities with ILC2s. The existence of an IL-10-producing ILC2 effector population was also described by Seehus et al., which

identified IL-10$^+$ ILC2s in the lungs of mice after, similar to the experimental set up of this study, treatment with IL-33 on 4 consecutive days. IL-2 increased the ability of IL-33 to elicit IL-10-producing ILC2s. In contrast to IL-10$^-$ ILC2s, IL-10-producing ILC2s lacked the surface marker Thy-1.1, thus Seehus et al. referred Thy-1.1$^-$ ILC2s as $ILC2_{10}$. IL-10$^+$ ILC2s were further associated with the downregulation of inflammatory genes, such as *Tnf*, *Lta*, *Il2* and *Ccll* and generation of IL-10-producing ILC2s by injection of IL-33 and IL-2 decreased eosinophil recruitment *in vivo* (Seehus et al., 2017). To confirm if IL-10-producing ILC2s from the liver correspond to $ILC2_{10}$ found in the lung hepatic IL-10$^+$ ILC2s should be further characterized regarding their expression of surface markers such as Thy-1.1 and their gene expression profile.
Besides representing a subpopulation of ILC2s, IL-10 production of ILC2s might be a mechanism of maintaining tissue homeostasis. IL-10 is an anti-inflammatory cytokine, which reduces production of pro-inflammatory cytokines by T cells and monocytes as well as T-cell activation (Iyer and Cheng, 2012). IL-10 was also described to reduce cytokine secretion by ILC2s and hereby facilitated suppressive effects of T_{regs} on ILC2s (Rigas et al., 2017). Further ILC2s were observed to produce IL-10 only upon an strong inflammatory stimulus such as treatment with IL-33 for several days or chronic inflammatory conditions as allergic responses to treatment with papain (Seehus et al., 2017). Therefore, IL-10 production might be part of a negative feedback mechanism of strongly activated ILC2s to prevent exaggerated immune responses. In the liver, macrophages, KCs (Knolle et al., 1995) as well as T and B cells (Alfrey et al., 1995) are main sources of IL-10 (Louis et al., 1997). In Con A-induced hepatitis, IL-10 regulates TNFα, IFNγ and IL-12 levels. Injection of exogenous IL-10 decreases levels of this pro-inflammatory cytokines and reduces hepatotoxicity of Con A. Furthermore, IL-10 also contributes to tolerance induction upon re-challenge with Con A (Erhardt et al., 2007). As these data shows that hepatic ILC2s can be sources of IL-10 upon IL-33 challenge, hepatic ILC2s might also contribute to the IL-10-mediated anti-inflammatory effects in the liver. This might be facilitated via autocrine or paracrine inhibition of pro-inflammatory cytokine production and inhibition of proliferation or activation of effector cells to prohibit inflammation or to induce tolerance (Fig. 22).

In this study, IL-22 was also detectable on very low but measurable levels in the supernatants of hepatic ILC2s, which were cultured in presence of IL-33 (Fig. 14 B).

IL-22 is typically associated with ILC3 function and in combination with IL-17, it is an indicator cytokine for group 3 ILCs (Cortez et al., 2015, Sawa et al., 2010). As both IL-17A and IL-22 expression were detected on very low levels in hepatic ILC2s, a contamination of ILC2s with ILC3s needs to be considered. The facts that IL-17A and IL-22 production was only seen upon stimulation with IL-33, which is not known to induce cytokine production of ILC3s, and cultures of hepatic ILC2s showed purities of 99.8 %, make this assumption implausible. However, IL-22 production of ILC2s were described by Mjösberg et al. They found a minority of prostaglandin D2 receptor $(CRTH2)^+$ ILC2s from the human blood to express IL-22. All IL-22^+ cells co-expressed IL-13 (Mjösberg et al., 2011). The appearance of IL-22 in the supernatants of hepatic ILC2s upon IL-33 challenge indicates that there might exists a cellular equivalent for this population in mice. To confirm this assumption IL-22 expression as well as co-expression of IL-13 of hepatic ILC2s should be investigated by flowcytometry upon activation with IL-33. In liver immunity, IL-22 mediates a hepatoprotective effect (Pan et al., 2014). IL-22 can be provided by T cells, RORγt^+ innate lymphoid cells, including LTi-like cells, and hepatic mononuclear cells (Pan et al., 2014). In Con A-induced hepatitis, increased IL-22 expression can be measured starting 1 h after injection of Con A. Neutralization of IL-22 aggravated Con A-induced hepatitis, whereas administration of exogenous IL-22 or transgenic mice, which constitutively overexpress IL-22, were prevented from Con A-induced injury (Pan et al., 2014). The protective effect in the liver is mediated by STAT3-dependent upregulation of anti-apoptotic genes, preventing the death of hepatocytes (Radaeva et al., 2004). As IL-22-production was preserved by hepatic ILC2s *in vitro* (Fig. 14 B), ILC2s might contribute to hepatoprotection upon liver damage in this way.

Regarding the cytokines IL-6, IL-9, IL-10 and IL-22, presence of IL-12 in the cultures did not influence the cytokine-inducing effect of IL-33. In a similar manner, IFNγ alone did neither induce nor modulate the production of these cytokines. However, in contrast to IL-5 and IL-13, hepatic ILC2s, which were cultured with IL-33 and IFNγ, showed decreased levels of IL-6, IL-9, IL-10 and IL-22 compared to the cytokine levels of ILC2s cultured with IL-33 alone. None of the conditions led to specific changes in the cytokine profile of hepatic ILC2s towards a Th1 or Th17 phenotype by markedly production of IFNγ or IL-17A (Fig. 15). Thus, none of the tested cytokines induced functional plasticity

in hepatic ILC2s. Taken together, these data underline the predominant role of hepatic ILC2s as producers of type 2 cytokines. Furthermore, the findings of the capacities of hepatic ILC2s to produce cytokines, such as IL-6, IL-9, IL-10 and IL-22, are congruent with other reports and in many cases, support the Th2-promoting character of hepatic ILC2s in an indirect manner or might facilitate a tissue-repairing effect of ILC2s.

4.5 Hepatic ILC2s in their Role as APCs

$CD4^+$ T cells are the main actors in Con A-mediated hepatitis (Tiegs et al., 1992) and ILC2s were shown to have the ability of antigen processing and presentation (Oliphant et al., 2014). Hence, the interplay of $CD4^+$ T cells and hepatic ILC2s are a key issue when it comes to investigating the role of ILC2s in immune-mediated hepatitis. Besides sufficient promotion of expansion, activation and type 2 cytokine production of hepatic ILC2s, IL-33 also influenced the expression of surface markers linked with T cell activation on hepatic ILC2s. This study showed an upregulation of MHC class II and ICOS expression upon activation via IL-33 (Fig. 16 A). IL-33-elicited hepatic ILC2s further showed high levels of OX40L intracellularly (Fig. 16 B). As IL-33 promotes the effector function of ILC2s, increased expression suggests a functional role of this surface markers. Antigen presentation via MHC class II and its binding with the TCR initiates antigen-specific T cell differentiation and proliferation of a naive T cells. Linage-specific differentiation further depends on the cytokine milieu, type of APC and co-stimulatory signals, which can be provided by the surface markers CD80 and CD86, ICOS-L and OX-40-L (Luckheeram et al., 2012). As ILC2s were described to have APC ability (Mirchandani et al., 2014, Oliphant et al., 2014), the question, if hepatic ILC2s might serve as APCs in the liver and upon liver inflammation was addressed by performing co-cultures of hepatic ILC2s and $CD4^+$ T cells. To determine if hepatic ILC2s could activate $CD4^+$ T cells by antigen presentation via MHC class II, ILC2s were cultured with OVA-specific $CD4^+$ T cells in absence and presence of OVA. Here antigen presentation of OVA by hepatic ILC2s to $CD4^+$ T cells was found to induce substantial proliferation and activation of both cell types (Fig. 17) and an increased cytokine response, displayed by elevated levels of type 2 cytokines IL-5 and IL-13 in the supernatants of the co-culture (Fig. 18). To identify the main sources of IL-5 and IL-13, cytokine expression on cellular levels was checked via flow cytometry and only hepatic

ILC2s, but not CD4$^+$ T cells, were found to express IL-5 or IL-13 in presence of OVA (Fig. 19, Fig. 21). This finding goes in line with many reports identifying ILC2s as main sources of type 2 cytokines upon interaction with CD4$^+$ T cells (Oliphant et al., 2014, Halim et al., 2014, Drake et al., 2014, Mirchandani et al., 2014).

In contrast, the inability of CD4$^+$ T cells to contribute to type 2 cytokine production contradicts with various reports of ILC2s to drive Th2-cell polarization of CD4$^+$ T cells (Oliphant et al., 2014, Mirchandani et al., 2014, Drake et al., 2014). Given that hepatic ILC2s expressed CD80/CD86 complex only on low levels (Fig. 16) and therefore co-stimulation via these molecules might not be sufficiently provided by hepatic ILC2s, aCD3/CD28-beads were added to some of the co-cultures, to see if this additional stimulation influences the phenotype of CD4$^+$ T cells. But again, CD4$^+$ T cells cultured in presence of aCD3/CD28 did not show significant increases in cytokine expression (Fig. 21). As additional co-stimulation via CD80/86 did not lead to increased cytokine expression in CD4$^+$ T cells, lack of co-stimulatory signal via CD80/CD86 is unlikely to be responsible for missing cytokine production of CD4$^+$ T cells.

As there was no significant cytokine production of CD4$^+$ T cells upon antigen-specific activation by hepatic ILC2s, a shift of CD4$^+$ T cells into a status of T-cell anergy is conceivable. Anergy is a state of T cells, in which T cells do not further exert inflammatory function by production of effector cytokines, but persist for an extended time period. This hyporesponsive state is caused by incomplete T-cell activation as missing co-stimulatory signals after antigen encounter. T-cell anergy is an important mechanism of peripheral tolerance (Schwartz, 2003). A shift into T-cell anergy by insufficient co-stimulation via CD80/86, can be ruled out, since the additional presence of aCD3/CD28 did not modulate T-cell proliferation or cytokine production.

Another mechanism to induce T-cell anergy is the presence of IL-10, which was also detectable on low levels in the supernatants of co-culture of hepatic ILC2s and CD4$^+$ T cells (Fig. 18). IL-10 was shown to inhibit T-cell proliferation and production of cytokines and in this way induced a long-lasting anergic state (Groux et al., 1996). As IL-10 was also observed in the supernatants of the co-culture (Fig. 18), induction of anergy besides sufficient co-stimulation is conceivable. Moreover, T-cell anergy can be also induced by co-inhibition (Schwartz, 2003). This could be facilitated by PD-L1, which

was detected on IL-33-elicited hepatic ILC2s (Fig. 16 B). PD-L1 is a co-inhibitory molecule, which is reported to inhibit cytokine response of $CD4^+$ T cells upon an inflammatory stimulus (Konkel et al., 2010). Thus, PD-1/PD-L1 signaling might suppress type 2 cytokine production of $CD4^+$ T cells. Blocking antibodies against PD-L1 could approve the option of a suppressing effect of PD-L1 by hepatic ILC2 co-cultures. Unlike Groux et al. or Konkel et al., inhibition of T-cell proliferation in the anergic state was not observed, but even a significant increased proliferation of T cells was shown (Fig. 17 B, D). This indicates that neither IL-10 nor co-inhibition via PD-L1 induced T-cell anergy in the co-cultures. Taken together, T-cell anergy can be ruled out as a reason for the lack of cytokine response by hepatic ILC2-stimulated $CD4^+$ T cells.

However, in the studies of Oliphant et al., Mirchandani et al. and Drake et al. only moderate levels of type 2 cytokine-producing T cells were reported. Mirchandani et al. reported that about 12% of $CD4^+$ T cells of BALB/c mice expressed IL-13 or IL-5 whereby the differences in the mice strains limits the comparability with the findings of this study, as BALB/c mice are known to be more likely to express a Th2 phenotype than C57Bl/6 mice (Hsieh et al., 1995, Mills et al., 2000, Watanabe et al., 2004).

For induction of a sufficient Th2 response in health and disease IL-4 is central, as it has a pivotal role in shaping the immune system by driving Th2 differentiation in $CD4^+$ T cells (Choi and Reiser, 1998). The absence of IL-4 signaling in IL-4-deficient mice abrogated Th2 cytokine response completely *in vitro* and significantly impaired Th2 cytokine production *in vivo* (Kopf et al., 1993). In this study, only small amounts of IL-4 were observed in the co-culture and monoculture of ILC2s. In the supernatants of co-cultures, IL-4 levels were only significantly increased upon presence of aCD3/CD28 (Fig. 18) and the frequency of $IL\text{-}4^+$ hepatic ILC2s was not significantly increased until day 5 of co-culture (Fig. 19). Upon IL-33 treatment, IL-4 expression of hepatic ILC2s was minor, displayed in low levels of $IL\text{-}4^+$ IL-33-activated ILC2s (Fig. 11). As IL-4 is pivotal for Th2 differentiation and ILC2-derived IL-4 has already been shown to be critical in ILC2/T cell interaction, as abrogating ILC2 derived IL-4 signaling decreased type 2 cytokine levels in ILC2/T cell co-cultures (Drake et al., 2014), the lack of Th2 cytokine producing $CD4^+$ T cells might be due to low IL-4 levels, which did not induce sufficient differentiation towards an Th2 phenotype. The importance of the genetic

background for IL-4 expression can be seen in a model of infection with the intracellular parasite *Leishmania major*. As C57Bl/6 mice resisted infection by initiation of Th1 immune response, BALB/c mice, predisposed for IL-4 production, did not withstand leishmaniosis. The importance of IL-4 for differentiation of T cells was further shown by treatment with anti-IL-4 antibodies, which enabled BALB/c mice to overcome the infection (Choi and Reiser, 1998). Thus, the restraint of $CD4^+$ T cells to produce type 2 cytokines might be linked to insufficient IL-4 production of hepatic ILC2s, but in Con A-mediated hepatitis, IL-4 might be provided by other innate immune cells such as basophils or mast cells.

In contrast, Oliphant et al showed $CD4^+$ T cells from transgenic OT II-C57Bl/6 mice to contribute to Th2 response after co-culture. On close inspection only a very low proportion of about 2% of $CD4^+$ T cells expressed IL-13 and from 1 x 10^4 T cells co-cultured with ILC2s only a total number of about 100 $CD4^+$ T cells were shown to express IL-13 or IL-4 (Oliphant et al., 2014). In this study, $CD4^+$ T cells were isolated via MACS and checked for purity reaching about 90%. Thus, other APCs such as DCs or macrophages might have been included. Besides the cytokine milieu, also biology and affinity of the antigen as well as biology of the presenting APC determine the Th differentiation of naive T cells (Kaiko et al., 2008). As type 2 cytokine production after co-culture with ILC2s was seen only in a small population of $CD4^+$ T cells from OT II-C57Bl/6- mice (Oliphant et al., 2014), the type 2 cytokine inducing effect in the culture might be missed, due to cells interfering ILC2/$CD4^+$ T cell-interaction. Consequently, a contribution of interfering cells to the induction of expansion and activation of $CD4^+$ T cells and ILC2s cannot be ruled out. Based on this experience, experiments should be repeated using FACS-sorted $CD4^+$ T cells for co-culture with hepatic ILC2s to further increase the purity of the $CD4^+$ T cell population. In this doing, confounding factors could be excluded and the resulting findings could exclusively be drawn back to either T cells or ILC2s and their antigen-specific interaction.

Notably, no differences of the cytokine levels were observed regarding the frequency or levels of cytokine expression in dependence of antigen-specific interaction via OVA or antigen-unspecific activation via aCD3/CD28. Additional presence of aCD3/CD28 did not further increase cytokine production, activation or proliferation, indicating, that

antigen-specific interaction of hepatic ILC2s and $CD4^+$ T cells is equivalently efficient in T-cell activation. This shows that antigen presentation via hepatic ILC2s was sufficient in inducing activation and proliferation of $CD4^+$ T cells on its own and further, as hepatic ILC2s did express CD80 and CD86 only on low extent, that this effect was presumably not facilitated by co-stimulation via these molecules. Furthermore, OX-40-L was detected by intracellular staining in flow cytometry. Given that OX-40/OX-40-L signaling is already described by Drake et al. to play a key role in the interplay of ILC2s and T cells (Drake et al., 2014), co-stimulation might be provided by these molecules. To evaluate the role of OX-40 and OX-40-L the expression should be quantified via flow cytometry upon co-culture on ILC2s and $CD4^+$ T cells. Further blocking antibodies for OX-40/OX-40-L signaling could be used to investigate their impact on activation and proliferation of both cell types upon co-culture.

As IL-33-elicited hepatic ILC2s expressed high amounts of ICOS (Fig. 16 A) but only low frequencies of ICOS-L, co-stimulation of $CD4^+$ T cells could have not been provided by ICOS-L. However, ICOS/ICOS-L-signaling might be a way of ILC2 activation. As ICOS-L expression is described for B cells, DCs, macrophages, epithelial cells and T cells (Khayyamian et al., 2002), $CD4^+$ T cells should be checked for ICOS-L expression after co-culture.

Importantly OVA-specific interaction of $CD4^+$ T cells and hepatic ILC2s also activated ILC2s to initiate a sufficient type 2 immune response. Besides sufficient activation and proliferation of hepatic ILC2s (Fig. 17 E, F) production of type 2 cytokines was displayed in significantly increased levels of IL-5 and IL-13 in the supernatants upon co-culture of hepatic ILC2s and $CD4^+$ T cells (Fig. 18). As flow cytometry showed increased percentages of $IL\text{-}5^+$ and $IL\text{-}13^+$ ILC2s, ILC2s could be identified as cellular sources of type 2 cytokines (Fig. 19). For ILC2 maintenance and their proliferation IL-2 and IL-7 are important factors (Moro et al., 2010). As these cytokines were not added to the cultures, these cytokines must have been provided from elsewhere. $CD4^+$ T cells are known to be predominant sources of IL-2 and further IL-2 production occurs upon TCR-signaling and co-stimulation of T cells (Malek, 2008). Thus, auto- and paracrine IL-2 signaling is a possible explanation for proliferation of ILC2s and $CD4^+$ T cells upon OVA-specific interaction. In contrast, IL-7 is mainly produced by stromal cells (Capitini

et al., 2009), which *in vivo* could be provided by hepatocytes (Sawa et al., 2009). As sufficient activation and proliferation of $CD4^+$ T cells and ILC2s was observed in *in vitro* co-culture, either IL-7 signaling plays a subordinate role in maintenance of hepatic ILC2s and $CD4^+$ T cells upon antigen-specific interaction or IL-7 could have been provided by either one of these cells. To clarify this, ILC2s and $CD4^+$ T cells could be examined for their mRNA expression of IL-2 and IL-7 after co-culture.

Taken together interaction between hepatic ILC2s and $CD4^+$ T cells can be facilitated via the humoral response, such as cytokine secretion, or direct cell-cell interaction. In this study MHC II-mediated antigen presentation of OVA by hepatic ILC2s was addressed. Given that antigen-specific interaction results in sufficient activation and proliferation of both cell types (Fig.17) and a sufficient type 2 cytokine response (Fig. 18), antigen-specific activation of T cells and ILC2s are shown to influence of the hepatic microenvironment. Besides a sufficient type 2 cytokine response, antigen-specific activation induced production of strongly proinflammatory cytokines such as IL-6, IFNγ and TNFα, but also anti-inflammatory IL-10 (Fig. 18), whose possible roles in hepatic inflammation is discussed in Chapter 4.6. To further confirm the importance of physical proximity, which is needed for cell-cell interaction, transwell experiments should be performed. Besides MHC class II, multiple surface markers such as ICOS and OX-40-L are detectable on IL-33-elicited ILC2s (Fig. 16), which are reported to facilitate proliferation of either T cells or ILC2s. Therefore, they could have enabled the promotion on proinflammatory effects by increasing the number of effector cells. To graduate their impact on ILC2/T-cell interaction experiments should be repeated with blocking antibodies against these surface markers.

In conclusion, antigen-specific activation by OVA in co-cultures of hepatic ILC2s and $CD4^+$ T cells showed a proinflammatory effects displayed by proliferation and activation of the involved effector cells and a powerful type 2 cytokine response. Therefore, this study shows that antigen-specific interaction of $CD4^+$ T cells and hepatic ILC2s was capable to contribute to the cytokine milieu in the liver, to reciprocally support proliferation and activation and therefore, to drive liver inflammation.

4.6 Proposed Role of Hepatic ILC2s in Con A-mediated Hepatitis

As this study revealed new insights regarding the immunoregulatory capabilities of hepatic ILC2s, this data needs to be integrated regarding their role in liver inflammation. Under physiological conditions, only a very small population of ILC2s were present in the liver. Upon treatment with Con A, hepatic tissue was enriched with the cytokines IL-33, IFNγ and IL-12 (Fig. 12). Especially IL-33 and IFNγ were described to have opposing effects on ILC2s as they promote and suppress ILC2s, respectively. By culturing hepatic ILC2s with the cytokines present in the inflamed liver, it could be shown that indeed IL-33 and IFNγ facilitate opposing roles on hepatic ILC2s. Most importantly, IL-33 was able to reverse the suppressing effect of IFNγ. This finding serves as an explanation why ILC2s have functional relevance in Th1 driven diseases such as Con A-induced hepatitis and goes in line with previous findings, which showed that ILC2s increase in the liver of Con A-challenged mice *in vivo* (Neumann et al., 2017).

Furthermore, Neumann et al. showed a functional connection between $CD4^+$ T cells and ILC2s in liver inflammation. In $Rag1^{-/-}$ mice, lacking T and B cells but not ILC2s, Con A injection failed to induce ILC2 proliferation and liver inflammation. Reconstitution of $Rag1^{-/-}$ mice with $CD4^+$ T cells before Con A administration restored susceptibility of mice for Con A and induced tissue injury, increased IL-33 levels and subsequently ILC2 proliferation, linked with increased GATA3 expression (Neumann et al., 2017).

To further address the connection of $CD4^+$ T cells and hepatic ILC2s co-cultures were performed, which indeed showed antigen-specific activation of hepatic ILC2s and $CD4^+$ T cells as the presence of OVA induced substantial cytokine response, cell activation and proliferation. In this study, hepatic ILC2s were confirmed as potent sources of IL-5 and IL-13 upon activation via IL-33 *in vitro* and *in vivo* as well as upon interaction with $CD4^+$ T cells. Thus, ILC2-derived type 2 cytokine production can contribute to microenvironmental changes of the cytokine milieu upon Con A-induced hepatitis. ILC2-derived IL-5 is closely linked with eosinophilia (Nussbaum et al., 2013) and the IL-5-driven recruitment of eosinophils was shown to be critical in the pathogenesis of Con A-mediated hepatitis (Louis et al., 2002). Likewise, IL-13 is an important

pathogenic factor in liver fibrosis by inducing expression of profibrotic genes in HSCs. IL-13 signaling has a pivotal role in fibrogenesis in different liver diseases such as schistosomiasis, hepatitis c virus infection and alcoholic and non-alcoholic liver disease (Liu et al., 2012). Furthermore, IL-33-dependent IL-13 production by ILC2s was shown to drive liver fibrosis by activation of pro-fibrogenic HSC in a STAT4-dependent manner (McHedlidze et al., 2013). In this study, IL-33-induced hepatic ILC2-derived IL-13 expression *in vivo* and *in vitro* was confirmed.

Not only activation via IL-33 but also interaction with $CD4^+$ T cells highly increased levels of ILC2-derived type 2 cytokines showing the functional importance of cell-cell interactions for a powerful ILC2-derived cytokine response. As ILC2s arise in Con A-mediated hepatitis as a result of hepatocyte damage and subsequent IL-33 release (Neumann et al., 2017), a role in initiation of Con A-mediated hepatitis is unlikely. Given, that depletion of ILC2s however, ameliorated liver inflammation (Neumann et al., 2017) strongly indicates a proinflammatory role for hepatic ILC2s as drivers of extent and progression of Con A-mediated hepatitis. In this study, antigen-specific activation of $CD4^+$ T cells by hepatic ILC2s were shown to lead to increased proliferation of lymphocytes and enhanced production of proinflammatory cytokines (Fig. 17, Fig. 18). Thus, in Con A-mediated hepatitis, ILC2s could present antigens of dead hepatocytes and cell debris via MHC class II to T cells and therefore drive hepatic inflammation. In this way, hepatic ILC2s could promote the effector population of $CD4^+$ T cells and contribute to the proinflammatory cytokine milieu in Con A-mediated hepatitis.

By analyzing hepatic ILC2s upon co-culture with $CD4^+$ T cells, more pro- but also anti-inflammatory capabilities were detected. Besides a predominant Th2 immune response, the cytokines TNFα, IL-6, IL-9, and IL-10 were released upon antigen-specific activation of hepatic ILC2s and $CD4^+$ T cells (Fig. 18). As these cytokines have differential effects on the effector cells of Con A-induced hepatitis, such as T cells, eosinophils and neutrophils, hepatic ILC2s have capabilities to orchestrate the immune response by influencing the micromilieu in the hepatic tissue.

IL-9 is critical for neutrophilia in Con A-induced hepatitis and while IL-9 is further known to promote expansion of Th2 cytokine response of T cells (Goswami and Kaplan, 2011) and ILC2s (Turner et al., 2013), whereas IL-10 is known to facilitate the opposing effect.

Similar to ILC2s, whose cytokine secretion was suppressed by IL-10 (Rigas et al., 2017), IL-10 has a directly inhibiting effect on $CD4^+$ T cells in mice (Ye et al., 2007) and human (de Waal Malefyt et al., 1993). Although ILC2-derived IL-9 and IL-10 were not the dominant cytokines in cultures of ILC2s, these cytokines may contribute to the orchestration of immune response in the liver.

The cytokines IL-6 and TNFα, which were detectable in the supernatants of the co-culture of hepatic ILC2s and $CD4^+$ T cells, have important roles in the pathogenesis of Con A-induced hepatitis. As the expression of IL-6 was not evaluated on cellular levels via FACS, the source of these cytokines cannot be determined with certainty, but as the production of IL-6 was seen in monoculture of hepatic ILC2s (Fig 14 B), they are likely be the source in the co-culture as well. Besides providing proinflammatory capacities, IL-6 is known to influence T-cell responses (Dienz and Rincon, 2009). IL-6 supports $CD4^+$ T-cell proliferation (Lotz et al., 1988) and T-cell survival by promoting expression of anti-apoptotic markers on T cells (Teague et al., 1997). In this doing, IL-6 can promote inflammation in immune-mediated hepatitis. Furthermore, as in Chapter 4.6 elaborated, IL-6 is a critical cytokine Con A-mediated hepatitis with proinflammatory but also anti-inflammatory capacities. In Con A-induced hepatitis, TNFα is an important effector cytokine, which is increased in the liver and the sera of Con A-challenged mice (Wang et al., 2012a). As pre-treatment with anti-TNFα completely protected against hepatitis, TNFα facilitates a strong hepato-destructive effect (Gantner et al., 1995). The proinflammatory effect of these cytokines and their functional relevance are underlined by the findings of Neumann et al. showing IL-6 and TNFα to be significantly decreased upon depletion of ILC2s in Con A-induced hepatitis, which was associated with amelioration of liver inflammation (Neumann et al., 2017).

In summary, it is likely that hepatic ILC2s do not play a leading role in the initiation of liver inflammation upon challenge with Con A, but once T-cell mediated tissue damage has begun and subsequently IL-33 is released from the necrotic hepatocytes, ILC2s strongly expand in the liver and contribute to the inflammation by providing the proinflammatory cytokines IL-6, TNFα and the Th2 cytokines IL-13 and IL-5. In this doing, the interaction of $CD4^+$ T cells and ILC2s might be part of a positive feedback loop, as ILC2s can act as APCs and present antigens from dead cells, which arise upon

Con A- mediated hepatitis to $CD4^+$ T cells inducing further activation and proliferation of $CD4^+$ T cells. Again, increased population of $CD4^+$ effector T cells can further contribute to tissue damage and subsequently increased levels of IL-33 levels in turn driving ILC2 proliferation and activation. Moreover, this interplay increases expression of cytokines, which might drive recruitment of other effector cells such as eosinophils and neutrophils. However, also further immunoregulatory function might be facilitated. ILC2s were also sufficient sources of IL-6, which also has anti-inflammatory capacities in Con A-hepatitis. Further hepatic ILC2s expressed the anti-inflammatory cytokine IL-10. Another possibility how hepatic ILC2s could facilitate anti-inflammatory effects is via PD-1/PD-L1 signaling, as ILC2s expressed PD-L1 and therefore could have suppressed $PD\text{-}1^+$ immune cells. However, the proinflammatory effect of ILC2s clearly dominated the phenotype of hepatic ILC2s upon antigen-specific interaction with $CD4^+$ T cells and in presence of Con A-induced cytokines (Fig. 22).

Taken together, this study shows that hepatic ILC2s reacted to the inflammatory stimulus of IL-33 with production of mainly proinflammatory cytokines. Hence, also ILC2s in the liver live up to their reputation as sentinels: by sufficient production of predominantly, but not exclusively, type 2 cytokines ILC2s can rapidly drive the immune response. Their function as gatekeeper is further increased by their ability of antigen presentation, which is shown for hepatic ILC2s for the first time. In this context, this study first showed hepatic ILC2s to influence the phenotype $CD4^+$ T cells, initiate T cell proliferation and vice versa hepatic ILC2s to be influenced by antigen-presentation. These findings underline the perception of ILC2s as important effector cells in immune-mediated hepatitis with reciprocal effects on other immune cells and the microenvironment shaping the cytokine milieu in the hepatic tissue and driving hepatic inflammation.

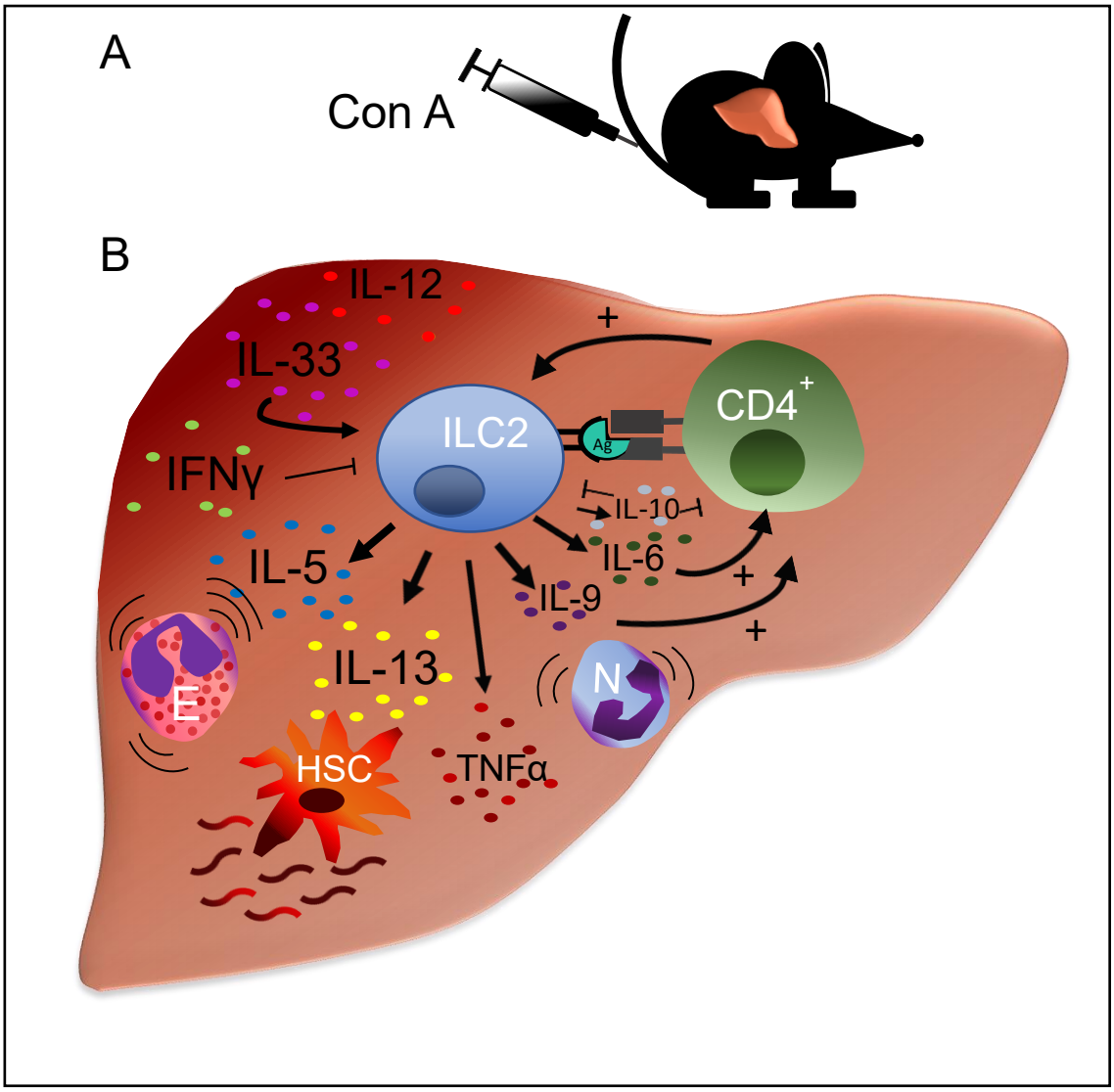

Figure 22: Proposed Role of ILC2s upon Con A-mediated hepatitis.
(**A**)Injection of Con A into the tail vein leads to immune-mediated hepatitis.
(**B**)Upon injection of Con A CD4+ T-cell mediated liver damage leads to release of the proinflammatory cytokines IL-33, IL-12 and IFNγ. IL-33-induced activation and cytokine production of hepatic ILC2s fuels inflammation and recruitment of further immune cells. ILC2s present antigen from dead cells to CD4+ T cells creating a positive feedback loop by driving activation and proliferation of ILC2s and CD4+ T cells, which further increases the release of proinflammatory cytokines. Ag, antigens; ILC2, type 2 innate lymphoid cells; CD4+, CD4+ T cells; E, eosinophils; HSC, hepatic stellate cells; N, neutrophils.

Summary

As newly identified players in type 2 immunity type 2 innate lymphoid cells (ILC2s) play an influential role in the pathogenesis of liver inflammation. In the model of Concanavalin A (Con A)-mediated hepatitis, a murine model for autoimmune hepatitis, ILC2s were shown to facilitate a strong proinflammatory effect. This study addressed the mechanisms how ILC2s exert their proinflammatory function and how they are regulated in liver inflammation. As ILC2s are highly influenced by environmental factors, this study characterized the phenotype of hepatic ILC2s in homeostasis and upon contact with liver inflammation-induced cytokines. Furthermore, their ability to interact with $CD4^+$ T cells, the main players of Con A-induced hepatitis, was investigated.

Flowcytometry analysis revealed that ILC2s are present in healthy C57Bl/6 mice but constitute only a small proportion of the hepatic leukocytes. In Con A-mediated hepatitis, mRNA analysis showed that the cytokines interleukin (IL)-33, interferon (IFN)γ and IL-12 were strongly induced in the hepatic tissue. As these cytokines were all described to have opposing effects on ILC2 phenotype and function, their influence on hepatic ILC2s was investigated. IL-12 was found to neither modulate the phenotype of hepatic ILC2s, nor to modulate the effects of IL-33 and IFNγ on ILC2s. IL-33, by contrast, was confirmed as a potent activator of hepatic ILC2s *in vivo* and *in vitro*. In *in-vitro* cultures, IL-33-activated ILC2s expanded greatly and flow cytometry analysis of the cells as well as ELISA-based analysis of their culture supernatants showed hepatic ILC2s to produce mainly, but not exclusively, the type two cytokines IL-5 and IL-13. Moreover, hepatic ILC2s were shown to be sources of IL-6 and tumor necrosis factor (TNF) α, which further enable proinflammatory effects in the hepatic tissue. Furthermore, IL-33-activated hepatic ILC2s also expressed the cytokines IL-9, IL-10 and IL-22. In contrast, IFNγ was shown to suppress proliferation and cytokine secretion of hepatic ILC2s *in vitro*. Importantly, IL-33 abrogated the suppressing effect of IFNγ, therefore explaining why hepatic ILC2s make an impact in the IFNγ-driven disease of Con A-mediated hepatitis. IL-33-mediated activation of hepatic ILC2s was associated with high expression of surface molecules enabling cell-cell interactions, such as major histocompatibility complex (MHC) class II, inducible T-cell costimulator (ICOS), OX-40,

its ligand OX-40-L, PD-1 and its ligand PD-L1. With focus of MHC class II, the ability of antigen-specific activation of $CD4^+$ T cells by hepatic ILC2s was investigated in co-cultures of transgenic Ovalbumin (OVA)-specific $CD4^+$ T cells and hepatic ILC2s in the presence of the antigen OVA peptide. Hepatic ILC2s presented OVA to $CD4^+$ T cells and this antigen-specific interaction resulted in activation and proliferation of both cell types and a powerful ILC2-derived type 2 cytokine response displayed in IL-5 and IL-13 production. Here, also proinflammatory cytokines such as IL-6, TNFα, IFNγ and the anti-inflammatory cytokine IL-10 were produced upon antigen-specific interaction of $CD4^+$ T cells and hepatic ILC2s.

Therefore, this study shows (1.) the effects of liver inflammation-induced cytokines on hepatic ILC2s enabling activation and cytokine production (2.) hepatic ILC2s to be sufficient producers of type 2 cytokines upon IL-33-mediated and antigen-specific activation, (3.) ILC2s to produce cytokines, which enable orchestration of the immune response and mediation of both hepato-destructive and protective effects, (4.) ILC2s to be capable of MHC class II-mediated antigen-specific interaction with $CD4^+$ T cells resulting in activation of these effector cells and a powerful cytokine response. In this way, ILC2s were shown to be highly influenced by environmental cytokines but vice versa are also able shape the hepatic microenvironment and immune responses upon an inflammatory stimulus, hereby birding innate and adaptive immunity in hepatic tissues.

Zusammenfassung

Als jüngst identifizierte Vertreter der Typ 2 Immunantwort nehmen angeborene Lymphozyten der Gruppe 2 (ILC2s) auch in der Pathogenese der Leberentzündung eine Rolle ein. In dem Model der Concanavalin A (Con A)-induzierte Leberentzündung, einem Mausmodel für autoimmune Hepatitis, zeigten ILC2s eine starke proinflammatorische Wirkung. Diese Studie adressiert die Mechanismen der proinflammatorischen Effektorfunktion von ILC2s und deren Regulation bei Leberentzündung. Da ILC2s stark von den sie umgebenden Botenstoffen beeinflusst werden, wurde der Phänotyp von hepatische ILC2s in Homöostase wie auch dessen Veränderungen bei Kontakt mit Zytokinen, welche in der Leberentzündung auftreten, näher charakterisiert. Desweitern wurde die Interaktion von ILC2s mit den Hauptakteuren der Con A-induzierten Hepatitis, $CD4^+$ T Zellen, untersucht.

In dieser Studie konnte mittels Durchflusszytometrie gezeigt werden, das hepatische ILC2s auch unter physiologischen Bedingungen in der Leber von C57Bl/6-Mäusen vorhanden sind, dort aber nur einen sehr kleinen Anteil der hepatischen Leukozyten ausmachen. In Con A-induzierter Hepatitis zeigten mRNA-Analysen, dass die Zytokine Interleukin (IL)-33, Interferon (IFN)γ und IL-12 im Lebergewebe induziert werden. Da sowohl IL-33 als auch IFNγ und IL-12 unterschiedlichste Effekte auf ILC2s haben können, wurde untersucht, inwiefern der Phänotyp von hepatischen ILC2s von diesen beeinflusst werden kann. Hierbei zeigte das Zytokin IL-12 weder einen Einfluss auf den Phänotyp von ILC2s noch modulierte es die Effekte von IL-33 oder IFNγ auf diese. Dahingegen konnte IL-33 als potenter Aktivator von ILC2s *in vivo* und *in vitro* bestätigt werden. *In in-vitro* Kulturen proliferierten ILC2s durch IL-33 Aktivierung stark und sowohl die Analyse der Zellen selbst mittels Durchflusszytometrie, als auch ELISA-basierte Analyse der Kulturüberstände zeigten eine potente Sekretion der Typ 2 Zytokine IL-5 und IL-13. Darüber hinaus produzierten ILC2s nach Aktivierung durch IL-33 die proinflammatorischen Zytokine IL-6 und Tumornekrosefaktor (TNF) α, aber auch IL-9, IL-10 und IL-22, welche diverse immunmodulatorische Effekte haben können. Im Gegensatz dazu konnte gezeigt werden, dass IFNγ die Proliferation und die Zytokinantwort von hepatischen ILC2s hemmt. IL-33 ist jedoch in der Lage den suppressiven Effekt von IFNγ aufzuheben. Dies erklärt, warum ILC2s in der von IFNγ

geprägten Erkrankung der Con A-induzierten Hepatitis eine wichtige Effektorfunktion übernehmen können.

Die Aktivierung von hepatischen ILC2s war mit der Expression von Oberflächenmarkern assoziiert, die Zell-Zell-Interaktionen ermöglichen. Dazu gehörte die Expression des Haupthistokompatibilitätskomplex (MHC) Klasse II, induzierbaren T-Zell Co-Stimulators (ICOS), OX-40, sowie dessen Liganden OX-40-L, PD-1 und dessen Liganden PD-L1. Mit Fokus auf das Oberflächenmolekül MHC Klasse II wurde die Fähigkeit von hepatischen ILC2s zur antigenspezifischen Aktivierung von $CD4^+$ T Zellen untersucht. Hierfür wurden transgene Ovalbumin (OVA)-spezifische $CD4^+$ T Zellen mit hepatischen ILC2s in Präsenz des OVA Peptids kultiviert und deren Phänotyp untersucht. Hepatische ILC2s präsentierten das Antigen OVA $CD4^+$ T Zellen und diese antigenspezifische Interaktion führte zur Aktivierung und Proliferation von beiden Zelltypen, ebenso wie einer starke Typ 2 Immunantwort durch ILC2s durch die Produktion Zytokine IL-5 und IL-13. Aber auch andere proinflammatorische Zytokine wie IL-6, TNFα, IFNγ und das antiinflammatorisch IL-10 wurden produziert.

Zusammenfassend zeigt diese Studie (1.) den Einfluss von den Zytokinen der Leberentzündung auf hepatische ILC2s, welcher zur Aktivierung und Zytokinproduktion führen kann, (2.) durch IL-33 aktivierte hepatische ILC2s als potente Produzenten von Typ 2 Zytokinen, (3.) ein weitgefächerteres Zytokinexpressionmuster von ILC2s nach IL-33- Aktivierung oder antigenspezifische Interaktion mit $CD4^+$ T Zellen, als bisher beschrieben, welches den proinflammatorischen Charakter dieser Zellen weiter stützt, aber auch weitere immunmodulatorische und gewebsregenative Aufgaben ermöglicht, (4.) MHC Klasse II-vermittelte antigen-spezifische Interaktion mit $CD4^+$ T-Zellen, welche zur Aktivierung beider Zelltypen und einer suffizienten Immunantwort führt und so ihrerseits wiederum in der Lage ist das Milieu der unmittelbaren Umgebung sowie Immunantworten zu beeinflussen. So konnte gezeigt werden, auf welche Weise ILC2s als potente Effektorzellen in der Leberentzündung fungieren und eine wichtige Brücke zwischen angeborenem und erworbenem Immunsystem darstellen können.

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Abbreviations

aCD3/CD28	anti-CD3/CD28 Dynabeads
ALT	alanine aminotransferase
apc	allophycocyanin
APC	antigen-presenting cell
AST	aspartate aminotransferase
ATP	adenosine triphosphate
bcl	B-cell lymphoma
CD	cluster of differentiation
cDNA	complementary DNA
c-Kit	tyrosine kinase KIT
CLP	common lymphoid progenitor
Con A	Concanavalin A
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
CT	cycle threshold
DAMP	danger associated molecular pattern
DC	dendritic cell
DNA	desoxyrivonucleic acid
Eomes	eomesodermin
FACS	fluorescence-activated cell sorting
Fc	fragment crystallizable
FMO	fluorescence minus one
FSC	forward scattering light
GATA-3	GATA-binding protein 3
HBSS	Hank’s balanced salt solution
i.p.	intraperitoneal
ICOS	inducible T-cell costimulator
ICOS-L	ICOS-ligand
ICOS-L	inducible T-cell costimulator ligand
ID2	DNA-binding protein inhibitor ID-2
IFNγ	interferon gamma
Ig	immunoglobulin
iILC2	inflammatory ILC2
IL	interleukin
IL-6R	IL-6 receptor
ILC	innate lymphoid cell
ILC1	group 1 innate lymphoid cell
ILC2	group 2 innate lymphoid cell
ILC3	group 3 innate lymphoid cell
KC	Kupffer cell

KLRG-1	killer cell lectin-like receptor subfamily G member 1
lin	linage
LN	lymph nodes
LSEC	liver sinusoidal endothelia cell
LTi	lymphoid tissue inducer
Ly	lymphocyte antigen
MACS	magnetic-activated cell sorting
MFI	median fluorescence intensity
MHC	major-histocompatibility complex
mRNA	messenger RNA
N. brasiliensis	Nippostrongylus brasiliensis
nd	not detectable
NK	natural killer cell
NKT cell	natural killer T cell
ns	not significant
OX-40L	OX-40-ligand
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death protein 1-ligand
PMA	phorbol-12-myristat-13-acetat
qRT-PCR	quantitative real-time polymerase chain reaction
RAG	recombination-activating gene
RNA	ribonucleic acid
RORα	retinoic-acid-receptor-related- orphan nuclear receptor alpha
RORγt	retinoic-acid-receptor-related- orphan nuclear receptor gamma
Rα	Receptor α
SA-PE	streptavidin-phycoerythrin
Sca-1	stem cell antigen- 1
SCID	serve combined immunodeficiency
SEM	standard error of the mean
SSC	sideward scattered light
STAT	signal transducer and activator of transcription
TCR	T-cell receptor
Th	T helper cells
Thy1	thymocyte antigen 1
TNF	Tumor necrosis factor
TNFα	tumor necrosis factor α
TRAIL	tumor necrosis factor related apoptosis-inducing ligand
T_{reg}	regulatory T cells
TSLP	thymic stromal lymphopoietin
TSLP bc	TSLP-binding chain

TSLPR	thymic stromal lymphopoietin receptor
γc	common gamma chain

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46th Annual Meeting of the German Society of Immunology, Hamburg, Deutschland

Curriculum Vitae

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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Unterschrift: .....