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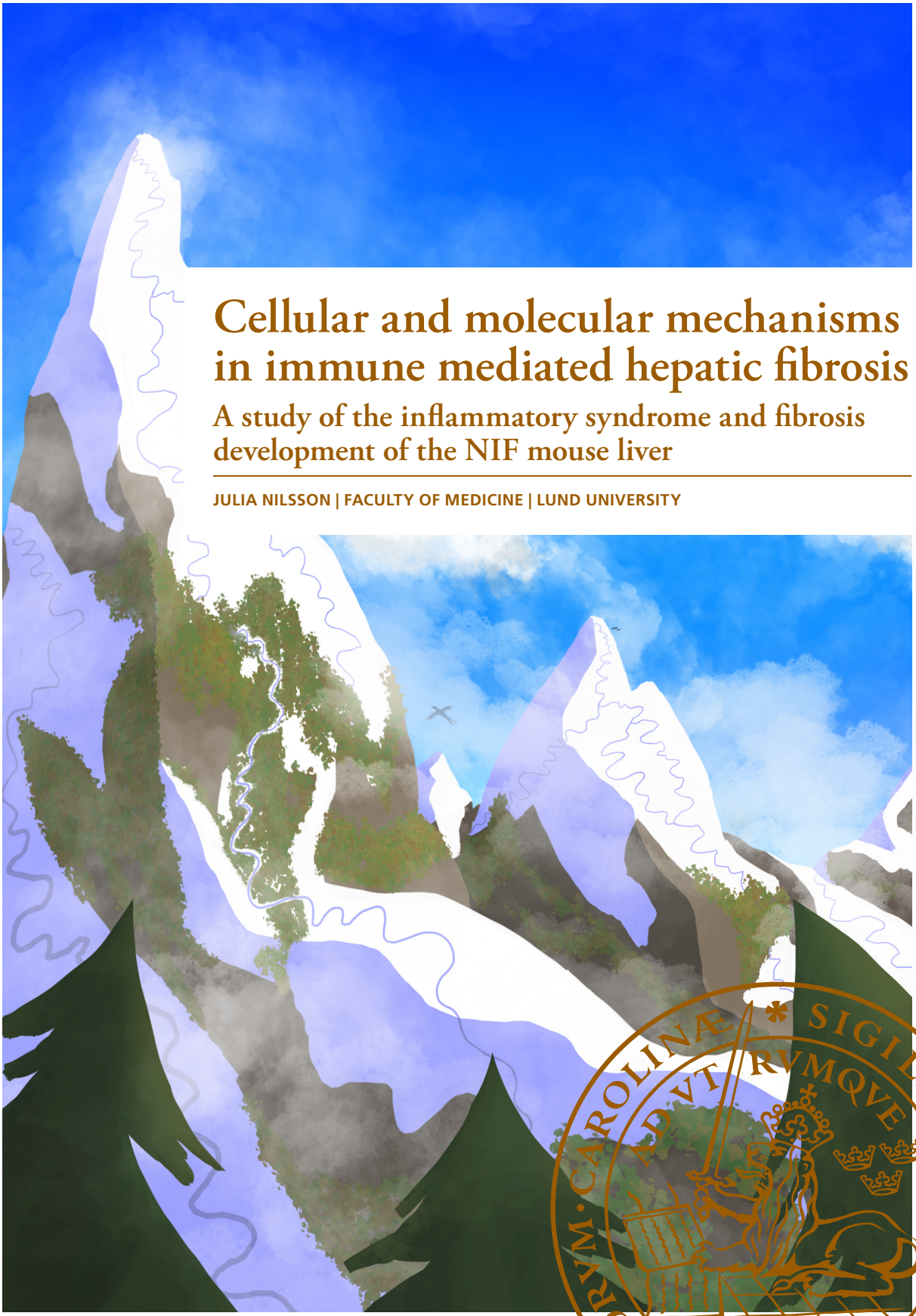

Cellular and molecular mechanisms in immune mediated hepatic fibrosis

A study of the inflammatory syndrome and fibrosis development of the NIF mouse liver

JULIA NILSSON | FACULTY OF MEDICINE | LUND UNIVERSITY

"I wish I could, but I don't want to."

– Phoebe Buffay

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Cellular and molecular mechanisms in immune mediated hepatic fibrosis

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LUND
UNIVERSITY

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Cellular and molecular mechanisms in immune mediated hepatic fibrosis
- A study of the inflammatory syndrome and fibrosis development of the NIF mouse liver

Abstract

Fibrosis is the result of dysregulated inflammation and tissue repair, and is characterized by the excessive accumulation of extra cellular matrix (ECM) proteins. It causes detrimental effects to the afflicted tissue and can subsequently lead to organ failure. Sterile liver inflammation and hepatic fibrosis are associated with many liver disorders of different etiologies. Both type 1 and type 2 inflammatory responses have been reported to contribute to the pathology, however, the mechanisms controlling the balance between them are largely unknown. A major limitation in the attempts to understand the underlying mechanisms leading to fibrosis development, and to establish efficient anti-fibrotic treatment protocols has been the restricted set of suitable animal models available.

In paper I, we characterize the NIF mouse, a recently established animal model that spontaneously develops chronic inflammation and fibrosis in the liver. The inflammatory syndrome is mediated by a transgenic population of Natural Killer T (NKT) cells induced on an immunodeficient NOD genetic background and is characterized by the combined production of both T_H1 and T_H2 cytokines. We show that the disease is transferrable to immunodeficient recipients, while polyclonal T cells from unaffected syngeneic donors can reverse the disease phenotype.

In paper II, we demonstrate that the transgenic NKT cells of the NIF mouse mediate the initiation of a chronic type 1 inflammatory response in the liver, involving the activation of the NLRP3 inflammasome. A subsequent shift into a type 2 inflammatory response, driven by the production of IL-33, activation of hepatic stellate cells (HSC) and production of anti-inflammatory/pro-fibrotic cytokines by the same transgenic NKT cell population, promotes the development of hepatic fibrosis. This data illustrates how plasticity in NKT cells can drive an initial type 1 inflammatory response, as well as promote the transition into a type 2 inflammatory response.

Paper III illustrates how the NIF mouse model can be used for efficacy testing of drug candidates against liver fibrosis.

It is imperative to develop new and improved techniques in order to be able to further investigate the pathophysiological tissue changes caused by fibrosis, as well as allowing for the possibility to monitor the effects of disease intervention protocols. In line with this, part of this dissertation work has been focused on the development of novel imaging technology for this purpose. In paper IV, we demonstrate how the anterior chamber of the eye (ACE) imaging technique can be utilized for the longitudinal in vivo study of structural changes in transplanted tissue.

The disease phenotype of the NIF mouse resembles human fibrotic conditions in several pertinent features, which offers a unique opportunity to gain further insight into the underlying mechanisms mediating transformation of chronic inflammation into development of pathological fibrosis.

Key words

Fibrosis, liver, mouse model, NKT-cells, type 2 inflammation, inflammasome

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To my fathers – Ulf & Bengt

In Omnia Paratus

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Abstract

Fibrosis is the result of dysregulated inflammation and tissue repair, and is characterized by the excessive accumulation of extra cellular matrix (ECM) proteins. It causes detrimental effects to the afflicted tissue and can subsequently lead to organ failure. Sterile liver inflammation and hepatic fibrosis are associated with many liver disorders of different etiologies. Both type 1 and type 2 inflammatory responses have been reported to contribute to the pathology, however, the mechanisms controlling the balance between them are largely unknown. A major limitation in the attempts to understand the underlying mechanisms leading to fibrosis development, and to establish efficient anti-fibrotic treatment protocols has been the restricted set of suitable animal models available.

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Populärvetenskaplig sammanfattning

Fibros betyder ärrbildning och är en viktig fysiologisk mekanism som läker och reparerar kroppens vävnader efter en uppkommen skada. Det är en strängt kontrollerad process som involverar flera olika stadier och många olika typer av immunceller, så väl som vävnadens egna celler. När sårläkningsprocessen kommer ur balans och blir kronisk utvecklas sjuklig, eller patologisk, fibros. Detta sker till följd av okontrollerad inflammation och kan utvecklas i praktiskt taget alla kroppens olika organ. Mer än 45 % av de naturliga dödsfallen i västvärlden tros bero på underliggande fibrotisk sjukdom och fibrosforskningsfältet har expanderat kraftigt de senaste årtiondena. Trots detta är de cellulära och molekylära mekanismerna som leder till okontrollerad inflammation, kronisk sårläkning och utveckling av patologisk fibros inte helt klarlagda och det finns inte heller några effektiva läkemedel mot fibros på marknaden.

I levern kan i stort sett alla kroniska sjukdomstillstånd leda till fibrosutveckling. Dessa sjukdomstillstånd kan uppkomma till följd av många olika anledningar, så som virus- eller bakterinfektioner, överkonsumtion av alkohol, metabol dysfunktion eller autoimmuna orsaker. Då immunförsvaret triggas igång till följd av faktorer som ovan så uppstår det en akut inflammation som syftar till att skydda levern och dess funktion. Den akuta inflammationen skadar dock i viss mån även leverns egna celler, och för att den akuta inflammationen ska läka ut och skadan i levern repareras, så aktiveras även en anti-inflammatorisk och sårläkande process. Denna anti-inflammatoriska process dämpar den akuta inflammationen, tar hand om och bryter ned de skadade cellerna, och ersätter dem med bindväv – ärrvävnad. Om dessa processer blir kroniska och/eller okontrollerade så fortsätter det att bildas mer och mer bindväv som ersätter allt mer av leverns egna celler. När de funktionella levercellerna byts ut mot ärrvävnad förlorar levern gradvis sin livsviktiga funktion – upptag av energi samt nedbrytning av kroppens slaggprodukter. Slutstadiet för leverfibros är skrumplever, cirros, som leder till en mycket förhöjd risk för leversvikt och även en ökad risk att utveckla levercancer. Det enda behandlingsalternativet för leversvikt idag är levertransplantation.

För att utveckla och öka förståelsen för mekanismerna som leder till leverfibros har flera olika djurmodeller använts och varit behjälpliga i att identifiera viktiga aspekter av sjukdomsförloppet. Det har dock varit svårt att översätta kunskapen från modell till människa då modeller ofta är begränsade i sin likhet med human fibrosutveckling. Vi har utvecklat och studerat NIF-musen som är en ny djurmodell

för kronisk inflammation och leverfibros, och utgör en förbättrad modell för human leverfibros då den uppvisar många viktiga likheter med det humana sjukdomsförloppet. Detta innebär en ökad potential för utvecklingen av vår förståelse av viktiga processer och aspekter inom kronisk inflammation och leverfibros. NIF-musen erbjuder också en möjlighet att användas som pre-klinisk modell för utveckling av potentiella fibrosläkemedel.

Olika tekniker för biologisk avbildning är viktiga hjälpmedel i studierna av leverfibros, både för den humana sjukdomen och i djurmodeller. Utvecklingen av nya och förbättrade tekniker för biologisk avbildning gör det möjligt att ytterligare öka kunskapen inom leverfibros. Som ett led i detta har vi även utvecklat en ny teknik för att undersöka aspekter så som vävnadsförändringar och cellulära händelser in vivo, på ett icke-invasivt och longitudinellt sätt i transplanterad vävnad.

Original papers included in this thesis

Paper I: A New Mouse Model That Spontaneously Develops Chronic Liver Inflammation and Fibrosis.

Nina Fransén-Pettersson, Nadia Duarte, Julia Nilsson, Marie Lundholm, Sofia Mayans, Åsa Larefalk, Tine D. Hannibal, Lisbeth Hansen, Anja Schmidt-Christensen, Fredrik Ivars, Susanna Cardell, Richard Palmqvist, Björn Rozell and Dan Holmberg.

PLoS ONE. 11(7), e0159850. doi:10.1371/journal.pone.0159850, 2016.

Paper II: NKT cells promote both NLRP3 inflammasome-mediated inflammation and IL-33 mediated fibrosis in a mouse model of liver fibrosis.

Julia Nilsson, Maria Hörnberg, Anja Schmidt-Christensen, Kajsa Linde, Marine Carlus, Saskia F. Erttmann, Sofia Mayans and Dan Holmberg.

Manuscript.

Paper III: The immunomodulatory quinoline-3-carboxamide paquinimod reverses established fibrosis in a novel mouse model for liver fibrosis.

Nina Fransén-Pettersson, Adnan Deronic, Julia Nilsson, Tine D. Hannibal, Lisbeth Hansen, Anja Schmidt-Christensen, Fredrik Ivars and Dan Holmberg.

PLoS ONE. 13(9), e0203228. Doi:10.1371/journal.pone.0203228, 2018.

Paper IV: Recruited fibroblasts reconstitute the peri-islet membrane: a longitudinal imaging study of human islet grafting and revascularisation.

Julia Nilsson, Rabiah Fardoos, Lisbeth Hansen, Håkan Lövkvist, Kristian Pietras, Dan Holmberg and Anja Schmidt-Christensen.

Diabetologia. 63, 137-148, 2020.

Papers not included in this thesis

Longitudinal In Vivo Imaging and Quantification of Human Pancreatic Islet Grafting and Contributing Host Cells in the Anterior Eye Chamber

Julia Nilsson, Dan Holmberg and Anja Schmidt-Christensen.

Journal of Visualized Experiments, In Press.

Optical projection tomography for rapid whole mouse brain imaging.

David Nguyen, Paul J. Marchand, Arielle L. Planchette, Julia Nilsson, Miguel Sison, Jérôme Extermann, Antonio Lopez, Marcin Sylwestrzak, Jessica Sordet-Dessimoz, Anja Schmidt-Christensen, Dan Holmberg, Dimitri Van De Ville and Theo Lasser.

Biomedical Optics Express. 8, 5637-5650, 2017.

Deficiency in plasmacytoid dendritic cells and type I interferon signalling prevents diet-induced obesity and insulin resistance in mice.

Tine D. Hannibal, Anja Schmidt-Christensen, Nina Fransén-Pettersson, Lisbeth Hansen, Julia Nilsson and Dan Holmberg.

Diabetologia. 60, 2033-2041, 2017.

Label-free fast 3D coherent imaging reveals pancreatic islet micro-vascularization and dynamic blood flow.

Corinne Berclaz, Daniel Szlag, David Nguyen, Jérôme Extermann, Arno Bouwens, Paul J. Marchand, Julia Nilsson, Anja Schmidt-Christensen, Dan Holmberg, Anne Grapin-Botton and Theo Lasser.

Biomedical Optics Express. 7, 4569-4580, 2016.

Abbreviations

ACE	anterior chamber of the eye
AGM	aorta/gonada/mesonephros
APC	antigen presenting cell
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
BCR	B-cell receptor
BM	bone marrow
CCL	CC-motif chemokine ligand
CCR	CC-motif chemokine receptor
ConA	concanavalin A
CSR	class switch recombination
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DAMP	danger associated molecular pattern
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
ECM	extra cellular matrix
αGalCer	α-galactosylceramide
βGlcCer	β-glucosylceramide
HMGB1	high mobility group protein B1
HSC	hepatic stellate cell
IFN	interferon
Ig	immunoglobulin

IL	interleukin
IL-1RAcP	IL-1 receptor accessory protein
ILC	innate lymphoid cell
iNKT	invariant NKT
KC	Kupffer cell
LAP	latency-associated protein
LOXL	lysyl oxidase homologue
LSEC	liver sinusoidal endothelial cell
MAIT	mucosal associated invariant T
mDC	myeloid DC
MMPs	matrix metalloproteinases
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NF-κB	nuclear factor κ-B
NIF	non-obese diabetic inflammation and fibrosis
NK	natural killer
NKT	natural killer T
NKTII	type II NKT
NLR	NOD-like receptor
OPT	optical projection tomography
PAMP	pathogen-associated molecular pattern
PBC	primary biliary cirrhosis
pDC	plasmacytoid DC
PDGF	platelet-derived growth factor
PD-L1	programmed cell death ligand 1
PET	positron emission tomography
2PM	two photon microscopy
PRR	pattern recognition receptor
PYD	pyrin domain

RAG1	recombinase activating gene 1
RAG2	recombinase activating gene 2
Rorγt	retinoic-acid-receptor-related orphan nuclear receptor γ t
α-SMA	α-smooth muscle actin
STAT	signal transducer and activator
T_C	effector cytotoxic CD8+ T cell
T_{FH}	T follicular helper cell
T_H	helper T cell
T_{reg}	regulatory T helper cell
T-bet	T cell associated transcription factor
TCR	T cell receptor
TGF-β	transforming growth factor-β
TIMP1	tissue inhibitor of metalloproteinase-1
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin
V, D, J	variable, diversity, joining segments

The immune system

Immunology is the study of the immune system, a complex network made up of signaling molecules, leukocytes – the immune cells – and the central and peripheral lymphoid tissues and organs. Together the different parts of the immune system form a highly efficient defense against pathogenic substances and their harmful effects.

The pluripotent hematopoietic stem cell give rise to almost all of the cells of the immune system. These cells develop and differentiate in the bone marrow or thymus – the central lymphoid organs – after which, they enter the blood stream and circulate the host patrolling for pathogens or enter tissues to become tissue resident leukocytes.

The immune system is classically divided into the innate and adaptive immune systems but they are, however, highly interdependent of each other. Most cells of the innate immune system develop from the common myeloid progenitor[1] and include macrophages, granulocytes (neutrophils, eosinophils and basophils), mast cells, dendritic cells and monocytes. The cells of the adaptive immune system; T lymphocytes (T cells) and B lymphocytes (B cells) develop from the common lymphoid progenitor[2].There are, however, additional cells developing from the common lymphoid progenitor that possess both innate and adaptive characteristics such as Natural Killer (NK) cells, Innate Lymphoid Cells (ILCs) and the unconventional T-cells that include γδ T cells[3], Mucosal associated invariant T (MAIT)[4] cells and Natural Killer T (NKT)[5] cells (figure 1).

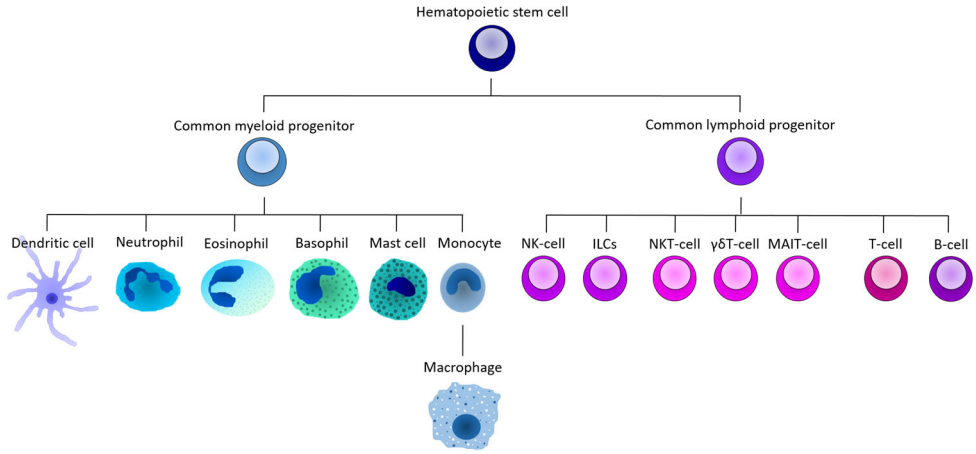

Figure 1: A simplified, schematic overview of the leukocytes and their progenitors in the innate and adaptive immune system. The myeloid cells arise from the common myeloid progenitor; dendritic cells, neutrophils, eosinophils, basophils, mast cells, macrophages and monocytes, while the lymphoid cells; T lymphocytes (T cells) and B lymphocytes (B cells) arise from the common lymphoid progenitor. The common lymphoid progenitor also gives rise to innate-like lymphocytes; natural killer (NK) cells, innate lymphoid cells (ILCs), the unconventional T cells that include natural killer T (NKT) cells, γδ T cells and mucosal associated invariant T (MAIT) cells.

Innate immunity

The innate immune system is the first line of defense against threat, be that pathogens which have breached the physical barriers of the body, or harmful internal risk factors such as cancer cells or necrosis. Innate immunity is characterized by an immediate and non-specific inflammatory response upon detection of an injurious substance, and with no development of immunological memory. The innate immune response is triggered and propagated by a range of different cells such as the myeloid cells of the hematopoietic lineage as well as lymphoid cells like NK cells and NKT cells, but also cells of non-hematopoietic lineage such as epithelial cells[6] and parenchymal cells[7]. Additionally there is a range of circulating proteins and smaller molecules aiding the cells of the innate immune system in identifying, disabling and clearing pathogenic material which include complement protein, LPS-binding protein and anti-microbial peptides. These components make up the humoral part of the innate immune system[6].

Pathogen recognition by innate leukocytes

The cells of the innate immune system detect pathogens by means of a limited number of pattern recognition receptors (PRRs). PRRs will identify pathogen-associated molecular patterns (PAMPs), highly conserved molecular structures found on pathogenic microbes[8], or danger associated molecular patterns (DAMPs),

derived from tissue damage as a consequence of inflammation[9]. Transmembrane PRRs, such as Toll-like receptors (TLRs), will elicit an inflammatory response to extracellular or phagocytosed pathogens while cytoplasmic PRRs, such as the NOD-like receptors (NLRs), will evoke an inflammatory response to intracellular pathogens[6,10]. The activation of PRRs propagates the inflammatory reaction by initiating signaling cascades that subsequently results in the production of pro-inflammatory mediators such as cytokines and chemokines.

The cells of the innate immune system

The different myeloid cells making up the innate immune system have distinct roles in the inflammatory response. The mononuclear phagocytes – macrophages and dendritic cells (DCs) – develop from bone marrow derived circulating monocytes[11] or, as in the case of certain tissue resident macrophages, from embryonic progenitors[12]. While DCs kill and degrade pathogens by phagocytosis their main role is activation of the adaptive immune response by means of antigen presentation[13]. Macrophages, however, efficiently clear large quantities of invading microorganisms through phagocytosis[11,13] but are also important orchestrators of the inflammatory response. They are highly plastic cells and can switch functional activation state depending on the microenvironment[14] mediating a large range of functions. Macrophages can be considered to be on a polarizing spectrum[15] where classically activated macrophages (M1, interferon (IFN)-γ induced) important in mounting immune response to bacterial and viral pathogens are on one end, while alternatively activated macrophages (M2, interleukin (IL)-4 induced) crucial in mediating wound repair[16,17] are on the other.

The polymorphonuclear cells, the granulocytes, comprise neutrophils, eosinophils and basophils[18]. The neutrophils are the most abundant of the innate immune cells[13], efficient eliminators of pathogens and are usually the first cells to be recruited in an inflammatory reaction[19]. The eosinophil population is generally more scarce than the neutrophils, but in response to inflammation, can be produced in abundance thereby causing eosinophila. Eosinophils are recruited by Th2 associated cytokines such as IL-5 and IL-33, and are generally associated with parasitic immunity and allergic immune responses, where they enhance the Th2 response by the secretion of IL-4[20]. Mast cells are large tissue resident mononuclear myeloid cells mainly found in skin, intestines and the airway mucosa, and together with basophils, they are like the eosinophils, key mediators in parasitic and allergic immunity[13,18].

Adaptive immunity

While the innate immune system will react immediately and non-specifically, adaptive immunity takes longer to mount a response, but with the advantage of specific and targeted effector functions and the development of immunological memory. The cells of the adaptive immune system is, as previously mentioned, composed of lymphocytes; T and B cells, developed from the common lymphoid progenitor. The specificity of lymphocytes relies on a nearly endless repertoire of unique antigen receptors, the T cell receptor (TCR) and the B cell receptor (BCR), which allow for specific antigen recognition.

T cell development and the TCR

T cells can be categorized into two distinct subtypes – conventional T cells and unconventional T cells. The conventional T cells express αβ TCR variants, while the unconventional T cells, including γδ T cells, MAIT and NKT cells, express γδ TCR variants and αβ TCR variants but with a limited receptor diversity, respectively[21] (reviewed in ref. 21).

Progenitor cells from the bone marrow or fetal liver migrate to the thymus, where the T-cells develop and acquire their antigen specific TCR. The complete TCR complex is made up of two separate polypeptide chains – α and β or γ and δ – together with the signal transducing complex CD3. The generation of these two distinct polypeptide chains during the T cell development is what ultimately gives rise to the extraordinarily diverse antigen binding sites of the TCR[22,23]. This great receptor diversity is achieved through somatic rearrangement – a series of random genomic rearrangements that in the end result in functional genes encoding the α and β or γ and δ chains[24]. The genes that are subject to rearrangement, encoding the TCR polypeptide chains, contain variable (V), joining (J) and diversity (D) segments and the random recombination of one V, one D and one J segment is mediated by the enzyme complex V(D)J recombinase. This enzyme complex is essential for the successful assembly of the TCR, and mutations in the genes coding for the proteins making up this enzyme complex – recombinase activating genes 1 and 2 (*RAG1* and *RAG2*) – will result in severe immunodeficiency due to the lack of mature T and B cells[25].

To ensure the development of functional, mature T cells, thymocytes undergo a positive selection and differentiation process, where conventional T cells result in single positive naïve $CD4^+$ T cells or $CD8^+$ T cells, and the unconventional T cells in single positive $CD4^+$ or $CD8^+$ cells or double negative (unconventional) T cells[13,26-28]. To ensure self-tolerance of the T cell compartment, potentially autoreactive lymphocytes, displaying excessively strong reactivity to self-peptides, are eliminated by apoptosis through negative selection, a process termed central tolerance[29].

T cell activation

After development in the thymus mature T cells emerge and enter the circulation as antigen-naïve cells. Upon activation through antigen recognition the T cell will undergo clonal expansion and differentiate into effector or memory cells. T-cell activation is mediated by TCR interaction with an antigen - MHC complex along with co-stimulatory signals such as CD80/CD86 - CD28 interaction. $CD8^+$ T cells recognize antigen on MHC class I, expressed ubiquitously in the body, and differentiate into effector cytotoxic $CD8^+$ T (T_C) cells. $CD4^+$ T cells, on the other hand, recognize antigen on MHC class II, a molecule expressed by activated antigen presenting cells (APCs) of the innate immune system, and differentiate into one of several phenotypically distinct $CD4^+$ helper T (T_H) cells[27,28,30]. Antigen recognition by the unconventional T cells – γδ T cells, MAIT cells and NKT cells – is not MHC restricted but relies on interactions with other non-polymorphic antigen-presenting molecules, and the subsequent activation of these cells lead to innate-like rapid cytokine secretion[21].

The cytotoxic $CD8^+$ T cells effectively kill infected host cells as well as tumor cells by the induction of apoptosis in the target cell[13]. The T_H cells are potent orchestrators of the inflammatory response through cognate cell-cell interactions and cytokine production. Characterized by the cytokines they produce and the developmental transcription factors needed, the T_H cells are subdivided into T_H1, T_H2, T_H17, T follicular helper (T_{FH}) and regulatory T (T_{reg}) cells that each play a distinct role in the immune response. T_H1 cells producing IFN-γ and IL-2 are important components in the immunity against intracellular pathogens and T_H2 cells expressing IL-4, IL-5 and IL-13 are crucial in parasitic immunity and tissue repair while T_{regs} are important in limiting inflammatory reactions by secreting IL-10 and TGF-β[13,31-33].

B cells

B-cells are the mediators of humoral adaptive immunity, a central aspect of the adaptive response, due to their effector function of antibody production. Antibodies, or immunoglobulins, are heterodimeric glycoproteins secreted in large quantities by the differentiated effector B cells – the plasma cells. They circulate the body and specifically target pathogens and toxins, efficiently neutralizing or targeting them for destruction through antibody-dependent cellular cytotoxicity[34].

The B-cells develop in the bone marrow where their receptor, a membrane-bound immunoglobulin (Ig) – the BCR – is assembled using somatic rearrangement[24], equivalently to TCR development. Upon antigen recognition in the periphery the BCR can then undergo further diversification through somatic hypermutation[35] of the variable region as well as class switch recombination (CSR). CSR causes the B cell to change their original expression of IgM and IgD to other isotypes through a

gene rearrangement mechanism similar to V(D)J gene rearrangement[36]. Upon activation of the B cell, which requires the combination of antigen recognition and cognate T cell interaction, it will proliferate and differentiate into plasma cells or memory B cells[31].

The liver

While the liver is not commonly considered a lymphoid organ it is, in fact, one of the most important hematopoietic organs during fetal development. The first hematopoietic stem cells of the developing mammal migrate to the fetal liver, from the yolk sac and the hemogenic endothelium of the AGM (aorta/gonada/mesonephros) region, where they originate. The fetal liver thus becomes the main site of hematopoiesis in the embryo. From the liver the hematopoietic stem cells then colonize the bone marrow (BM) and at the end of gestation the BM replaces the hematopoietic function of the fetal liver[37,38]. The adult liver is no longer the body's main hematopoietic organ, but in addition to being the central organ for metabolism, it also retains critically important immunological functions. The liver is positioned right in the center of the circulation and receives both richly oxygenated arterial blood as well as highly nutrient dense portal blood[7,39,40]. The nutrient rich portal blood flow also carries large amounts of gut-derived microbial material – antigens and toxins – directly from the gut to the liver making the liver a second line of defense against pathogens breaching the gut epithelial barrier[39,41].

The anatomical architecture of the liver allows for efficient uptake of nutrients as well as effective clearance of harmful substances, and the mounting of rapid immune responses[7,39,40]. It is a densely vascularized organ where the parenchymal cells – the hepatocytes – are arranged in nodules surrounding the small, specialized capillaries called sinusoids. The sinusoids in turn are lined with a highly permeable and fenestrated vessel wall of liver sinusoidal endothelial cells (LSECs), separating the sinusoidal lumen from the parenchyma and the hepatic stellate cells (HSCs), found in the perisinusoidal space (space of Dissé)[42] (figure 2). The blood flow through the sinusoids is slow, which in combination with the highly permeable vessel wall, result in prolonged exposure of antigens to APCs, extended lymphocyte – APC interactions and the promotion of lymphocyte extravasation[43]. In addition to hepatocytes, LSECs and HSCs the liver is also selectively enriched in cells of hematopoietic origin such as the tissue resident macrophages, Kupffer cells (KCs), hepatic DCs, NK cells and NKT cells[44,45]. KCs and hepatic DCs reside within the sinusoidal lumen where they, together with LSECs, constitute the reticulo-endothelial system responsible, through endocytosis, for the clearance of gut-derived antigens and toxins as well as endogenous waste products from the circulation[7]. The NK and NKT cell populations migrate through the extensive sinusoidal network where they upon interaction with e.g. KCs or LSECs are able to modulate hepatic immune responses[39].

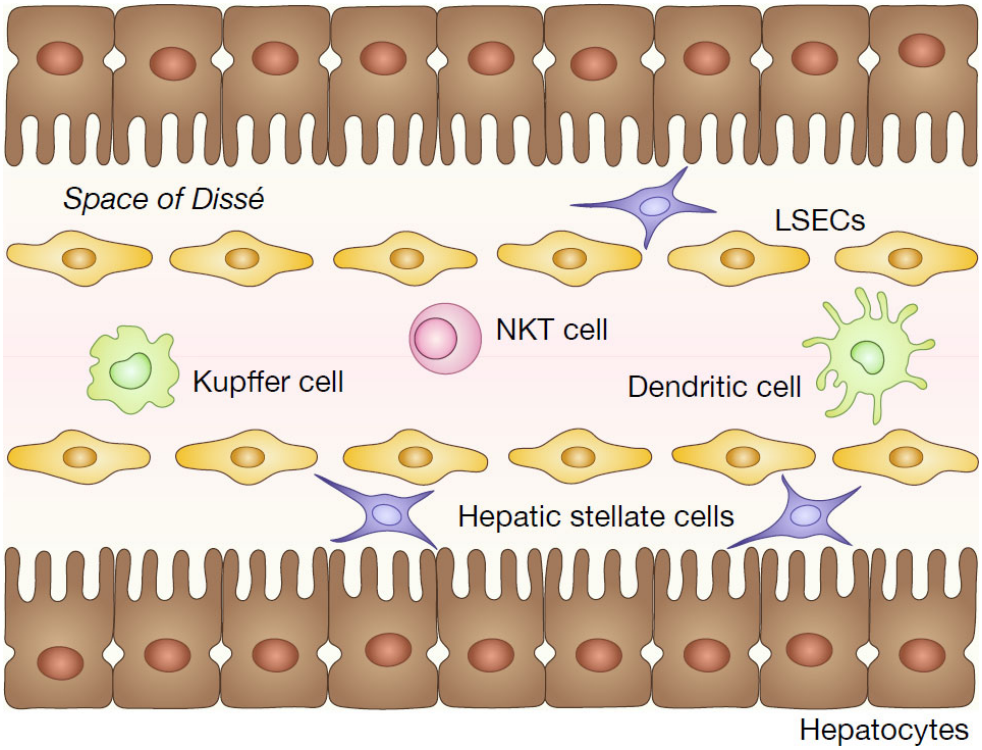

Figure 2: A simplified schematic overview of the liver sinusoid illustrating; the hepatocytes, liver sinusoidal endothelial cells (LSECs), sinusoid lumen, the enriched leukocytes of the liver; Kupffer cells, NKT-cells and dendritic cells (NK-cells not shown), and the perisinusoidal space (space of Dissé) where the hepatic stellate cells reside.

Hepatic immunity

The hepatic immune system is constantly challenged by nutrient-derived antigens as well as pathogenic material, toxins and endogenous waste products, carried by the portal blood from the gut. Due to this continuous stimulation by potential PAMPs and DAMPs there is always a basal level of cytokine expression in the homeostatic liver, both pro- (e.g. IL-2, IL-12 and IFN-γ) and anti-inflammatory (e.g. IL-10, IL-13 and TGF-β1)[46]. Due to this unusual microenvironment a complex interplay between resident leukocytes, circulating lymphocytes, the parenchyma and other structural cells results in a wide repertoire of different tolerance mechanisms during liver homeostasis[41,43].

Hepatic tolerance

The KCs are the largest tissue resident macrophage population in the body[43] and by constantly removing pathogenic material from the circulation they, in practice, constitute a primary immune surveillance filter in the liver. However, during homeostasis, PRR stimulation of KCs does not induce a pro-inflammatory response, but instead results in the production of the general immunosuppressive cytokine IL-10[41,47]. KCs also promote tolerance in the homeostatic liver by activation of T_{regs}[48,49] and the inhibition of T-cell activation through secretion of the prostaglandins PGE_2 and 15-deoxy-delta12,14-PGJ_2[50].

The hepatic myeloid DCs (mDCs) are poor stimulators of T cells compared to their counterparts in other tissues such as the spleen, and – like KCs – produce significant amounts of IL-10 during homeostasis, contributing to hepatic tolerance[51]. Hepatic plasmacytoid DCs (pDCs) are equally poor activators of T cells[46] and maintain an immature phenotype in spite of PRR stimulation[40,52], thus also contributing to the tolerogenic hepatic environment. Additionally NK cell primed mDCs[53], as well as the poor T cell stimulatory capacity of pDCs are associated with development of T_{regs} in the liver[54].

The non-hematopoietic cells of the liver also contribute to the tolerogenic environment through various mechanisms. Hepatocyte priming of naïve $CD8^+$ T cells by low level expression of MHC class I, in combination with a lack of co-stimulatory signals lead to tolerance by clonal deletion of $CD8^+$ T cells[55]. LSEC – T cell interactions during hemostasis also result in $CD8^+$ T cell tolerance, by the lack of development of cytotoxic effector functions, however, not clonal deletion, due to the interaction between co-inhibitory molecules programmed death ligand 1 (PD-L1) and PD1[56]. Priming of $CD4^+$ T cells by LSECs induces T_{reg} differentiation[57].

Hepatic inflammation

Despite the many immune tolerance mechanisms displayed by the cells of the liver – hematopoietic as well as structural – they are also capable of mounting robust and rapid inflammatory responses to pathogenic invasion, tumors or injury[41]. The tolerogenic environment can be suppressed or shifted into an inflammatory state by a variety of triggers[41]. Strong enough signaling by PAMPs and DAMPs through the specific PRRs, expressed on both the resident innate immune cells such as the KCs and on the structural cells of the liver – the hepatocytes[58], LSECs and HSCs[59] –, result in the induction of hepatic inflammation[46,60]. PRR stimulation prompts downstream signaling pathways resulting in the activation of crucial pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB)[13,60].

Important triggers of hepatic inflammation are the two DAMPs – or alarmins – IL-33, a member of the IL-1 cytokine family[61], and high mobility group protein B1 (HMGB1), released from hepatocytes upon cellular stress or injury[62]. HMGB1 has been shown to be important in neutrophil recruitment in both sterile and toxin induced liver inflammation[63], while stimulation of IL-33 receptors, expressed on mast cells and T_H2 cells, will generate a T_H2 inflammatory response[41]. Metabolic factors such as triglycerides and cholesterol are also important triggers of hepatic inflammation with the ability to promote TLR signaling and inflammasome activation, leading to the production of various pro-inflammatory cytokines[46].

During inflammatory conditions hepatocytes will upregulate MHC class II expression and are consequently able to prime naïve $CD4^+$ T cells, resulting in T_H2 differentiation, while interaction with differentiated T_H1 cells results in a decreased IFN-γ secretion by the T_H1 cells[64]. Hepatocytes also express the MHC-like molecule CD1d allowing for activation of NKT cells leading to rapid release of a wide range of pro- and anti-inflammatory cytokines[46].

LSEC stimulation of naïve T cells during homeostasis results in tolerance[57], a mechanism that can be overcome in an inflammatory setting. Increased co-stimulation through CD28 or increased TCR signaling strength will result in an inflammatory response[65].

KCs play an important role in hepatic inflammatory responses and can, upon activation, become potent producers of both pro- and anti-inflammatory cytokines[66]. In addition to inflammatory promoting cytokine production, TLR stimulation of KCs can also lead to T cell proliferation, rather than T cell suppression, overriding the tolerogenic mechanisms of KCs during homeostasis[40]. Activated KCs will also upregulate expression of cell surface adhesion molecules and chemo-attractants, resulting in leukocyte recruitment and promotion of inflammation[67].

Inflammasomes

Inflammasomes are important signaling platforms in innate immunity and the inflammatory response, facilitating the auto-processing of caspase and downstream activation of pro-inflammatory mediators such as IL-1β and IL-18[68]. They are macro-molecular complexes[68] highly expressed in leukocytes – especially in macrophages and monocytes – as well as in various other cells in a number of tissues. Inflammasomes are initiated and assembled as a result of PAMP and DAMP recognition by intracellular PRRs such as the NLRs[69].

The most commonly studied and therefore also the best characterized inflammasome is the NLRP3 inflammasome[70]. Inactive NLRP3 resides in the cytoplasm[13] and a unique feature of NLRP3, in regards to other NLRs, is that its full activation requires a two-step process where just expression of NLRP3 is not sufficient for activation[71]. For NLRP3 inflammasome assembly and activation to

take place a priming step – the transcription of the inflammasome component genes – is needed[72]. The transcription of these genes, such as *Nlrp3* and *Il1b*, is regulated by NF-κB, generally induced by TLR activation. Following priming, a second signal is required for NLRP3 activation[73] which can be triggered by a wide range of compounds, both exogenous and endogenous[73], such as nucleic acids[74], pore-forming toxins[75] and adenosine triphosphate (ATP)[76]. What ultimately induces NLRP3 inflammasome formation is controversial but K^+ efflux is generally considered to be a common pathway convergence, which indicates that NLRP3 reacts to cellular stress[72,77].

NLRP3 consists of a central oligomerization domain, an N-terminal pyrin domain (PYD) and a C-terminal LRR[78]. Canonical activation of NLRP3 inflammasome formation involves the aggregation of the LRR domains of several NLRP3 molecules which in turn induces the PYDs to interact with apoptosis-associated speck-like protein containing a CARD (ASC)[13]. ASC then recruits pro-caspase-1 via their CARD domains resulting in the polymerization of pro-caspase-1 into active caspase-1[68]. The subsequent proteolytic cleavage of pro-IL-1β and pro-IL-18, by active caspase-1, generates the mature secreted forms of these pro-inflammatory cytokines[13,79].

The inflammasomes have numerous implications in the inflammatory response. The downstream effects of its activation – cleavage and maturation of IL-1β and IL-18 – are important for the recruitment and activation of immune cells, and critical in the type 1 inflammatory defense against invading pathogens[80]. However, inflammasome activation has also been shown to contribute to uncontrolled inflammation resulting in excessive tissue damage[81]. Inflammasomes also play a role in type 2 inflammation and tissue repair, where the activation of e.g. caspase and IL-18 are critical to wound healing[82]. Additionally, inflammasome activation has also been linked to the development of pathological fibrosis[83-85].

Type 1 inflammation

Type 1 inflammation is an important protective immune response against infectious microorganisms like bacteria and virus, while also playing an important role in tumor surveillance. Type 1 inflammatory reactions are mediated by an extensive group of effector cells such as T_H1 and T_H17 cells, cytotoxic T-cells, type 1 innate lymphoid cells (ILC1s), classically activated macrophages and specific immunoglobulins like IgM and IgA[86].

$CD4^+$ T cell differentiation into T_H1 cells is controlled by the transcription factor T-bet via activation of signal transducer and activator 4 (STAT4) in the presence of IL-12[87]. Upon differentiation these T_H1 effector cells produce pro-inflammatory cytokines, primarily IFN-γ and IL-2[88]. IFN-γ, a dominating cytokine in type 1 inflammation[16], subsequently augments further T_H1 differentiation in a positive

feedback loop as well as promoting type 1 immunity by the suppression of T_H2 differentiation[89]. Macrophages are another important cell population in type 1 inflammation[16]. The majority of macrophage TLR signaling will result in activation of NF-κB leading to induction of pro-inflammatory cytokine expression such as tumor necrosis factor-α (TNF-α), IL-1β and IL-6, as well as various chemotactic factors[13]. In addition to PRR stimulation macrophages can also be activated by IFN-γ, which promotes classical macrophage activation[90]. IL-1β production by classically activated macrophages, an inflammasome dependent process, induces the expression of various other pro-inflammatory cytokines such as IL-6 and IL-8 further promoting type 1 immunity[91].

Dysregulated type 1 inflammation leads to significant cell death and tissue damage due to apoptosis[16] and excessive production of T_H1 cytokines are associated with autoimmune disease[92].

Type 2 inflammation

It was originally believed that type 2 inflammation was merely a regulatory mechanism designed to limit the potential tissue damaging consequences of type 1 inflammation[93]. However, it has become clear that type 2 immunity, while an important regulator of type 1 inflammation e.g. in various autoimmune diseases[94-96], also plays a critical role in the protection against pathogens, in particular extracellular parasites[97]. Another crucial mechanism of type 2 inflammation is the restoration of tissue homeostasis following injury, by the promotion and regulation of important tissue repair and regeneration pathways[16].

Type 2 immunity involves a plethora of cells and cytokines, particularly characterized by T_H2 cells, eosinophils, ILC2s, mast cells and basophils, as well as the type 2 cytokines IL-4, IL-5, IL-9, IL-13[86], IL-33 and thymic stromal lymphopoietin (TSLP)[98]. IL-4 is the archetypical type 2 cytokine and serves a variety of essential functions in the induction and amplification of type 2 inlammation. The origin of "primary" IL-4 is debated, however, several cell populations have been identified as significant IL-4 sources during type 2 inflammation[90]. One such population is NKT cells that possess the innate ability for rapid secretion of various cytokines in response to cellular activation, and are substantial producer of IL-4[99]. Another population is basophils that have been shown to produce large quantities of IL-4 in response to cytokines such as IL-33 and IL-18[90]. A crucial mechanism of IL-4 mediated immunity is its role in the T_H2 differentiation of $CD4^+$ T cells[100]. IL-4 upregulates the expression of GATA3, an essential transcription factor for T_H2 differentiation[101], in a STAT6-dependent manner[98]. Upon differentiation, T_H2 cells themselves become a significant source of IL-4, in addition to their production of other important type 2 cytokines such as IL-5 and IL-13[98]. Moreover, IL-4 is also an important mediator in the regulation of B cell class switching to IgE[98], which plays a crucial role in the propagation of type

2 inflammation through the activation of innate immune cells such as mast cells and basophils. IgE activation of these cells results in vascular permeability and inflammatory cell recruitment, due to the secretion of a range of mediators upon their degranulation[98]. IL-4, as well as IL-13 and IL-33[102], are also able to alternatively activate macrophages into an M2 phenotype[103], which are thought to play an important role in the orchestration of tissue repair, regeneration and restoration of homeostasis[66]. However, the mechanisms regulating tissue repair and regeneration are not yet fully elucidated[104].

Apart from its host protective effector mechanisms, type 2 immunity can also display pathogenic functions. The regulatory suppression of type 1 inflammation can result in pathogenesis when proper type 1 responses are hindered and infections are allowed to persist and become chronic[86], or when cytotoxic T cell development is suppressed, promoting tumorigenesis[105]. Dysregulated type 2 inflammatory responses will also lead to various diseases and disorders such as allergic reactions – a result of T_H2 cytokine overproduction[106]. Another consequence of dysregulated type 2 inflammation is the development of progressive, pathological fibrosis due to chronic activation of tissue repair pathways[107].

Fibrosis

Tissue fibrosis – the formation of scarring – is a pathological condition that can manifest in virtually any tissue or solid organ and is a leading cause of mortality in the western world. It is estimated that over 45% of deaths[16] in the US are attributed to an underlying fibrotic condition. Pathological fibrosis development has varying etiologies such as bacterial, viral or parasitic origin, toxic damage, metabolic dysregulation, sterile inflammation as a result of trauma or autoimmune triggers[16]. It is, however, generally believed that key components of the pathology of fibrogenesis are shared in spite of differing etiologies.

Fibrosis is characterized by the overproduction and excessive deposition of extra cellular matrix (ECM) proteins which result in disproportionate connective tissue formation. This leads to the remodeling of normal tissue architecture and the replacement of functional parenchymal cells, which subsequently causes a loss of function of the afflicted organ, and in the end organ failure[107].

Physiological tissue repair following cellular injury is a fundamental process needed in order for tissue homeostasis to be reestablish. It is a tightly regulated process which requires a complex interplay of many different cells and mediators. Tissue trauma elicits an acute inflammatory response which serves to eliminate the cause of injury, upon which subsequent tissue repair mechanisms follow[108]. The process of biological repair essentially involves two overlapping phases – first the regeneration of damaged cells allows for tissue functionality to be restored and then a second phase ensues, where connective tissue replaces parenchymal cells that can't be regenerated[16]. Pathological fibrosis develops as a consequence of the dysregulation or chronicity of these tissue repair mechanisms.

Currently there are no effective anti-fibrotic drugs and the ultimate treatment in many end-stage fibrosis cases is organ transplant.

Fibrogenic pathways

The underlying mechanisms of pathological fibrosis development are not yet fully elucidated but a number of key cells, cytokines and signaling pathways have been identified. Dysregulated or chronically activated inflammation and wound repair involves numerous components, such as alarmins, cytokines, chemokines, matrix

metalloproteinases (MMPs) and various other molecules, which all contribute to the subsequent development of fibrosis. Chemokines such as CC-motif chemokine ligand 3 (CCL3) and CCL2, important for monocyte recruitment, are critical pro-fibrotic mediators and their inhibition consequently leads to reduced fibrosis development[109,110]. The type 2 cytokine TSLP has also been linked to fibrosis development[111,112] and implicated in the initiation of collagen synthesis[113], a major component of the ECM. Another identified important pro-fibrotic mediator is the enzyme lysyl oxidase homologue 2 (LOXL2). LOXL2 promotes cross-linking of collagen I, and subsequently, antibody blocking of LOXL2 activity leads to decreased fibrosis development[114]. There are a number of important effector cells involved in promoting fibrosis development but the most critical is the myofibroblast, a proliferative and contractile cell, characterized by an excessive production of ECM[115,116]. Many of the identified pro-fibrotic pathways involve myofibroblast engagement and activation.

TGF-β1

Transforming growth factor-β1 (TGF-β1) is a regulatory cytokine with important anti-inflammatory functions[13]. It is also arguably the most studied cytokine in fibrosis development[107] and is considered one of the most potent pro-fibrotic mediators[117], with implications in a range of different fibrotic diseases[118-120].

TGF-β1 control is mediated primarily through the activation of latent TGF-β1[16,121], which is stored in cells[121] and the ECM[122], bound to latency-associated protein (LAP)[107], keeping it in its inactive form. The subsequent activation of TGF-β1 requires the dissociation from LAP, a process mediated by a number of varying agents, such as different MMPs, thrombospondin and integrin-αvβ6[107,123]. The primary role for TGF-β1 in fibrogenesis is the activation of resident meschenchymal cells into myofibroblasts – potent producers of vast amounts of ECM[107]. TGF-β1 receptor binding leads to downstream SMAD3 signaling, which upon phosphorylation, translocates into the nucleus where it regulates expression of fibrogenic target genes[124]. Important upregulated target genes, following TGF-β1 activation of myofibroblasts include pro-collagen I and III, tissue inhibitor of metalloproteinase-1 (TIMP1) and TIMP2[121,125].

The T_H2 cytokines – IL-4 and IL-13

The type 2 cytokines IL-4 and IL-13, produced by a multitude of different cells such as ILC2s, T_H2 cells, eosinophils and NKT cells[86,126], are both potent pro-fibrotic cytokines and share many fibrogenic functions due to the fact that they share a common signaling receptor, IL-4α[127]. Both IL-4 and IL-13 can alternatively activate macrophages to upregulate MMP12, an essential driver of fibrosis, which in turn

suppresses the expression of the proteolytic MMPs MMP2, MMP9 and MMP13 which leads to reduced ECM degradation[128].

The extent to which IL-4 contributes to different fibrotic diseases varies, however, inhibiting IL-4 has proved to significantly decrease fibrosis development in a number of fibrotic conditions[129-131]. IL-4 has also, *in vitro*, been shown to stimulate the fibroblast synthesis of the ECM components fibronectin and collagen type I and III[132].

IL-13 has, despite sharing the IL-4α receptor pathway with IL-4, shown to exhibit non-redundant roles in fibrosis development, using IL-13 transgenic knock-out mice[133,134] and IL-13 antagonists[135,136]. Additionally IL-13 has proved to be the more potent pro-fibrotic cytokine, compared to IL-4, in several experimental models[108,135,137]. IL-13 can also promote fibrogenesis by inducing the production of latent TGF-β1 in macrophages, as well as upregulating the expression of proteins that catalyze the cleavage of LAP from TGF-β1, resulting in increased activation of TGF-β1[138,139].

The alarmin IL-33

Alarmins are characterized as specific DAMPs released from necrotic cells in an unconventional fashion[140] and the cytokine IL-33, a member of the IL-1 family[141], indeed constitutes such a DAMP[62]. IL-33 is localized in the nucleus[142] of, mainly endothelial, epithelial and fibroblast-like cells[143] and is released upon cellular injury or necrosis[144].

The cytokine function of IL-33 is mediated through its interaction with the receptors ST2 (IL-1 receptor-like 1) and IL-1 receptor accessory protein (IL-1RAcP)[62]. The ST2 receptor is primarily expressed on type 2 leukocytes, such as T_H2 cells, mast cells, eosinophils, NK cells and NKT cells[145-147], thus IL-33 stimulation of ST2 leads to the production of important type 2 cytokines, such as IL-5 and IL-13[148]. In support of this it has been shown that administration of IL-33 to naïve mice induce splenomegaly, eosinophilia, increased IgE in serum, as well as IL-5 and IL-13 production[141]. The mechanism by which IL-33 promotes fibrosis development is also believed to be through the induction of IL-13 production by ILC2s, macrophages[149] and eosinophils[150]. However in a study by Vannella et al. inhibition of IL-33 alone did not reduce fibrosis development in mice but blocking of IL-33, as well as IL-25 and TSLP, significantly reduced fibrosis and inflammation, ILC2 recruitment and eosinophilia[148], suggesting that IL-33 cytokine effector functions in fibrosis development can be compensated by other fibrogenic mediators.

The IL-33 stimulation of ST2 also leads to downstream activation of transcription factors such as NF-κB, resulting in upregulation of pro-inflammatory cytokines like IL-1β, IFN-γ and TNF-α[62]. However, it has also been shown that IL-33 interacts with NF-κB impairing its binding to DNA leading to decreased NF-κB-dependent pro-inflammatory gene transcription in fibroblasts[151].

In the murine liver, activated HSCs are the major source of IL-33 during fibrosis development but also hepatocytes are a source of IL-33 during liver injury, an expression that in a model of concanavalin A (ConA)-induced hepatitis is NKT-cell dependent[152].

Hepatic fibrosis

Virtually all chronic liver diseases are associated with the development of hepatic fibrosis[153] and can be triggered by a wide variety of different factors, including viral (e.g. hepatitis C virus), toxic (e.g. alcoholic steatohepatitis), metabolic (e.g. non-alcoholic fatty liver disease and non-alcoholic steatohepatitis) or autoimmune (e.g. primary biliary cirrhosis) causes[154].

As hepatic fibrosis progresses the excessive accumulation of connective tissue eventually bridges portal tracts and central veins together, compromising the function of the liver sinusoids[155]. This distortion of the hepatic architecture impairs liver function and is defined as cirrhosis – the end stage of hepatic fibrosis[156]. The development of cirrhosis leads to the risk of liver failure, as well as an increased risk of developing hepatocellular carcinoma[155].

An extensive number of cells contribute to the initiation and development of hepatic fibrosis, structural liver cells, as well as resident and recruited immune cells. LSECs release both pro-inflammatory and pro-fibrotic cytokines upon injury, which leads to the recruitment of various immune cells[154]. T_H2 cells promote fibrogenesis through the activation of macrophages by the secretion of pro-fibrotic cytokines such as IL-4 and IL-13[135] and ILC2s through IL-13 production[157].

Liver macrophages

Macrophages are important regulators of hepatic fibrosis and during inflammation the macrophage population in the liver significantly increases due to the recruitment of monocyte derived macrophages[154], which has been shown to promote the progression of hepatic fibrosis in CCl_4-treated mice[158]. The resident KCs are believed to play an important role in the recruitment of monocyte derived macrophages, through their production of chemokines such as CCL2, a main monocyte chemo-attractant[159]. Deletion of the CCL2 receptor CCR2 in mice leads to decreased macrophage infiltration upon injury, and significantly inhibits hepatic fibrosis[160,161]. KCs also secrete C-X-C motif ligand 16 (CXCL16), important for the recruitment of NKT cells[162] (discussed further under NKT cells).

Both resident KCs and recruited monocyte derived macrophages contribute to the development of hepatic fibrosis[159] and have been shown to play dual roles in the

fibrogenesis[163]. Interestingly, liver macrophages can express both M1 and M2 markers simultaneously[164] and it has been indicated that the role of macrophages in liver depend on origin, rather than activation state[165].

In the fibrotic liver, macrophages are found in close proximity to activated HSCs[163,166] and promote fibrogenesis by the secretion of pro-fibrotic cytokines such as TGF-β1, the TGF-β-activating protein thrombospondin 1[164] and platelet-derived growth factor (PDGF), which leads to the activation and proliferation[15] of HSCs – the major ECM protein source[167]. Macrophages also produce pro-inflammatory cytokines such as TNF-α and IL-1β, which contribute to upregulation of NF-κB, stimulating activated HSC survival, further promoting hepatic fibrogenesis[167]. However, liver macrophages also contribute to the resolution of fibrosis through their production of a number of ECM-degrading MMPs that promote fibrosis resolution[166]. This functional duality is demonstrated by macrophage depletion leading to an attenuated fibrotic response in several studies[163,167,168] but the depletion of macrophages in the recovery phase of fibrosis inhibits both ECM degradation as well as the destruction of HSCs[163].

Hepatic stellate cells (HSCs)

Activated HSCs are, undeniably one of the most important cell populations in the development of hepatic fibrosis. In their quiescent, non-activated state, HSCs store retinol esters in lipid droplets in the cytoplasm, and make up about 10% of resident liver cells[117]. However, upon activation, these cells transdifferentiate into proliferative, contractile and fibrogenic myofibroblasts that are characterized by an excessive production of ECM components[116].

HSCs can be activated by a wide range of cytokines and growth factors, as well as reactive oxygen species[169]. They are a primary target for TGF-β1[121], which through SMAD3 activation promotes transcription of ECM proteins such as type I and type III collagen[83,170] during HSC activation. TGF-β1 also upregulates α-smooth muscle actin (α-SMA)[171] in activated HSCs and promotes myofibroblast survival[172]. Conversely, quiescent HSCs are negatively regulated by TGF-β1, through SMAD7 signaling[121]. Activated HSCs lack SMAD7 and this has been suggested to be a reason for excessive pro-fibrotic TGF-β1 effects during hepatic fibrosis development[173]. One of the most important growth factors regulating activated HSCs is PDGF, a critical factor for HSC proliferation and migration, where depletion of its receptors (PDGF ligand and PDGF receptor-β) on HSCs has been shown to decrease murine hepatic injury and fibrosis[174].

Moreover, HSCs can be stimulated by TLR engagement, leading to downregulation of the TGF-β1 pseudo-receptor BAMBI, which in turn sensitizes the HSCs to TGF-β1 stimulation. Additionally, TLR stimulation also leads to HSC activation and chemokine production[59].

NKT cells

NKT cells are emerging as key mediators in hepatic immune regulation[175] and have been demonstrated to play diverse roles in liver injury, inflammation and fibrosis[169,176-178].

As previously mentioned, the NKT cells are innate-like lymphocytes, a subset of unconventional T cells developing from the common lymphoid progenitor[5]. They recognize antigen in the context of a fully rearranged TCR but possess the ability to rapidly secrete large amounts of cytokines and chemokines upon activation[179]. Previously NKT cells were defined as $CD3^+$ T cells with a co-expression of NK markers such as NK1.1, however a more inclusive definition of NKT cells are their specificity for the highly conserved protein class I MHC-like molecule CD1d[5,180,181].

NKT cells are naturally enriched in the liver[44,45], constituting 30-40% of total liver lymphocytes in mice and 15-25% in humans[169]. NKT cells naturally home to the liver[182,183], in large part due to their expression of the chemokine C-X-C motif receptor 6 (CXCR6). The CXCR6 ligand CXCL16 is a chemotactic chemokine expressed by LSECs which subsequently leads to the recruitment of NKT cells into the liver[184,185].

There are two main categories of NKT cells, type I or invariant NKT (iNKT) cells and type II NKT (NKTII) cells with differing distribution in mice versus humans. iNKT cells are the prevalent cell type in mice while NKTII cells are the more abundant subset in humans[186]. The division of the NKT cells is based on their TCR expression as well as lipid-antigen specificity and antigen recognition mechanism[186]. iNKT cells express a semi-invariant TCR encoded by the invariant Vα14Jα18[187] gene segment paired with, in mice, one of a limited number of Vβ chains (Vβ8.2, Vβ7 or Vβ2)[188,189] and, in humans, with the Vβ11 chain[190,191], while NKTII cells express a more varied, yet restricted, set of TCR configurations[192].

Invariant NKT cells

iNKT cells where the first NKT-cells to be discovered[193] and is also the most studied of the two subsets. iNKT cells are strongly activated by the marine sponge-derived antigen α-galactosylceramide (αGalCer)[194] but can also be activated by both microbial and self-derived lipid antigens[195]. TCR mediated activation of iNKT cells

can be enhanced by IL-12 and type I IFN produced by TLR activated DCs[196], but they can also be activated by cytokines alone (e.g. IL-12 and IL-18), without TCR signaling[175,186]. The iNKT cell population can be further be divided into subsets based on their description factors and/or secreted cytokines, mimicking the effector subsets of $CD4^+$ T cells; iNKT1 (T-bet/IFN-γ), iNKT2 (GATA3/IL-4), iNKT10 (IL-10) and iNKT17 (Rorγt/IL-17)[197-199].

iNKT cells display diverse roles in hepatic immunity depending on factors such as microenvironment and type of activation. In many experimental models of chronic liver disease such as ConA-induced hepatitis[178,200], non-alcoholic fatty liver disease (NAFLD)[201-203] and primary biliary cirrhosis (PBC)[204], iNKT cells display a predominantly pro-inflammatory role. The pro-inflammatory effects of iNKTs, in the liver, are in large part most likely mediated by their capacity to produce great amounts of various pro-inflammatory cytokines such as IFN-γ and TNF-α[179,205], however iNKT cells also have the ability to directly kill hepatocytes through Fas/FasL interactions[178]. While iNKT cells exhibit pro-inflammatory roles in many chronic liver diseases, in acute hepatic injury models such as acute CCl_4-induced fibrosis they display a protective role, confirmed by increased inflammation upon iNKT cell depletion[177]. Additionally, in the chronic CCl_4-induced liver injury model, iNKT cells exhibit a protective phenotype in the early stages of inflammation and fibrosis development, but not in the later stages, in part accredited to inhibition of hepatic stellate cell activation[177].

NKTII cells

The NKTII cells are less explored than the iNKT cells, therefore not as much is known about their effector function diversity nor their varying roles in hepatic immunity. As opposed to iNKT cells NKTII cells do not recognize αGalCer but is activated by a, continually increasing, range of other antigens, such as the sulfated glycolipid sulfatide[206], β-glucosylceramide (βGlcCer) and βGalCer[207,208]. NKTII cells appear to be, primarily, reactive to self-lipid antigen but can, however, also recognize structurally similar microbial-derived lipids[209]. In contrast to iNKT cells, NKTII cells seem to be activated, mainly, by TCR signaling and not in response to TLR signaling in APCs[208,210,211].

NKTII cells have been shown to have various roles in non-hepatic immunity, where their immune modulatory role in different diseases seem to be highly dependent on their context as they can display both protective and pathogenic roles depending on the setting. NKTII cells have been reported to play a protective role in several autoimmune disease models such as in the development of autoimmune diabetes in the NOD mouse[212,213] and in the experimental autoimmune encephalomyelitis (EAE) model[206], where IL-4 secretion by NKTII cells has been suggested to mediate

protection from disease development[194]. Yet, in other autoimmune models such as the mouse model for Gaucher disease, NKTII cells have displayed a pro-inflammatory, pathogenic role[214], and NKTII cells have also been reported to mediate autoimmune colitis in mice[215]. Moreover, several studies suggest NKTII cells to have a suppressive effect on tumor immunity[216-219], thereby promoting tumorigenesis, however, there has also been a report where NKTII cells contribute to protective tumor immunity[220]. The protective mechanisms of NKTII cells in autoimmune disease, as well as their suppressive function on tumor immunity, is believed to be dependent on the induction of iNKT cell anergy and the inhibition of conventional Tcells[221-223].

In the liver, the ability of NKTII cells to suppress inflammatory responses induced by iNKT cells has shown importance in liver disease such as ConA-induced hepatitis[224,225] and ischemic liver injury[226] where the NKTII mediated iNKT cell anergy results in protection from liver injury. However, NKTII cells have also been shown to promote chronic liver disease and hepatic inflammation, e.g. in a high fat diet induced obesity model[227] and in a transgenic model overexpressing CD1d, where the increased crosstalk between NKTII cells and conventional T-cells results in the promotion of chronic autoimmune liver disease[228].

The NIF mouse

The NIF (non-obese diabetic inflammation and fibrosis) mouse, 2,4αβNOD.*Rag2*$^{-/-}$, is a novel mouse model for spontaneous development of inflammation and fibrosis. It develops inflammation and fibrosis in multiple organs, such as skin, liver and kidney, at an early age. The disease phenotype displayed in the liver of the NIF mouse resembles human fibrotic conditions in several pertinent features, including the temporal heterogeneity of the fibrotic lesions, the progressive nature of the disease and the preceding chronic inflammation (paper I).

The disease phenotype of the NIF mouse is mediated by an overexpressed, transgenic, monoclonal αβ TCR NKT cell population. This αβ TCR NKT cell transgene was originally isolated from the NKT cell line VIII24[229,230] and expressed in the NOD mouse in order to study NKT cell involvement in autoimmune diabetes[212]. The isolated NKT cell is classified as a type II NKT cell due to its TCR rearrangement – Vα3.2/Vβ9 – and when expressed in a *Rag2*-deficient mouse (NOD.*Rag2*$^{-/-}$)[231], leads to the spontaneous development of chronic inflammation and subsequent severe hepatic fibrosis.

In the liver, the NIF mouse displays inflammation and fibrosis particularly evident around portal tracts and central veins and is accompanied with evidence of abnormal intrahepatic bile ducts. Characterization of this inflammatory syndrome and hepatic fibrosis revealed that the extensive cellular infiltration consists mainly of granulocytes, particularly eosinophils, but also macrophages and monocytes and is accompanied by the presence of high levels of both pro-inflammatory and pro-fibrotic cytokines (paper I). A maximum infiltration level of the liver is reached at 8 weeks of age whereupon the leukocyte recruitment decline, yet remaining at increased levels compared to controls. However pro-inflammatory and pro-fibrotic cytokines in the NIF liver remain elevated after cellular infiltration has diminished (paper II).

The transgenic NKT cell population drives the NIF liver phenotype, which is illustrated by the NKT cell infiltration preceding the recruitment of the myeloid cells in the liver (paper II). This is confirmed by adoptive transfer experiments where transgenic NKT cells from NIF mice can induce the inflammation and fibrosis in the liver of immunodeficient recipients. The expression of the transgenic NKT cell population in heterogeneous NOD.*Rag2*$^{+/-}$ mice, with a fully functioning adaptive immune system, does not lead to development of inflammation and fibrosis, and it has been shown that the NIF disease phenotype can be inhibited and reversed by the

presence of an, as of yet, unidentified T cell population (paper I). Additionally it is believed that the hepatic fibrosis of the NIF mouse is mediated, in part, by IL-4-independent fibrogeneic pathways, such as IL-33 signaling (paper II).

The disease phenotype displayed by the NIF mouse is also thought to be promoted by one or more dominant gene(s) of the NOD genome, as illustrated by NIF backcrossing. It is, however, not elucidated what gene(s) or what role it plays in the development of the inflammation and fibrosis in the NIF mouse (paper I).

Treatment of the NIF disease phenotype

In order to use the NIF mouse model as an effective pre-clinical tool in the efficacy testing of potential anti-fibrotic drugs and the evaluation of therapeutic treatment protocols, the fibrosis needs to be treatable and reversible. The efficient reduction and reversal of both the hepatic inflammation and the subsequent fibrosis of the NIF mouse is also an advantage when using the NIF mouse to further elucidate important and crucial mechanisms of hepatic fibrosis development and resolution.

The inflammatory and hepatic phenotype of the NIF mouse can be inhibited and reversed by cell therapy. Using adoptive transfer of splenocytes from syngeneic immunocompetent mice, both liver inflammation and fibrosis is effectively reversed (paper I).

The quinoline-3-carboxamide compound Paquinimod, an anti-inflammatory compound[232,233] that has also shown possible anti-fibrotic effects in the treatment of scleroderma[234], can be used to successfully treat the inflammatory syndrome as well as the hepatic fibrosis of the NIF mouse (paper III). One of the targets of Paquinimod has been reported to be the chemotactic and pro-inflammatory mediator S100A9[235], involved in the recruitment of myeloid cells during inflammation. The anti-inflammatory effects of Paquinimod are believed to be the result of its inhibitory action on S100A9 receptor interactions[236], thereby affecting myeloid cell recruitment. Comparatively however, treating NIF mice with the small molecule compound ISI[237], with a specific and high affinity for S100A9 interaction, results in reduced inflammation but not fibrosis[238,239]. This suggests that the anti-fibrotic effects displayed by Paqinimod, in the NIF mouse, might be mediated by, at least in part, a separate mode of action from its anti-inflammatory mechanism(s). One such possible anti-fibrotic effect could be the inhibition of the transgenic NKT cells, which upon Paquinimod treatment, become less granular and thus, appear less activated (paper III).

The hepatic fibrosis development of the NIF mouse is TGF-β1- dependant and can therefore also be successfully treated by using a TGF-β1 blocking antibody. Hence, administering the blocking anti-TGF-β1 antibody (1D11), leads to significant decrease of already established hepatic fibrosis in the NIF mouse (paper II).

Hepatic imaging

Classical biological techniques to study tissue structure, cellular quantification and distribution, such as histology, flow cytometry and immunohistochemistry are excellent investigative tools for studying many aspects of inflammation and fibrosis. However, they do not provide 3-dimensional information or the possibility of studying cellular dynamics. To complement the classical 2-dimensional techniques numerous 3D-imaging technologies have been developed. Ex vivo techniques such as optical projection tomography (OPT)[240] or light sheet microscopy[241] give high resolution 3D images of whole murine organs, and can be combined with fluorescence labeling for visualization of various cell populations or anatomical micro-structures in end-point analyses. Intravital imaging, however, offers the possibility to study cellular dynamics and longitudinally follow disease progression in vivo. Longitudinal monitoring of progressive disease processes such as chronic inflammation and fibrosis is important for diagnosis and prognosis of hepatic disease conditions such as NAFLD/NASH, and constitutes an important area of development in clinical hepatology.

There are several non-optical based, intravital imaging techniques available for studying the liver, such as magnetic resonance imaging (MRI) and positron emission tomography (PET), which are used in both mouse and human pre-clinical and clinical studies, have high imaging depths and can be used for whole body imaging[242-246]. Although these techniques offer many advantages they have yet to reach sufficient resolution to study single cell dynamics. Intravital, high resolution imaging in the single cell range presents a problem due to spatial resolution being inversely correlated to imaging depth, i.e. the deeper in the tissue you reach, the lower the resolution. High resolution in situ imaging of liver, therefore, poses a number of challenges due to its embedded location in the peritoneal cavity. In vivo imaging of cellular events in the liver thus requires the invasive procedure of surgically exposing the organ, directly to the lens or to an “imaging window”.

The ACE technique

To circumvent the need for invasive surgery in high resolution imaging of tissues in disadvantaged locations, the anterior chamber of the eye (ACE) transplantation method has previously been developed[247]. The ACE method exploits the transparent

cornea as a natural imaging "window", and thereby offers the possibility of non-invasive imaging of transplanted grafts. When combined with two photon microscopy (2PM)[248], the ACE offers a non-invasive, optical, high resolution imaging technique with the possibility of repetitive, longitudinal in vivo imaging. Transplantation using the ACE as graft site has been used in the research of several different tissues[247,249,250] (paper IV) and constitutes an optimal transplantation site, due to the densely vascularized iris and oxygen rich environment. The ACE technique has since been modified, in order to study the cellular dynamics in inflammation, by transplanting pancreatic islets into NOD mice, whereupon the inflammatory progression of autoimmune diabetes could be followed[251].

We are currently adapting the ACE technique presented in paper IV to study liver grafts. By transplanting syngeneic liver anlage into the anterior eye chamber, we can follow the revascularization process and subsequent liver development for up to two months by repetitive, longitudinal imaging (figure 3). The subsequent aim is to use this technique in the NIF mouse, in order to further investigate the initiation and progression of the inflammatory syndrome, as well as the hepatic pathophysiological tissue changes caused by the fibrosis development. This adaptation of the ACE technique will also allow for the possibility to monitor the effects of disease intervention protocols and potential anti-fibrotic drug candidates.

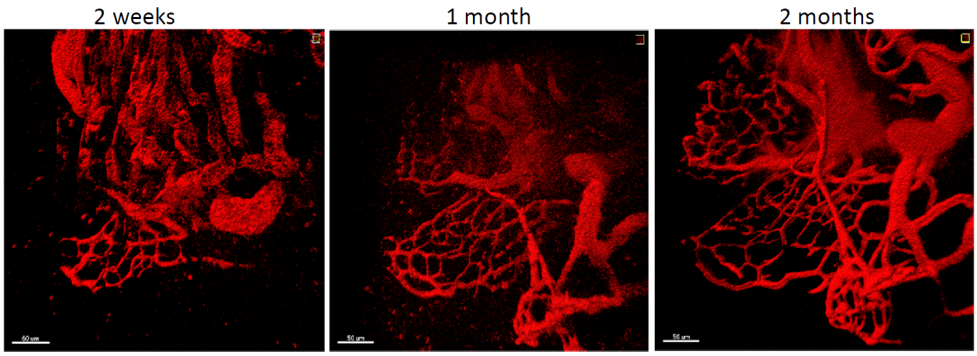

Figure 3: Two photon microscopy images of liver grafts in the ACE, illustrating the developing vascularization. E13 embryonic liver transplanted into the anterior eye chamber of a NOD.*Rag2*$^{-/-}$ mouse. Imaged repeatedly for 2 months using 2PM. Vessel tree visualized by i.v. injection of 70-kDa-FITC-Dextran during imaging.

Synopsis of original work

Summary and discussion of papers

Paper I:

A New Mouse Model That Spontaneously Develops Chronic Liver Inflammation and Fibrosis.

Nina Fransén-Pettersson, Nadia Duarte, Julia Nilsson, Marie Lundholm, Sofia Mayans, Åsa Larefalk, Tine D. Hannibal, Lisbeth Hansen, Anja Schmidt-Christensen, Fredrik Ivars, Susanna Cardell, Richard Palmqvist, Björn Rozell and Dan Holmberg.

Summary:

We characterize the NIF (2,4αβNOD.*Rag2*$^{-/-}$) mouse, a recently established animal model that spontaneously develops inflammation and fibrosis in several different organs. The phenotype is particularly evident in the liver where the fibrosis development is preceded by chronic inflammation. The NIF mouse overexpress a monoclonal αβ TCR transgenic NKT-cell population, which induces the disease phenotype, on an immunodeficient NOD genetic background. Its control counterpart, the 2,4αβNOD.*Rag2*$^{+/-}$ mouse, overexpress the same transgenic NKT-cell population but in the presence of a functioning adaptive immune system, inhibiting the inflammatory and fibrotic syndrome from developing.

Key findings:

- The NIF mouse develops hepatic inflammation, with extensive cellular infiltration dominated by eosinophils, macrophages, mast cells and multinucleated giant cells, as early as 3 weeks of age.
- The hepatic inflammation was accompanied by fibrosis, primarily localized to the portal tracts and central veins, with varying degrees of periportal and bridging fibrosis.
- Transgenic NKT cells accumulating in inflammatory areas produce TH2 cytokines such as IL-4, IL-5 and IL-13, as well as the TH1 cytokines IFN-γ and IL-2.

- The monoclonal transgenic NKT cells induce the inflammatory syndrome and fibrotic disorder of the NIF mouse, and can be suppressed by other T-cell populations.
- The disease phenotype of the NIF mouse is dependent on the 2,4αβ TCR transgenic NKT-cell population and the NOD genome contains dominant gene(s) promoting this development.

Discussion:

One of the major caveats in using animal models for studying the molecular mechanisms of fibrosis development, has been the difficulty in reproducing the process of persistent and chronic inflammation, commonly observed in human fibrotic disorders. The NIF mouse, however, develops a chronic hepatic inflammation that precedes the development of a severe fibrotic condition. The extensive cellular infiltration observed in the hepatic inflammation in the NIF mouse is dominated by type 2 associated leukocytes such as eosinophils, macrophages, mast cells and multinucleated giant cells.

The overexpressed transgenic NKT-cells in the NIF mouse display a mixed T_H1/T_H2 cytokine profile whereas they, in the 2,4αβNOD.*Rag2*$^{+/-}$ control mouse, express a T_H1 cytokine profile. While the molecular mechanisms for this shift still remains to be fully elucidated, it stands to reason that the mixed cytokine profile of the NIF NKT-cells underlie the inflammatory syndrome observed in the NIF mouse.

The NIF mouse is a homogenous *Rag2*$^{-/-}$ mouse – thereby lacking an adaptive immune system – while the 2,4αβNOD.*Rag2*$^{+/-}$ control mouse possess a fully functioning adaptive immune system. Hence, it can be concluded that the inflammatory syndrome of the NIF mouse can be inhibited by a functioning adaptive immune system. Further investigation into what could constitute possible inhibitory component(s) revealed that the inflammatory syndrome of the NIF mouse can be suppressed by other T-cell populations, possibly T_{regs}. Adoptive transfer of polyclonal T-cells from unaffected syngeneic donors to phenotypic NIF mice results in a decrease in inflammation and fibrosis, while adoptive transfer of T-cell depleted splenocytes were unable to show the same reduction.

The NIF disease phenotype is transferrable to NOD.*Rag2*$^{-/-}$ recipients by adoptive transfer of NIF splenocytes, while NKT-cell depleted NIF splenocytes cannot induce inflammation and fibrosis in an immunodeficient recipient. This shows that the pathology of the NIF mouse is initiated and driven by the transgenic NKT cells, suggesting a possibly important role for NKT-cells in the orchestration of fibrotic disorders.

An interesting observation is that, while IL-4 is an important mediator of fibrogenesis, adoptive transfer of polyclonal syngeneic T-cells to NIF mice, which reduced both hepatic inflammation and fibrosis, did not decrease the expression of IL-4. This suggests that the fibrosis development of the NIF mouse depends, at least

in part, on alternative IL-4-independent fibrogenic pathways, such as IL-33 signaling.

Despite the efforts made, and the subsequent identification of key mediators and mechanisms in the development of pathological fibrosis, there are still no efficient anti-fibrotic treatments available to date. The disease phenotype of the NIF mouse resembles human fibrotic conditions in several pertinent features, including the temporal heterogeneity of the fibrotic lesions, the progressive nature of the disease and the preceding chronic inflammation. It also provides several previously unmet demands on animal models for fibrosis, compared to most available animal models for fibrosis today, such as its spontaneous nature, early on-set and its reproducibility. This provides a rare opportunity to gain further insight into the underlying mechanisms mediating transformation of chronic inflammation into pathological fibrosis, as well as the possibility of identifying potential new drug therapy targets. It also offers the potential for the NIF mouse to be used as a unique tool for the evaluation of potential drug candidates and intervention protocols for treating fibrotic disorders.

Paper II:

NKT cells promote both NLRP3 inflammasome-mediated inflammation and IL-33 mediated fibrosis in a mouse model of liver fibrosis.

Julia Nilsson, Maria Hörnberg, Anja Schmidt-Christensen, Kajsa Linde, Marine Carlus, Saskia F. Erttmann, Sofia Mayans, and Dan Holmberg.

Summary:

We demonstrate that the transgenic NKT cell population of the NIF mouse, in the absence of a functional adaptive immune system, can mediate the initiation of a chronic type 1 inflammation, involving the NLRP3 inflammasome, in the liver. The subsequent shift into a type 2 inflammatory response, driven by an altered NKT cell cytokine profile, in combination with IL-33 secretion, promotes the development of hepatic fibrosis, and points to a plasticity in the NKT cells. This data illustrates that the pathogenesis in the NIF mouse largely overlap the initial stages of sterile liver inflammation associated with human liver diseases.

Key findings:

- The NIF mouse develops chronic liver inflammation, which is followed by the development of progressive hepatic fibrosis.
- NKT cells drive an initial type 1 inflammatory response in the liver of NIF mice, promoted by the activation of the NLRP3 inflammasome pathway.

- The fibrogenesis of the NIF liver is TGF-β dependent and is associated with activation of HSCs as well as increased IL-33 expression.
- A switch to NKT cell-derived type 2 cytokine production promotes the transition into type 2 inflammation in NIF mice.

Discussion:

Sterile liver inflammation and fibrosis are associated with many liver disorders of different etiology, such as NAFLD and NASH. Both type 1 and type 2 inflammatory responses have been reported to contribute to the pathology, however, the processes controlling the balance between them are largely unknown. Relevant animal models are essential to extend our understanding of the mechanisms underlying sterile liver inflammation and fibrosis, including NAFLD and NASH, and to provide pertinent tools for preclinical drug testing. Recently, several novel models that reflect the metabolic phase of NAFLD/NASH pathogenesis have been established, however models that accurately reproduce the inflammatory and fibrosis phase of the disease are still largely lacking.

Here we show that the NIF mouse displays a chronic liver inflammation with a progressively increasing leukocyte infiltration accompanied by limited necrosis up until 8-12 weeks of age, whereupon the inflammatory progression is halted. The initial inflammatory reaction in the NIF liver is followed by an observed development of progressive liver fibrosis.

NKT cells, naturally homing to the liver, have been repeatedly associated with chronic liver inflammation and fibrosis. In the NIF liver we found that the accumulation of NKT cells preceded that of the accumulation of $CD45^+$ leukocytes, suggesting that the NKT cell population mediates the initial events in the NIF liver inflammatory pathogenesis. Previous studies have shown that NKT cells in both NIF and 2,4αβNOD.*Rag*$2^{+/-}$ control mice express the pro-inflammatory cytokines IFN-γ and TNF-α. Here we noted that the levels of IFN-γ were decreased in the NIF liver from 6 weeks of age compared to those of the 2,4αβNOD.*Rag*$2^{+/-}$ control livers, while the expression levels of TNF-α were increased. Moreover, significant levels of GM-CSF were expressed in NIF livers, but not in 2,4αβNOD.*Rag*$2^{+/-}$ control livers. We believe that this increased expression of pro-inflammatory cytokines such as TNF-α and GM-CSF, by the NKT cell population, in combination with observed hepatocyte damage, albeit limited, are the drivers of the initial type 1 inflammatory response in the liver of NIF mice.

Accumulating evidence indicate that activation of the NLRP3 inflammasome pathway can play a critical role in sterile liver inflammation. We observed that the inflammation in the NIF liver was found to be associated with an increased expression of the *Nfkb* gene, as well as increased expression of the NF-κB regulated *Nlrp3* and *Il1b* genes. We could also confirm the increase in protein levels of the inflammasome components NLRP3 and pro-IL-1β. In addition to the NF-κB

mediated induction of NLRP3 and pro-IL-1β, full activation of the NLRP3 inflammasome requires additional signaling resulting in assembly of the inflammasome complex, activation of caspase-1 and the subsequent production of biologically active IL-1β, is required. We could verify cleavage of pro-caspase-1 and pro-IL-1β through detection of increased levels of processed caspase-1 and IL-1β in the NIF liver, thus confirming that a full activation of the NLRP3 inflammasome occur during NIF liver inflammation. Hence, we concluded that the NKT cell-driven inflammation in the liver of NIF mice is, at least, partially driven by the activation of the NLRP3 inflammasome pathway.

A pro-inflammatory response will also trigger the immune system to prepare for a transition into a repair mode in which key inflammatory cells, such as KCs and monocyte-derived macrophages switch from a type 1 pro-inflammatory to a type 2 anti-inflammatory/reparative response. TGF-β1 is known to be an important mediator in pro-repair/pro-fibrotic type 2 inflammation, and a critical consequence of TGF-β1 signaling in hepatic fibrogenesis is the activation of HSCs – the most potent fibrogenic cells and mediators of the secretion and deposition of ECM. The NIF mouse can be observed to express significantly elevated levels of hepatic TGF-β1, which in combination with an accumulation of cells, in the fibrotic areas of the liver, expressing markers upregulated by activated HSCs (PDGFRα^{+} and α-SMA^{+}), indicates the pathogenic activation of HSCs. Following treatment with a blocking anti-TGF-β1 antibody (1D11), the NIF liver fibrosis was significantly reduced, which led us to conclude that the development of hepatic fibrosis in the NIF mouse is TGF-β1 dependent.

Type 1 inflammation and its resulting tissue damage are assumed to be driving forces behind the transition to a type 2 inflammatory response, promoting subsequent tissue repair, but also fibrogenesis. Alarmins, such as IL-33, constitute some of the earliest type 2 cytokines to be produced and contribute to this transition. Significant upregulation of IL-33 expression can be detected in the NIF liver and accumulation of IL-33-expressing cells can be observed specifically localized to the areas of hepatic inflammation, indicating its involvement in the NIF liver fibrogenesis. IL-33 is preferentially expressed in endothelial and epithelial cells but has been reported to be expressed in injured hepatocytes and activated HSCs during inflammation. IL-33 expression in the NIF liver was not observed to be preferentially expressed in vascular endothelial cells or leukocytes, hence we concluded that hepatocytes and activated HSCs are the main source of IL-33 in the NIF liver.

The transition into an anti-inflammatory and pro-fibrotic type 2 inflammation in the NIF liver was accompanied by an observed switch in cytokine profile of the NKT cells in the NIF mouse, leading to the production of type 2 cytokines such as IL-4, IL-5 and IL-13. Additionally we observed that the transgenic NKT cell population, defined as type 2 NKT cells based on the receptor specificity, in the presence of a mature adaptive immune system produces a predominantly pro-inflammatory

cytokine profile. In contrast, in the immunodeficient background of the NIF mouse, this cytokine profile shifts into a mixed type 1/type 2 profile. While type 2 NKT cells have previously, mainly been associated with an anti-inflammatory and regulatory role, this mixed cytokine profile appears to drive the development of a chronic type 1 liver inflammation as well as support the shift into a type 2 inflammatory response driving the development of progressive liver fibrosis in the NIF mouse. This observed plasticity in the NKT cell population suggests that this cell population, dependent on the local environment, can fulfill different roles in the orchestration of the immune response.

In summary we here demonstrate that overexpressing a transgenic population of NKT cells, in the absence of a functional adaptive immune system, induces chronic liver inflammation and promotes fibrogenesis in the NIF mouse model. We show how the accumulation of NKT cells in the NIF liver precedes the recruitment of other inflammatory cells, suggesting that this NKT-cell population mediates the initial events in the pathogenesis. We propose that the observed hepatocyte damage, albeit limited, together with the increased expression of pro-inflammatory cytokines such as TNF-α and GM-CSF by the NKT cell population are driving the initial type 1 inflammatory response, involving the activation of the NLRP3 inflammasome. A subsequent shift into a type 2 inflammatory response, driven by an altered NKT cell cytokine profile – into anti-inflammatory/pro-fibrotic cytokine production – in combination with IL-33 secretion, and the activation of hepatic stellate cells (HSC), promotes the development of hepatic fibrosis. This pathogenesis largely overlap the initial stages of sterile liver inflammation associated with human liver diseases such as ASH and NAFLD/NASH. Thus the NIF mouse offers an improved model for elucidating the mechanisms involved in sterile liver inflammation and associated hepatic fibrosis development, as well as constituting a useful tool for testing specific targets represented in the type 2-inflammatory response and fibrogenesis.

Paper III:

The immunomodulatory quinoline-3-carboxamide paquinimod reverses established fibrosis in a novel mouse model for liver fibrosis.

Nina Fransén-Pettersson, Adnan Deronic, Julia Nilsson, Tine D. Hannibal, Lisbeth Hansen, Anja Schmidt-Christensen, Fredrik Ivars and Dan Holmberg.

Summary:

By using the quinoline-3-carboxamide compound Paquinimod, an anti-inflammatory compound that has previously shown anti-fibrotic effects in the treatment of scleroderma, we demonstrate that the chronic inflammation and hepatic fibrosis of the NIF mouse can be effectively treated. This illustrates that the NIF mouse model can be successfully used for efficacy testing of drug candidates against hepatic fibrosis. Additionally this also provides further evidence of the anti-fibrotic properties of Paquinimod and the potential for possible use in treatment of fibrotic conditions.

Key findings:

- Paquinimod treatment leads to reduced inflammation in the NIF mouse liver and altered composition of infiltrates – reduced frequency of Siglec-F^+ cells but increased frequency of $Ly6G^+$ cells – as well as reduced levels of both pro- and anti-inflammatory cytokines.
- Paquinimod treatment significantly reduces already established fibrosis in the NIF mouse.
- Paquinimod treatment leads to decreased granularity of the transgenic NKT cells and decreased expression levels of T_H2 cytokines in the liver of the NIF mouse.
- Paquinimod treatment reduces the number of M2-like $CD11b^+$ $F4/80^+$ $CD206^+$ macrophages – as well as the frequency of $CD206^+$ cells within the $CD11b^+$ $F4/80^+$ population – and also reduces $CD115^+$ $Ly6C^{hi}$ cells in the liver of the NIF mouse.

Discussion:

The NIF mouse spontaneously develops a chronic inflammation and a severe fibrosis of the liver, recapitulating many of the features of human fibrotic conditions. Due to this, the NIF mouse has the potential to be used as a unique tool in pre-clinical drug development for potential anti-fibrotic compounds.

Q-compounds, such as Paquinimod, have been reported to bind to the pro-inflammatory mediator S100A9. S100A9 is a protein with chemotactic effects, expressed in, primarily neutrophils and monocytes, and is involved in the recruitment of myeloid cells during inflammation. Paquinimod inhibits the

interaction of S100A9 with its receptors, a mechanism suggested to underlie the anti-inflammatory effects of Paquinimod.

Paquinimod treatment of the NIF mouse led to a significant reduction of $CD11b^+$ cells in the liver and the numbers of the major $CD11b^+$ subpopulations; monocytes ($CD11b^+Ly6C^{hi}$), neutrophils ($CD11b^+Ly6G^{hi}$), and eosinophils ($CD11b^+$Siglec-F^+), were all significantly reduced. This corresponds to previous studies where Paquinimod has been shown to reduce accumulation of myeloid cells in acute inflammation, due to its S100A9 binding properties. Paquinimod treatment also led to an altered composition of the $CD11b^+$ infiltrating cells – a reduced frequency of Siglec-F^+ cells but an increased frequency of $Ly6G^+$ cells – as well as reduced levels of both pro- and anti-inflammatory cytokines. Also this in line with previous studies of Paquinimod specifically reducing monocytes and eosinophils but not neutrophils.

Paquinimod treatment of NIF mice with fully established hepatic fibrosis resulted in a significant reduction of the observed fibrosis confirming the anti-fibrotic therapeutic potential of Paquinimod. The fibrotic condition of the NIF mouse is driven by an overexpressed transgenic NKT-cell population, which displays a mixed T_H1/T_H2 cytokine profile. Paquinimod treatment led to decreased granularity of the transgenic NK- cells and an observed decrease of both T_H1 and T_H2 cytokine expression levels in the liver, as well as in the spleen. This suggests that the NKT-cells were less activated after treatment, hence indicating that the disease-inhibiting effect of Paquinimod was, at least in part, mediated by modifying the NKT-cells in the NIF mouse. The reduction in absolute numbers of the NKT-cells in the spleen, after treatment, supports the notion that Paquinimod directly inhibits the NIF NKT-cell population. The absence of a corresponding decrease of NKT-cells in the liver could be due to preferential homing of NKT-cells to the liver, thus a larger proportion of the remaining NKT cells may be recruited to the liver.

Alternatively activated macrophages play a crucial role in fibrogenesis, primarily through their production of TGF-β1. The increased numbers of M2-like macrophages ($CD11b^+$ $F4/80^+$ $CD206^+$) in the NIF liver was reduced upon Paquinimod treatment and the frequency of $CD206^+$ cells within the $CD11b^+$ $F4/80^+$ macrophage population decreased. Classically activated macrophages are important in fibrolysis and the change in macrophage polarization as a result of Paquinimod treatment, together with the observed decrease in expression levels of T_H2 cytokines constitutes an expected change in the microenvironment, from fibrotic to anti-fibrotic, in the NIF liver.

This study provides further evidence that the Q- substance Paquinimod, in addition to its properties as an anti-inflammatory agent, constitutes a potential drug candidate for the treatment of fibrotic conditions. It also demonstrates that the NIF mouse model can be successfully used as a pre-clinical tool for efficacy testing of anti-fibrotic drug candidates.

Paper IV:

Recruited fibroblasts reconstitute the peri-islet membrane: a longitudinal imaging study of human islet grafting and revascularisation.

Julia Nilsson, Rabiah Fardoos, Lisbeth Hansen, Håkan Lövkvist, Kristian Pietras, Dan Holmberg and Anja Schmidt-Christensen.

Summary:

Using a non-invasive, longitudinal and high-resolution imaging approach we investigate the dynamics of pancreatic islet engraftment in syngeneic mouse, as well as xenogeneic human islet grafts. Pancreatic islet transplantation offers the potential to provide physiological glycemic control for type 1 diabetes patients, however, despite early promise, it has had limited clinical impact. Rapid and adequate islet revascularization and restoration of the islet–extracellular matrix (ECM) interaction are significant factors influencing islet graft survival and function, and understanding these processes are essential. We demonstrate that grafted islets are ensheathed by fibroblast-like stromal cells, aiding the reconstitution of a peri-islet-like basement membrane, which may have implications for the understanding of long-term graft rejection and future design of novel strategies to interfere with this process.

Key findings:

- Revascularized islet grafts regenerate an ECM capsule similar to the peri-islet basement membrane lost upon islet isolation.
- Recruited recipient fibroblasts are responsible for the secretion of the ECM proteins comprising the reconstituted peri-islet-like basement membrane.
- Human islets retain their species-specific anatomical structure and vascular network density when transplanted into a non-human host.
- Syngeneic islet transplantation results in chimeric donor/recipient intra-islet endothelium, whereas inter-species transplantation results in mainly recipient-derived intra-islet endothelium.

Discussion:

We applied a longitudinal and high-resolution 3D imaging approach to investigate the dynamics of the pancreatic islet engraftment process after transplantation. Human and syngeneic mouse islet grafts were inserted into the anterior chamber of the mouse eye, using a NOD.ROSA-tomato.*Rag2*$^{-/-}$ or B6.ROSA-tomato host, and imaged repeatedly for up to 11 months. This allowed for the investigation of the

expansion of host vs donor cells, and the contribution of host cells to aspects such as promotion of encapsulation and vascularization of the graft.

The islet basement membrane is a sensitive biomarker of islet damage, resulting from enzymatic isolation and islet repair after transplantation. The peri-islet basement membrane in mice comprises a range of different ECM glycoproteins, while the peri-islet basement membrane in humans has a similar composition it also contains additional laminins, such as laminin α5, not found in the mouse peri-islet basement membrane. We observed little or no laminin staining in the periphery of isolated islets prior to transplantation, from either human or mouse, suggesting a complete degradation of the islet basement membrane due to islet isolation. Stained sections of islet grafts, several weeks post transplantation, demonstrated that the surface, of both human and mouse grafts, was progressively ensheathed by a laminin-containing ECM capsule. We show that this peri-islet-like basement membrane structure contains ECM proteins such as collagen IV, nidogen-2, laminin γ1, perlecan and fibrillary collagen I, as well as laminin α5 chains specifically in human islet grafts. This agrees with the major peri-islet basement membrane components that have been reported for the pancreas and demonstrates that revascularized islet grafts, in this model, regenerate an ECM capsule similar to the peri-islet basement membrane lost upon islet isolation.

Following islet transplantation, we observed cell nuclei outside the peripheral laminin staining. These spindle-shaped cells, identified as recipient-derived by expression of tomato fluorescence protein, could be detected encapsulating the transplanted donor islet grafts in both syngeneic mouse islet transplantations, as well as in inter-species human to mouse transplantations. Similar observations were also made in the widely used renal islet transplantation model. Histological analyses allowed us to define a generic cell surface antigen profile for these cells, as $CD31^{-}$, $CD45^{-}$, $NG2^{-}$, $EpCAM^{-}$, $E\text{-}Caherin^{-}$, $Sca\text{-}1^{-}$, $PDGFR\alpha^{+}$ and $PDGFR\beta^{+}$ in combination with a cytoplasmic expression profile of αSMA^{-}, $GFAP^{-}$, $gp38^{+}$ and $vimentin^{+}$, indicating a fibroblast-like cell population. Cryo-sections of the islet grafts co-stained for ECM proteins demonstrate that only in areas where host cells have formed a single outer layer of fibroblasts, a corresponding basement membrane-like structure is also formed. The co-localization of the recipient fibroblast-like cells with basement membrane proteins indicates that these cells constitute the main producers of the restored peri-islet-like basement membrane.

We found that endothelial cells from the transplant recipient are recruited into the islet graft, creating new islet vessel networks already within several days. Both human and mouse islets displayed a similar initial vessel density increase, reaching a plateau at approximately 2 months post transplantation. Despite the similar initial vessel density increase in syngeneic and interspecies transplantations, the mouse islet grafts reached a vessel density of approximately 17%, while the newly formed vessel network of human islet grafts reached a density of only 9%. This is, however, in agreement with the substantially lower capillary density found in human vs mouse

endocrine pancreas, and in accordance with the vessel densities of islets previously recorded in situ in mouse pancreas and human live pancreas sections. This shows that human islets retain their species-specific vascular network density when transplanted into a non-human host. Additionally we observed an intact species-specific anatomical organization of the transplanted pancreatic islet grafts for both mouse and human islets.

The recruitment of recipient endothelial cells generated a chimeric endothelium in the syngeneic mouse islet graft, including vessel sections of either donor or recipient origin or chimeric vessel sections of both donor intra-islet cells and recipient derived cells. This chimeric pattern showed dynamic changes over time, which was accompanied by a gradual decrease of recipient cells in later time points. The progressive replacement of the endothelial cells of donor origin in the initial revascularization phase, as well as the gradual disappearance of recipient cells at later stages are, at least in part, a result of cellular migration, supported by the observation of migrating cells along the iris blood vessels. Conversely, in our model, inter-species transplantation of human islets resulted in mainly recipient-derived intra-islet endothelium. Initially, intra-islet endothelial cells constituted an alternative vascular source, which exists in isolated islets and can account for up to 20% of the endothelial cells lining functional capillaries, within the first weeks of revascularized xenogeneic human grafts. However, fully revascularized human islet grafts contained vessels of a purely mouse recipient origin. Human endothelial cells rapidly disappeared, at least parts of them migrating out of the graft, as could be seen by the frequent detection of human $CD31^+$ cells in the iris vasculature of the recipient. From this, in combination with the observed retained species-specific vessel network, we conclude that even though recipient endothelial cells are the main cell population involved in the revascularization process, cues from the donor islet determines the structure and density of the vessel network, possibly by providing complex matrices and pre-formed paths that the recipient cells might navigate along.

In summary, this data provides evidence of a fibroblast population as the main organizer of the ECM encapsulation of transplanted islets of Langerhans and of islet-derived factors acting as cues for the architecture of the revascularization of these islets. Additionally this demonstrates how the anterior chamber of the eye (ACE) imaging technique can be utilized for the longitudinal *in vivo* study of structural and vascular changes in transplanted tissue.

Acknowledgements

Before we get any further: I admire you for making it this far in your, obviously committed, reading of my thesis! And now, here's what you came for:

First and foremost I would like to thank my supervisor **Dan Holmberg** for taking me on as a PhD student and remaining positive in the face of all our administrative let downs! Your passion for science is remarkable and your can-do attitude is both motivating and incredibly frustrating! Thank you for always encouraging scientific discussions, supporting conference participation and for inspiring a fun-loving work-environment. Thank you also for having had patience with my "occasional" temper tantrums, though I have, of course, had patience with your obnoxious teasing and belief that you're always right, so I'd say we're even! I feel lucky to have had such a caring and genuine person as my supervisor during these years, thank you!

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