The role of immune mechanisms in development of metabolic syndrome in obesity

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DOCTORAL THESIS

Zagreb, 2018

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET
BIOLOŠKI ODSJEK

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ULOGA IMUNOSNIH MEHANIZAMA U RAZVOJU METABOLIČKOGA SINDROMA U DEBLJINI

DOKTORSKI RAD

Mentori:
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This doctoral thesis was made at the Department of Histology and Embryology, Faculty of medicine, University of Rijeka under the mentorship of Prof. Bojan Polić, MD, Ph.D and Prof. Danka Grčević, MD, Ph.D within the postgraduate study of Biology at the Department of Biology, Faculty of Science, University of Zagreb.

Zahvala

Zahvaljujem se mentoru prof. dr. sc. Bojanu Poliću na pruženoj pomoći i savjetima pri izradi ovog doktorskog rada, ali i na cjelokupnom profesionalnom razvoju. Zahvaljujem se na pomoći i doc. dr. sc. Felixu Wensveenu koji mi je pomogao svojim savjetima i usmjerio me na pravi put u mom istraživanju.

Hvala i prof. dr. sc. Stipanu Jonjiću i izv. prof.dr.sc. Danki Grčević
na svoj iskazanoj pomoći i savjetima.

Kolegama i kolegicama Zavoda za histologiju i embriologiju, a posebno Marku, Ingi, Vedrani, Maji, Sali, Ani, Anti, Miru, Miju i Tini zahvaljujem na savjetima, pomoći pri izradi dijela rezultata ovog istraživanja i, najbitnije, ugodnom okruženju tijekom zajedničkog rada.

Hvala mojim roditeljima na beskrajnoj ljubavi i podršci što mi je pružaju cijeli život i što su mi tokom odgoja usadili vrijednosti bez kojih znanje i doktorat ništa ne vrijede.

I naposljetku, hvala mojem suprugu Edvardu na ljubavi, strpljenju, potpori i razumijevanju prilikom izrade ovog rada.

University of Zagreb
Faculty of Science
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Doctoral thesis

THE ROLE OF IMMUNE MECHANISMS IN DEVELOPMENT OF METABOLIC SYNDROME IN OBESITY

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Summary

More than billion of obese and overweight people live currently in the world. A significant number of them develop diabetes mellitus type 2 (DM2) and non-alcoholic fatty liver disease (NAFLD). Mechanisms that induce inflammation and trigger transition from pre-diabetes to DM2 or from hepatic steatosis to non-alcoholic steatohepatitis (NASH) are still unclear. In this research I found that γδ T cells are enriched in visceral adipose tissue and they produce proinflammatory cytokines. In response to high fat diet (HFD), there is increase in number of γδ T cells that produce IL-17A. Research on NAFLD revealed that initiating event, in response to high calorie diet, is the increase in expression of the NKG2D ligand H60 on hepatocytes. Upregulation of ligands leads to mobilisation of γδ T cells through an NKG2D engagement that induces an increase in IL-17A. Lack of either γδ T cells, NKG2D or IL-17A significantly decreases development of liver fibrosis.

(111 pages, 23 figures, 3 tables, 264 references, original in: English)

Keywords: diabetes mellitus type 2, γδ T cells, IL-17A, non-alcoholic fatty liver disease, NKG2D receptor, stress induced molecules.

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Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Biološki odsjek

Doktorski rad

ULOGA IMUNOSNIH MEHANIZAMA U RAZVOJU METABOLIČKOGA SINDROMA U DEBLJINI

SONJA MARINOVIĆ

Medicinski fakultet, Sveučilište u Rijeci

Sažetak

Trenutno na svijetu živi više od milijarde pretilih ljudi i onih s prekomjernom težinom. Značajan broj njih obolijeva od šećerne bolesti tipa 2 (DM2) i nealkoholne bolesti masne jetre (NAFLD). Mehanizmi koji induciraju upalu u debljini i uzrokuju prijelaz iz stadija predijabetesa u DM2 ili iz stadija jetrene steatoze u nealkoholni steatohepatitis (NASH) su još uvijek nejasni. U ovom istraživanju otkrila sam da se γδ limfociti T nalaze u velikom broju u visceralnom masnom tkivu i da mogu sintetizirati proupalne citokine. Kao odgovor na visokokalorijsku prehranu bogatu mastima dolazi do povećanja broja γδ limfocita T koji proizvode IL-17A. Istraživanja na NAFLD-u pokazala su da kao odgovor na visokokalorijsku dijetu, dolazi do povećanog izražaja NKG2D liganda H60 na hepatocitima. Povećani izražaj NKG2D liganda dovodi do mobilizacije γδ stanica kroz aktivaciju receptora NKG2D koji potiče sintezu IL-17A. Nedostatak γδ limfocita T, receptora NKG2D ili citokina IL-17A značajno smanjuje razvoj fibroze jetre.

(111 stranica, 23 slike, 3 tablice, 264 literaturna navoda, jezik izvornika: engleski)

Ključne riječi: diabetes mellitus tip 2, γδ limfociti T, IL-17A, nealkoholna masna bolest jetre, receptor NKG2D, stresom inducirane molekule.

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Prošireni sažetak

Prevalencija debljine drastično je porasla u zadnjih par desetljeća te je postala veliki javnozdravstveni problem. Debljina uzrokuje brojne kliničke poremećaje i povećava rizik za razvoj inzulinske rezistencije koja doprinosi razvoju metaboličkog sindroma (Stevens et al., 2012). Metabolički sindrom je skupina metaboličkih poremećaja poput povišene razine glukoze u krvi, dislipidemije te hipertenzije što može dovesti do razvoja kardiovaskularnih oboljenja, dijabetesa tipa 2, s pridruženim komorbiditetima i komplikacijama, te nealkoholne masne bolesti jetre. Bitna podloga za razvoj inzulinske rezistencije leži u kroničnoj sistemskoj upali niskog intenziteta. Dugotrajna prisutnost proupalnih citokina u krvi uzrokuje oslabljen prijenos signala putem inzulinskog receptora u tkivima koja su osjetljiva na djelovanje inzulina, kao što su masno tkivo, jetra i mišići (Rui et al., 2002). Danas se zna da je masno tkivo glavni izvor sistemske upale povezane s debljinom.

Ono što se kolokvijalno naziva masno tkivo zapravo sačinjavaju različite nakupine tkiva koji imaju različitu ontologiju i funkciju (Sanchez-Gurmaches et al., 2016). Bijelo masno tkivo se nalazi na raznim mjestima u tijelu. Jedan dio se nalazi pod kožom i čini potkožno ili supkutano masno tkivo, dok se visceralno masno tkivo nalazi u trbušnoj šupljini (npr. omentum) i može oblagati mezenterij i bubrege (Sethi and Vidal-Puig, 2007). Za razvoj metaboličkog sindroma, posebno je bitno visceralno masno tkivo (engl. *Visceral adipose tissue*, VAT) koje kao odgovor na pretilost nakuplja puno više upalnih stanica imunološkog sustava nego potkožno masno tkivo (Sluik et al., 2011).

Čak i u uvjetima normalne prehrane, i unatoč činjenici da nema ulogu tkivne barijere, visceralno masno tkivo naseljeno je s mnogim imunosnim stanicama. Većina tih stanica ima protuupalni ili Th2 fenotip koji je karakteriziran proizvodnjom protuupalnih citokina (Lumeng et al., 2007). U normalnom masnom tkivu, makrofazi su dominantna populacija imunosnih stanica, a većina tih stanica su M2 tipa ili tzv. alternativno aktivirani fenotip, eksprimiraju arginazu koja razgrađuje arginin i time inhibira aktivnost inducibilne dušik oksid sintetaze (iNOS) te proizvode protuupalne molekule, kao što su interleukin (IL-) 10 i antagonist IL-1 receptora (IL-1Ra) (Molofsky et al., 2013). Tkivno-rezidentni makrofazi masnog tkiva kod miševa normalne tjelesne težine održavaju se u ovom stanju od strane T-pomoćničkih stanica tipa 2, adipocita i stromalnih stanica koji proizvode protuupalne citokine (Barnes et al., 2015).

Kao odgovor na akumulaciju masti u adipocitima, profil imunosnih stanica u VAT-u bitno se mijenja. Kod ljudi, dakako, teško je odrediti početak debljanja, ali zato su mišji modeli veoma pomogli razjasniti ponašanje imunosnih stanica u ranima fazama debljanja. Jedan od

najčešće korištenih modela za pretilost, i posljedičnu inzulinsku rezistenciju, je mišji model dijetom inducirane debljine sa visoko-kalorijskom prehranom bogatom mastima (eng. *High-fat diet*, HFD). Rane faze HFD-a karakterizirane su povećanjem količine masti po adipocitu i akumulacijom imunosnih stanica koje su u početku ograničenog upalnog kapaciteta. U istraživanjima na tom modelu, pokazalo se da u prvim tjednima od početka HFD-a dolazi dok nakupljanja neutrofila, makrofaga i prirodnoubilačkih stanica NK (engl. *Natural killer cell,* NK) u VAT-u (Wensveen et al., 2015).

Iako nakupljanje makrofaga predisponira upalu u masnom tkivu, ipak sama za sebe nije dovoljna. Potrebno je stvoriti proupalne makrofage, pomoću različitih citokina, da bi oni sami mogli proizvoditi proupalne citokine (Goldszmid et al., 2012). Ovo zapravo znači da se stres u masnom tkivu detektira od strane nekih drugih stanica, a ne makrofaga. Te stanice onda prevode taj stresni signal i omogućuju polarizaciju prema M1 proupalnim makrofazima. Naša grupa je nedavno pokazala da stanice NK mogu imati tu ulogu posrednika (Wensveen et al., 2015). Stanice NK nadziru naše tijelo pregledavajući tkiva u potrazi za stanicama koje su inficirane, transformirane ili pak na drugi način „stresirane". Naoružane su različitim aktivirajućim i inhibirajućim receptorima koji su kalibrirani kako bi detektirali znakove citopatologije, ali istovremeno osiguravajući toleranciju vlastitih tkiva (Vivier and Ugolini, 2011). Pretili adipociti potiču povećanu ekspresiju liganada na svojoj površini koji aktiviraju NK-stanični receptor NKp46. Aktivacija receptora stanica NK stimulira proliferaciju i proizvodnju citokina interferona gamma (IFNγ) koji dalje pokreće pretvorbu makrofaga i promovira razvoj inzulinske rezistencije. Nedostatak stanica NK, receptora NKp46 ili IFNγ smanjuje nakupljanje proupalnih makrofaga u VAT-u i značajno poboljšava osjetljivost tkiva na inzulin (Wensveen et al., 2015).

Osim stanica NK, druge stanične populacije su sposobne detektirati stanični stres koji se događa u VAT-u. Dokaz tome je deplecija stanica NK koja odgađa konverziju makrofaga u proupalni fenotip i razvoj inzulinske rezistencije, ali ju ne sprječavaju u potpunosti. Stoga, moraju postojati još neke stanice u masnom tkivu koje prevode stanični stres adipocita prema makrofazima. VAT sadrži veliki broj drugih stanica urođene imunosti, kao što su stanice γδ limfocita T. Iako su neka istraživanja pokazala da postoji uloga za ove stanice u prepoznavanju stresa u debljini njihovi aktivirajući ligandi i mehanizami njihovog sudjelovanja su i dalje nepoznati. Još bitnije, jedan od glavnih načina na koji se gleda sudjeluju li neke stanice u razvoju dijabetesa tipa 2 je kroz promjenu broja stanica te populacije. Međutim, većini tkivno specifičnih imunosnih populacija broj se ne mijenja drastično, već imaju ulogu u aktivaciji drugih stanica kroz proizvodnju citokina (Walker et al., 2013). Dakle, njihova uloga se često

može previdjeti ako se samo fokusira na broj stanica, a ne na efektorske molekule koje proizvode.

U sklopu ovog istraživanja vidjeli smo su γδ limfociti T prisutni u velikom postotku u masnom tkivu u odnosu na druga tkiva te da se njihov broj povećava s primjenom HFD-a. Ovo bi moglo biti od velike važnosti s obzirom da ove stanice imaju obilježja urođene i adaptivne imunosti i jedne su od prvih staničnih populacija koje proizvode proupalni citokin IL-17 kao odgovor na stresne uvjete. Međutim, moji podaci upućuju na to da odsutnost γδ T stanica u prvih nekoliko tjedana nema bitnu ulogu u progresiji upale, već na homeostazu masnog tkiva. Naime, u razdoblju od samo tri tjedna na HFD-u, opaženo je povećanje mase tijela i povećanje težine visceralnog masnog tkiva kod životinja bez γδ limfocita T. Moguće je objašnjenje da IL-17A ima dvostruki učinak - jedan u homeostazi masnog tkiva, a drugi u disfunkciji adipoznog tkiva u pretilosti.

Danas se zna da je za razvoj NASH-a iz steatoze jetre, pored sistemske kronične upale i IR koje su povezane s VAT-om, neophodan i razvoj lokalnog upalnog procesa u jetri. Uslijed epidemije pretilosti, nealkoholna masna bolest jetre javlja se u jednoj trećini pretilih u razvijenim (zapadnim) zemljama (Younossi et al., 2016). Bitan je uzrok morbiditeta i mortaliteta te se smatra jetrenom manifestacijom metaboličkog sindroma. NAFLD predstavlja širok spektar bolesti jetre od jednostavne steatoze do steatohepatitisa (sa ili bez fibroze) koja može napredovati do ciroze i gubitka funkcije jetre bez konzumacije alkohola u količinama štetnim za jetru ili drugih rjeđih uzroka jetrene steatoze (Chalasani et al., 2018). Iako NAFLD, odnosno sama bolest jetre bez vaskularnih komplikacija ima relativno benignu prognozu, kod jedne petine oboljelih, bolest napreduje do NASH-a. Trećina bolesnika s NASH-om kroz 5 do 10 godina razvije cirozu jetre, a četvrtina bolesnika s cirozom jetre razvije komplikacije u smislu portalne hipertenzije i hepatocelularnog karcinoma (Younossi et al., 2016).

Mehanizmi koji su upleteni u patogenezu NASH-a nisu u potpunosti razjašnjeni. „Hipoteza dva udarca“ je jedna od trenutno prihvaćenih hipoteza. Za uspostavu NASH-a, prvi udarac“ je nakupljanje masti u adipocitima, uglavnom u obliku triglicerida. To je uzrokovano neravnotežom dva metabolička puta; prvog koji se odnosi na unos i sintezu te drugog koji potiče oksidaciju i izvoz masnih kiselina od strane hepatocita. Predloženo je da je inzulinska rezistencija najbitniji faktor u akumulaciji lipida i razvoju jetrene steatoze. IR dovodi do povišenja razine glukoze u krvi, periferne i hiperinzulinemije. Lipoliza zatim povećava razinu cirkulirajućih slobodnih masnih kiselina što uzrokuje njihovo povećano spremanje u hepatocite. Hiperinzulinemija pojačava jetrenu sintezu slobodnih masnih kiselina kroz povećanu glikolizu, smanjujući mogućnost da se trigliceridi reesterificiraju i izađu van te na taj način pogoduje

nakupljanju triglicerida u hepatocitima (Mehta et al., 2002). Kao posljedica jetrene steatoze, hepatociti postaju osjetljivi na oksidativni stres što je vjerojatno uzrok progresije bolesti od jednostavne steatoze do steatoze povezane s upalom i fibrozom. Dakle, predloženo je da je oksidativni stres „drugi udarac“ (Rolo et al., 2012). Međutim, specifična uloga lokalnih imunosnih stanica i citokina u indukciji i progresiji upale jetre je još uvijek nedovoljno razjašnjena.

Upala jetre u kontekstu NASH uglavnom je praćena mononuklearnim infiltratom (Kupfferove stanice, monociti). Međutim, γδ limfociti T su također brojni u jetri te se njihov broj i funkcija znatno mijenjaju kod bakterijskih ili virusnih upala jetre (Papotto et al., 2017). U ovom istraživanju sam pokazala da su u našem modelu masne bolesti jetre, γδ limfociti T aktivirani u ranoj fazi razvoja NAFLD-a i da je proupalni citokin IL-17A proizveden od ovih stanica važan za razvoj upale jetre i fibroze. Također, pokazala sam da IL-17A nije važan za aktivaciju monocita i neutrofila, već je vjerojatnije da IL-17A ima izravan utjecaj na neke druge stanice. Dodatno sam pokazala da je u našem modelu povećana ekspresija mRNA liganda NKG2D te da je stimulacija kroz NKG2D glavni aktivirajući faktor koji je doprinio proizvodnji IL-17A od strane jetrenih γδ limfocita T.

Ukratko, moje istraživanje je pokazalo da su γδ T stanice imunosne stanice koje povezuju stanični stres uzrokovan viškom hranjivih tvari u ranom stadiju razvoja NAFLD. Ovaj proces je posredovan NKG2D induciranom IL-17A proizvodnjom pomoću γδ limfocita T. Stoga, blokiranje NKG2D ili IL-17A signalizacije može biti od velike važnosti za sprječavanje prijelaza iz jednostavne steatoze u NASH i razvoja fibroze.

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1. INTRODUCTION

In last four decades, the worldwide incidence of obesity has nearly tripled. A recent World Health Organization (WHO) report has shown that in 2016, around 2 billion adults were overweight and roughly one third of them were obese and it is expected that these figures will increase in the forthcoming years (WHO, 2017). The Croatian population appears to follow this global trend. Approximately 25 % of men and around 35 % of women in Croatia are overweight or obese (Medanić D, 2012). Currently, obesity is the fifth leading cause of global deaths with around 3 million people dying each year due to complications resulting from being obese or overweight. Even more concerning is the fact that over 340 million children are overweight or obese which makes them more likely to develop cardiovascular diseases and diabetes and at a younger age. Obesity can therefore be considered as one of the most important health challenges of 21st century (Medanić D, 2012; WHO, 2015).

Obesity is connected with a large number of comorbidities including insulin resistance (IR), increase in blood pressure, blood sugar levels and triglycerides and decrease in high-density lipoprotein levels (Grundy et al., 2004). Clustering of at least three of the five mentioned symptoms leads to a condition called metabolic syndrome (MetS). Importantly, metabolic syndrome is recognized as an important risk factor for the development of diabetes mellitus type 2 (DM2) and non-alcoholic fatty liver disease (NAFLD) (Harley et al., 2014; Zhang et al., 2010). According to recent estimates, in 2015 one quarter of the United States population had metabolic syndrome. MetS was indirectly responsible for a loss of around $ 38 billion due to reduced labour productivity in the USA alone (Danaei G, 2011). It is considered that these numbers are similar in the European Union (WHO, 2017).

An important underlying cause of obesity-induced insulin resistance and DM2 is chronic low-grade systemic inflammation. The long-term presence of proinflammatory cytokines in the blood blunts the signal transduction capacity of the insulin receptor in insulin-sensitive tissues (Neels and Olefsky, 2006). Obesity-induced systemic inflammation is thought to originate predominantly in visceral adipose tissue (VAT), which is why an important part of this thesis is focused on this organ. Despite the fact that VAT does not have a barrier function, it is populated with a surprisingly large number of immune cells even under lean conditions. The majority of these cells have an anti-inflammatory or Th2 type, characterized by anti-inflammatory cytokines (Lumeng et al., 2007). However, the immune cell profile of VAT changes significantly in response to fat accumulation in adipocytes.

With an increasingly obese population NAFLD has become the leading cause of chronic liver disease in western countries (Younossi et al., 2016). Even though NAFLD, without vascular complications, has a relatively benign prognosis, somewhere around 20% of patients with fatty liver eventually develop NASH that can lead to the development of liver cirrhosis within five to ten years. It is now commonly accepted that for the transition from simple steatosis to NASH, apart from chronic systemic inflammation and IR that start in VAT, liver inflammation is also crucial (Tosello-Trampont et al., 2012). As in adipocytes, accumulation of lipids in hepatic cells leads to metabolic and oxidative stress, which induces local immune activation and inflammation.

This thesis starts from the **hypothesis** that obesity is associated with inflammatory responses in liver and VAT and that the innate immune system is important for induction of these inflammatory processes. The innate immune system initiates inflammatory processes in response to diet induced stress signals and eventually lead to the development of DM2 and NASH.

The general objective was to characterize interactions of γδ T cells with lean and obese adipose and hepatic tissues and determine their role in the development of obesity-induced metabolic disease.

Specific objectives were:

I. **Identification of new stress induced molecules** in adipose tissue and liver that activate innate immune cells in murine model of diet induced obesity
II. **Characterization of innate immune cells and their cytokine profiles** that respond to these stress induced molecules, to promote VAT and liver inflammation in diet induced obesity
III. **Block interaction between stressed adipocytes and hepatocytes with the innate immune system** in order to prevent obesity-induced inflammation, insulin resistance, DM2 and NASH development.

The **goal** of this thesis was to demonstrate that cell stress-induced molecules are important for activation of immune cell subsets and that cytokines that these cells produce are responsible for the initiation of inflammation in DM2 and NASH in obesity. Altogether, by generating relevant high-impact data this thesis might help to define new targets for therapy of DM2 or NAFLD patients.

In humans, the onset of obesity is difficult to characterize. However, mice models have helped much to analyse the first phases of immune cell behaviour following adipose tissue expansion. Diet induced obesity (DIO) is the most commonly used animal model for the induction of obesity and obesity-induced pathology. By using HFD and modified high calorie diet model called steatosis-steatohepatitis diet (SSD) (Clapper et al., 2013) I was able to follow the development of DM2 and NAFLD, respectively. Identification of new stress-induced molecules was conducted by qPCR. Characterization of immune cells and their cytokine profiles in VAT and liver was done by flow cytometry. Blocking interactions between stressed adipocytes and hepatocytes with the innate immune system was done by conditional depletion of receptors with the help of established Cre-loxP system.

2. LITERATURE REVIEW

2.1 Obesity and metabolic syndrome

Body mass index (BMI) is a simple index of weight-for-height that is currently used to classify overweight and obesity in adults. Of all the known metrics, the body mass index is most closely connected with the amount of excess fat in the body. It is calculated by dividing person's weight in kilograms by the square of his height in meters ($BMI = \frac{weight\ (kg)}{height\ (m^2)}$). There are four categories: underweight (BMI from 15 to 19.9), normal (BMI from 20 to 24.9), overweight (BMI from 25 to 29.9) and obese (BMI 30 to 35 or greater). The values of the recommended BMI are the same for both sexes; they range from 18.5 to 24.9 kg / m^2 according to the WHO classification for the European population (Nuttall, 2015). In the last 30 years, the percentage of adults with a BMI higher than 25 kg / m^2 increased around 20 % in both men and women. Currently, the total number of adults who are obese or overweight in the world is greater than that of normal-weight adults and adverse health consequences of obesity represent greater threats for public health than hunger or malnutrition (Nuttall, 2015).

Obesity is a complex medical condition, characterized by an increased accumulation of fat. Fat is mainly stored in adipocytes in adipose tissue, however, it can also be found in other organs including liver and muscles. The accumulation of fat develops under the influence of genetic and metabolic factors, but most importantly by unhealthy life habits. The most frequent cause of obesity is therefore a chronic energy imbalance. Increased intake of high caloric food rich in fat and refined sugar with reduced physical activity generates surplus of energy that is stored in the body in the form of fat (Heymsfield and Wadden, 2017). Based on the anatomic location of adipose tissue accumulation, there are two types of obesity: subcutaneous and visceral. Subcutaneous obesity is more common in women and it is characterized by excess of subcutaneous fat located around the hip and thighs („pear - like“ body shape). In visceral obesity fat is concentrated in the abdominal region („apple - like“ body shape) and is more common in men (Nguyen-Duy et al., 2003; Wajchenberg et al., 2002). More importantly, VAT is connected to worse clinical prognosis (Nguyen-Duy et al., 2003).

Metabolic syndrome (MetS) is a disorder connected to obesity prevalence. As the commonness of obesity rises, the predominance of MetS increases in parallel. The exact numbers are similar like in obesity, somewhere between 20 - 30 % of the population is affected in the middle and older age. Studies indicate that the prevalence of MetS in persons younger

than 30 is 6.7 %, while in persons between 60 and 69 years it is rising to 43.5 % (O'Neill and O'Driscoll, 2015).

WHO first time defined MetS in 1998 as "glucose intolerance, impaired glucose tolerance (IGT) or diabetes mellitus (DM), and/or insulin resistance, together with two or more of the components listed below:

- Raised arterial pressure, i.e., ≥ 140 / 90 mm of Hg
- Raised plasma triglyceride (≥ 150 mg/ dl) and/or low high-density lipoprotein cholesterol (HDL-C) (< 35 mg / dl in men and < 39 mg / dl in women)
- Central obesity, i.e., BMI > 30 kg / m^2
- Microalbuminuria, i.e., urinary albumin excretion rate ≥ 20 μgm / minute or albumin / creatine ratio ≥ 30 μgm / mg" (WHO, 1999)

Abdominal adiposity and insulin resistance, as a result of obesity, seem to be at the basis of the MetS pathophysiology and its individual components. Even though not all obese persons are metabolically unhealthy, substantial number of them are insulin resistant (Stefan et al., 2008). Under normal conditions, insulin inhibits adipose tissue lipolysis. However, in the setting of insulin resistance, insulin is unable to properly suppress lipolysis, resulting in relatively more free fatty acids (FFA) being liberated into the plasma (Eckel et al., 2005). In the liver of IR patients, FFA influx is high and it impairs hepatic insulin action, increases triglyceride synthesis and storage. Since excess of triglycerides is secreted as very low-density lipoprotein (VLDL) triglycerides, it is considered that the dyslipidaemia associated with IR is a direct consequence of increased VLDL secretion by the liver (Ginsberg et al., 2005).

One of the first observations regarding the clustering of metabolic disorders in MetS was the connection of these features with an increased risk of developing type 2 diabetes [28]. In fact, obesity, IR and dyslipidaemia precede the progression to DM2 in around 80 % of individuals (Lebovitz, 1999). Additionally, many studies have shown that people with MetS have fatty liver (especially in obesity) which can progress to NAFLD (Bloomgarden, 2007). This has been explained by the fact that deposition of fat in the liver is mainly the result of high FFA influx to the liver, due to increased lipolysis connected with IR but as well as increased liver lipogenesis (Kotronen et al., 2007). Interestingly, pervasiveness of NAFLD is around 25 % in the general population, but it has been suggested that 95 % of obese individuals and up to 70 % of those with DM2 have some form of NAFLD. Thus, a common pathophysiology appears to underlie metabolic syndrome, DM2 and NAFLD in the context of obesity.

2.2 Diabetes mellitus type II and non-alcoholic fatty liver disease

Diabetes mellitus type II

Obesity has an adverse effect on organs with endocrine and metabolic functions such as fat tissue, liver, pancreas and muscles (Shoelson et al., 2007). Maybe the most important complication of obesity is development of IR. IR induces a reduced glucose transportation to the cells of fat, muscle and liver and as a result glucose levels rise. In parallel, there comes to a release of FFA from fat due to impaired ability of insulin to prevent it. Pancreatic beta (β-) cells try to make up for reduced insulin sensitivity through higher insulin production. As IR progresses, pancreas is not capable to produce sufficient amount of insulin to decrease glucose levels leading to hyperglycaemia and hyperlipidaemia. With time, β-cells exhaustion and dysfunction occur leading to the relative insulin deficiency, a key hallmark of DM2. This is opposite to diabetes mellitus type 1 (DM1). DM1 is an autoimmune disease that commonly affects children and it is caused by an absence of insulin production due to ablation of pancreatic β cells (Polonsky et al., 1996). People with DM2 comprise around 90 % of all diabetes cases (Odegaard and Chawla, 2012). Individuals with DM2 are at high risk for numerous micro- and macro-vascular complications, like diabetic retinopathy, kidney failure, heart disease and deficient blood circulation in the extremities that can lead to amputation (WHO, 2013). The acute complications due to ketoacidosis, characteristic for DM1, are relatively rare. However, a hyperosmolar hyperglycaemic state may occur.

For the diagnosis of diabetes, doctors most often use the fasting plasma glucose (FPG) levels, oral glucose tolerance test (OGTT) or the glycosylated haemoglobin (HbA1c) levels. The WHO definition of diabetes is either an FPG ≥ 7.0 mmol / l or plasma glucose levels ≥ 11.1 mmol / l two hours after the OGTT, measured on two separate occasions. Another method of diagnosing diabetes includes random blood glucose value ≥ 11.1 mmol / l together with typical symptoms or levels of glycated haemoglobin (HbA1c) greater than 48 mmol / mol.

Main characteristic of DM2 is insulin insensitivity as a consequence of IR, decreased insulin production and failure of pancreatic β-cells. The first phase of diabetes development comprises a long period when IR starts, which is compensated by elevated levels of insulin and an increase in the mass of pancreatic β-cells (Polonsky et al., 1988).The second phase is the adjustment stage when β-cells can not completely make up for the increased IR. This results in slightly increased FPG levels. This period probably begins when FPG levels are inside the normal range (around 5.6 mmol / L) and is followed by a decline in postprandial insulin secretion (Weir and Bonner-Weir, 2004). Therefore, the first and second stages in this manner

happen before the prediabetic stage. In the third phase, the β-cells can no longer compensate for IR and subsequently glucose values begin to rise rapidly. This period marks the transition from prediabetes to diabetes.

Before an individual develops DM2, people may suffer for years to decades from prediabetes (or "transitional hyperglycaemia"), a state where glycaemic parameters are above ordinary but beneath diabetes threshold values. Prediabetes is considered a high-risk state for diabetes development with a conversion rate of around 5 - 10 % per year. However, for some individuals with prediabetes, initial use of medications can reverse blood glucose values to the normal range.

2.2.1 Insulin signalling and insulin resistance

Insulin binding to the insulin receptor causes activation of very complex signalling cascades. Insulin receptor activation causes phosphorylation of several proteins, most notably Insulin receptor substrate 1-4 (IRS1-4), Proto-oncogene tyrosine-protein kinase Src and Growth factor receptor-bound protein 2 (Grb-2). Phosphorylation of IRS-1 or IRS-2 provides docking sites for the Phosphatidylinositol 3-kinase pathway (PI3K) and phosphorylation of Grb-2 activates Ras and leads to the activation of the Mitogen-activated protein kinase (RAS-MAPK) pathway. PI3K products are phosphatidylinositol bisphosphate (PIP2) and phosphatidylinositol triphosphate (PIP3) which provides docking sites for phosphoinositide-dependent kinase-1 (PDK1) and Protein kinase B (PKB or Akt). This close vicinity binding enables that PDK1 phosphorilates Akt. Phosphorilation of Akt has a key role in uptake of glucose in adipocytes and skeletal muscle cells but also in activation of other pathways that lead to glycogen synthesis, lipogenesis, inhibition of lipolysis in fatty tissue and liver, and inhibition of liver gluconeogenesis. The RAS-MAPK signalling pathway mediates the effects of insulin on mitosis, cell growth and differentiation (Saltiel and Kahn, 2001).

IR is the result of reduced signalling via insulin receptors in tissues important for glucose homeostasis. It is manifested in reduced glucose uptake, unsuccessful restriction of hepatic gluconeogenesis and uncontrolled hydrolysis of triglycerides in fat tissue, leading to elevated glucose and fatty free acids levels (Vrhovac, 2008). IR is initially compensated by an increase in insulin production in the pancreatic Langerhans islets. However, endoplasmic reticulum stress and metabolic overload of mitochondria, eventually lead to β cell failure and hypo-insulinemia (Muoio and Newgard, 2008). Various molecular mechanisms have been shown to be involved in reduced insulin receptor signalling. Of these, proinflammatory

cytokines have been shown to be of particular importance, since they abrogate insulin signalling at several steps in the downstream insulin signalling cascade.

Inflammation can disrupt insulin receptor signalling via different transcription and post-transcription mechanisms. Serine kinases JNK and IKKβ, activated by inflammation, are able to phosphorylate IRS proteins at inhibitory sites, thus inhibiting downstream signalling. Salicylates, which inhibit the action of IKKβ and cyclooxygenases, prevent the inhibitory phosphorylation of IRS-1, thus preventing IR (Gao et al., 2002; Ozes et al., 2001). Furthermore, NF-κB and AP-1 transcription factors can promote inflammation by inducing transcription of inflammatory genes as well as directly inhibit some parts of insulin signalling pathways. These inflammatory mediators can then promote the expression of suppressor of cytokine signalling (SOCS), proteins that bind to the insulin receptor and prevent the phosphorylation of IRS-1 and IRS-2 proteins (Figure 1) (Emanuelli et al., 2000; Ueki et al., 2004). Inflammation can also reduce insulin sensitivity by changing several metabolic pathways, resulting in the emergence of secondary messengers that promote development of IR. One example is TNF that promotes lipolysis in adipocytes resulting in increased levels of FFA, which reduce insulin sensitivity (Aggarwal, 2003). Furthermore, inflammatory signals induce the expression of genes important for lipid metabolism, including the enzyme involved in the synthesis of ceramides and sphingolipids, both of which inhibit insulin PKB activation (Holland et al., 2011).

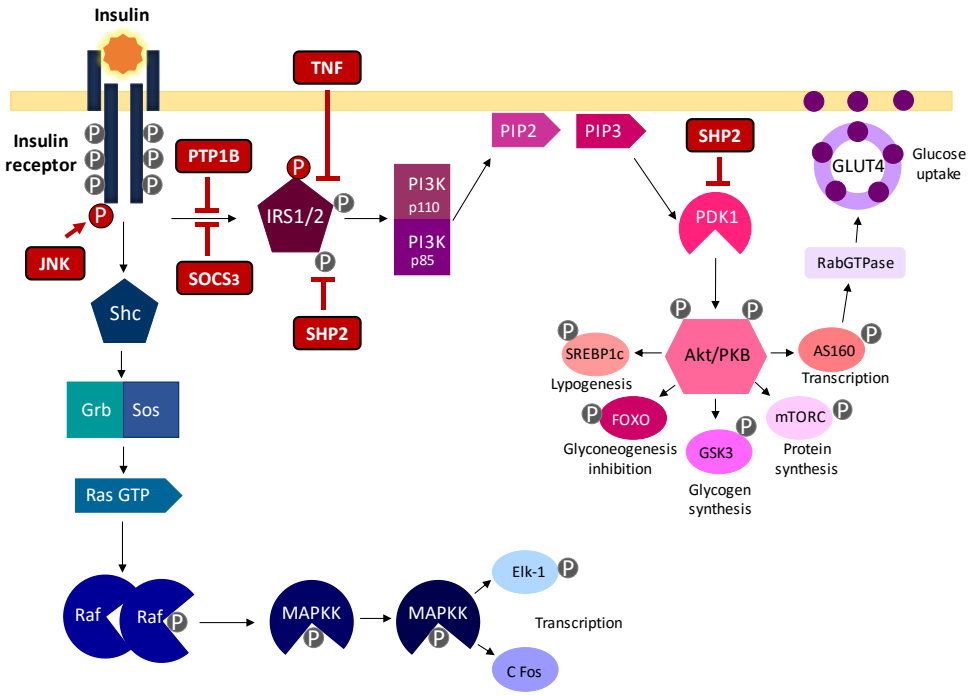


Figure 1: Insulin signalling pathways linking inflammation and insulin resistance. Key branches of the insulin signalling cascade that are normally triggered by insulin. Activation of the Mitogen-activated protein kinase (RAS-MAPK) pathways is indicated in blue and PDK1 /

Akt pathway is in pink/purple. Specific sites that become phosphorylated (P) on insulin stimulation are indicated within grey circles. Red boxes and flat arrowheads represent recognized alterations of insulin signalling pathways in IR and DM2 subjects.

Under normal metabolic conditions, insulin regulates glucose disposal in peripheral tissues, like adipose tissue and skeletal muscles that store around 80 % insulin-stimulated glucose (Osborn and Olefsky, 2012). In addition, insulin takes part in lipogenesis and adipose tissue lipolysis (Frayn, 2001). However, both effects are impaired in IR suggesting that reduced insulin sensitivity of muscle and adipose tissue are very important in the formation of hyperglycaemia and hyperlipidaemia during diet-induced obesity.

2.2.2 Obesity and inflammation

Obesity and DM2 in humans and in mice is connected with chronic low-grade inflammation. This is mainly marked by high inflammatory gene expression as well as macrophage infiltration (D'Souza et al., 2013; Pascual et al., 2007).

In muscles, macrophages accumulate between the muscle fibers in the intercellular clusters of adipocytes ("crown-like structures") and around the blood vessels. These macrophages produce tumour necrosis factor (TNF) and other proinflammatory cytokines that can activate inflammatory signalling pathways in the myocytes, leading to reduced signalling via the insulin receptor (Martins et al., 2012; Turcotte and Fisher, 2008; DeFronzo and Tripathy, 2009). However, whether muscle inflammation mediated by macrophages is functionally important for systemic IR is still unknown.

On the other hand, adipose tissue inflammation is recognized as a critical player in systemic IR through secretion of cytokines and FFAs that regulate hepatic, skeletal muscle, and vascular insulin signalling. Low-grade adipose tissue inflammation, as a result of obesity, is associated with infiltration and expansion of macrophages. These macrophages produce proinflammatory cytokines, among others TNF and interleukin (IL-) 1β, that interfere with insulin signalling (Hotamisligil et al., 1993) and lead to a reduction in cells that promote adipose tissue homeostasis. Therefore, it is now obvious that VAT inflammation contributes to the development of DM2 but also to other obesity-induced conditions including NAFLD (Weisberg et al., 2003; Xu et al., 2003).

2.2.2.1 **Visceral adipose tissue homeostasis**

Formerly considered a static depot for energy storage, VAT is now recognized as an endocrine organ that has a crucial part in local and systemic homeostasis. It is now known that adipocytes have the capacity to produce and secrete many hormones termed ‘adipokines’ (Fain et al., 2004). Friedman’s group was the first to discover leptin, an adipokine that is important for the adipose tissue-brain communication but also for the sustenance of energy homeostasis (Zhang et al., 1994). Chronically increased levels of leptin in obesity lead to an occurrence of central leptin resistance, further weight gain and accumulation of VAT immune cells (Haas et al., 2008; Tian et al., 2002). Adiponectin is another notable adipokine with opposing effects compared to leptin. It is an anti-inflammatory hormone that improves insulin sensitivity (Yamauchi et al., 2001) and low levels of adiponectin have been connected to development of MetS (Kadowaki et al., 2008).

Apart from adipocytes, VAT is composed of numerous different cell types. These cells are pre-adipocytes, fibroblasts, endothelial cells and immune cells. Specifically, immune cells participate in both, maintenance of proper VAT homeostasis and obesity-induced inflammation and development of IR. The status of tissue resident macrophages appears to be a crucial factor. Macrophages exist in various subtypes, with different phenotypes and functions (Gordon, 2007). ‘M1’ macrophages are known as proinflammatory. They can be activated through proinflammatory signals and molecules like lipopolysaccharide (LPS) or type 1 T-helper (Th1) cytokines like interferon (IFN) γ and TNF (Lumeng et al., 2007). Conversely, 'M2' (or alternatively activated) macrophage differentiation is induced by type 2 T-helper (Th2) cytokines like IL-4, IL-5 and IL-13 (Gordon and Martinez, 2010; Martinez et al., 2009) and they are the dominant population in lean adipose tissue. They express arginase-1 and are believed to be engaged in tissue repair through the generation of anti-inflammatory mediators, such as IL-1 receptor agonist or IL-10 (Lumeng et al., 2007) (Figure 2).

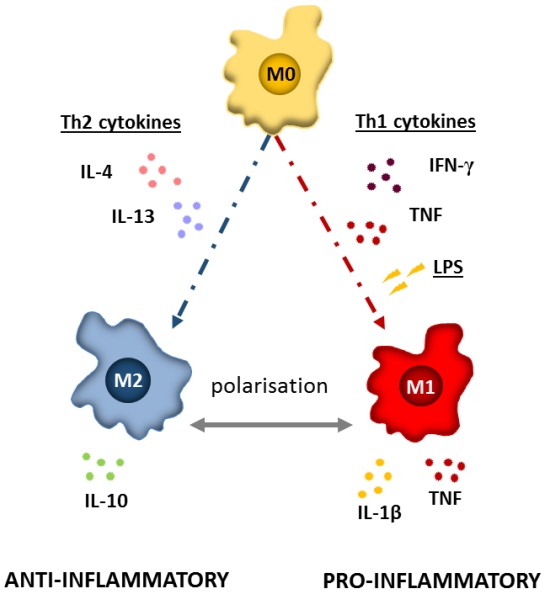


Figure 2: Graphical representation of macrophage polarisation. Macrophage polarisation is a process finely regulated by environmental stimuli. Th1 cytokines or LPS drive the M1 macrophage phenotype. In the presence of Th2 cytokines, such as IL-4 and IL-13, monocytes differentiate into M2 macrophages. Macrophage polarisation between M2 and M1 subsets determines effector response as regulatory versus proinflammatory. M2 function is associated with expression of suppressive cytokines while M1 phenotype is connected with expression of proinflammatory cytokines.

Notably, under lean conditions, M2 macrophages have a major role in the inhibition of immune cell activation. In mouse studies, where adipose tissue macrophages failed to react to M2-polarizing stimuli because of genetic deficiency of cytokine receptors, an increase in production of TNF and IL-1β was detected (Mauer et al., 2014; Wu et al., 2011). A similar anti-inflammatory role for M2 macrophages was observed in other tissues important for the control of glycemia, such as muscle and liver (Lumeng et al., 2008; Odegaard et al., 2007). The M2 phenotype of adipose tissue macrophages (ATMs) is preserved by various immune cells but also adipocytes. In lean VAT, the main source of IL-4 are eosinophils (Mauer et al., 2014). Either deficiency of these cells or hyper-eosinophilia in VAT, results in reduction or increase in the M2 numbers, respectively (Wu et al., 2011). Survival of VAT-resident eosinophils depends on IL-5, a cytokine that is primarily secreted by type 2 innate lymphoid cells (ILC2s) (Molofsky et al., 2013). Removal of any segment of the ILC2 / eosinophil / M2 axis results in VAT inflammation and subsequent progression to obesity-induced development of IR. Therefore, this specific axis is one of the most important regulatory mechanisms of VAT homeostasis (Molofsky et al., 2015; Patsouris et al., 2008; Wu et al., 2011) (Figure 3).

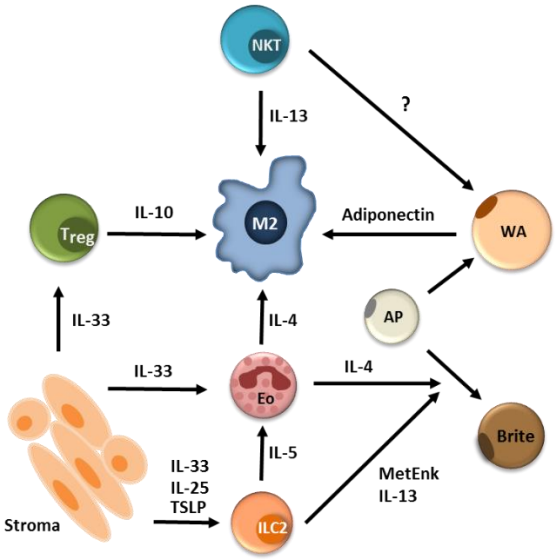


Figure 3: Adipose tissue homeostasis axis. Lean adipose tissue is populated by a type 2-polarised immune system. Dominant immune cell subsets are eosinophils, iNKT cells, and Treg cells, which produce Th2 cytokines. Adipocytes also contribute through the production of adiponectin. ILC2s regulate eosinophil numbers through the production of IL- 5. In addition to sustaining a type 2 immune cell environment, adipose tissue cells engage in extensive crosstalk to (re)model adipose tissue structure and phenotype. (Taken from (Wensveen et al., 2015))

Regulatory T cells (Treg) are also important for the prevention of macrophage polarisation. They secrete IL-4 and IL-10, the Th2 type cytokines, and are directly involved in control of macrophages and inhibition of VAT inflammation and insulin resistance (Ilan et al., 2010). Even though Tregs are one of the main populations in lean VAT, their number decrease with the development of obesity (Feuerer et al., 2009; Winer et al., 2011). Experimental depletion of Tregs in murine models of diet induced obesity, resulted in increased production of the proinflammatory cytokines RANTES, TNF and IL-6 and decreased insulin sensitivity (Feuerer et al., 2009).

Invariant-chain natural killer T (iNKT) represent an additional subset that preserves the M2 phenotype of macrophages in VAT through secretion of Th2 M2-polarizing cytokines. They are highly enriched in lean VAT but their number decreases in obesity (Lynch et al., 2009). Lack of iNKT cells causes reduction in cytokines levels of IL-4 and IL-13, along with an increase in M1 macrophage numbers (Mathis, 2013). Some studies report that iNKT can play a role in promoting IR in obesity (Wu et al., 2012), but the mechanisms through which iNKT cells induce inflammation in obesity are still not known.

2.2.2.2 **Chronic low grade obese VAT inflammation**

The early periods of diet induced obesity have been studied extensively in mice. The most frequently used model to provoke insulin resistance is DIO (Li et al., 2008). In this model 50 - 60 % of calories are derived from animal fat which results in the formation of IR and glucose intolerance after 10 - 12 weeks from the beginning of feeding. The early stages of DIO in VAT are characterized by an adipocyte hypertrophy and by aggregation of immune cells that have restricted proinflammatory capacity.

The first few days of DIO are marked by neutrophil accumulation and production of proteinase elastase (Talukdar et al., 2012). However, after a longer period of HFD feeding, neutrophils become a minor cell population in VAT. Still, it has been shown in 12 week-experiments of HFD feeding that the lack of elastase or its inhibition leads to improved insulin sensitivity in comparison to animals fed with a standard diet. The trigger for neutrophil accumulation in VAT is still not known, but in humans, intense lipid burden leads to an inflammatory boost that increases the levels of monocyte chemoattractant protein (MCP-1) and C-reactive protein in VAT (Kanda et al., 2006). Alternately, DIO can induce intense changes in the adipose tissue microenvironment, for example, changes in oxygen consumption that can lead to expansion of pre-adipocytes (Netzer et al., 2015). This acute stress might take part in accumulation of neutrophils to VAT in first few days of HFD feeding.

Early DIO is further characterized by an increase of natural killer (NK) cells and macrophages in VAT. In contrast to neutrophil accumulation, infiltration and expansion of VAT NK cells and macrophages seems to be deferred, happening in weeks, instead of days after the beginning of DIO. While VAT neutrophils are believed to be essentially of peripheral origin (Talukdar et al., 2012), adipose tissue macrophages and NK cells seem to increase in part or totally due to proliferation of tissue resident cells (Kanda et al., 2006). HFD-fed animals pulsed with BrdU showed a significant increase in BrdU incorporation of VAT-resident NK cells compared to splenic NK cells (Wensveen et al., 2015). This suggested that the rise in numbers of NK cells is due to proliferation rather than ingress from the periphery. Increase in ATMs as a result of HFD seems to be more complicated. One study showed that local proliferation is the prevalent source of ATM increase (Kanda et al., 2006), while others claim that chemo-attractants in VAT are responsible for a major peripheral influx (Amano et al., 2014; Li et al., 2015). Likely, both ways contribute to the increase in ATM numbers in VAT in obesity.

Apart from the aggregation of proinflammatory cells in VAT, diet induced obesity is additionally connected with a decline in number of anti-inflammatory cells. Studies showed that in both humans and mice, VAT resident ILC2s are decreased in obese individuals, both in absolute numbers and per gram of fat tissue (Brestoff et al., 2015). This seems to be due to restriction of ILC2s to react to the IL-33 cytokine in the presence of IFNγ (Molofsky et al., 2015). Reduction in numbers of ILC2, results in decrease of IL-5, since they are the main producers of this cytokine. IL-5 is considered crucial for the maintenance of eosinophils, therefore their numbers also decrease (Molofsky et al., 2013; Wu et al., 2011). As mentioned previously, in response to DIO the number of Tregs declines and therefore their inhibitory capacity also decreases. The ensuing increase of VAT inflammation and subsequent systemic presence of proinflammatory cytokines greatly contribute to impairment of insulin signalling in VAT, muscle and liver (Feuerer et al., 2009; Vasanthakumar et al., 2015).

With the expansion of VAT mass, major changes also happen in adipokine production, which has an important effect on the immunological micro-environment in this tissue. Most importantly, production of adiponectin goes down, which leads to a decrease in glucose uptake and impairs suppression of immune cell activation. In obesity, adipocytes produce more leptin. The leptin receptor, otherwise called LEP-R or CD295, is present on great number of immune cells including NK cells, neutrophils and macrophages. As a result, increased adiposity locally stimulates the immune system. Apart from leptin, VAT also produces different adipokines that influence immune cells, most prominently IL-6 and MCP-1. MCP-1 chemokine attracts blood monocytes and T cells at the site of inflammation (Zietz et al., 2005). IL-6 has a versatile role in obesity and it shares some parallels with IL-33. Generally, it is thought to be a proinflammatory mediator and like in the case of MCP-1, its levels are increased in individuals with DM2 (Eder et al., 2009).

Even though accumulation of immune cells leads to inflammation of VAT, itself it is not enough for the immune cells activation. The most important thing in the initiation of VAT inflammation and advancement to IR is macrophage polarisation from an anti-inflammatory to proinflammatory-phenotype (Lumeng et al., 2007). Until 2015, the process of macrophage accumulation was still not clarified, and the assumption was that the macrophages enter VAT due to 'stress' signals coming from hypertrophic adipocytes (Neels and Olefsky, 2006; Wellen and Hotamisligil, 2003). In 2015, our group showed that VAT resident NK cells represent a crucial connection between obesity-induced adipose tissue stress and M1 polarisation of macrophages. We have shown in our research that obesity leads to the upregulation of NCR1 ligands on adipocytes and that these ligands are crucial for the activation of NK cells through

NCR1 activating receptor. This upregulation stimulates proliferation of NK cells and production of IFNγ that prompts the proinflammatory macrophages differentiation (Wensveen et al., 2015). These proinflammatory macrophages then become a dominant source of proinflammatory cytokines which can be detected in the blood of DM2 patients and are considered to have a major role in development of systemic IR (Lumeng et al., 2008) (Figure 4).

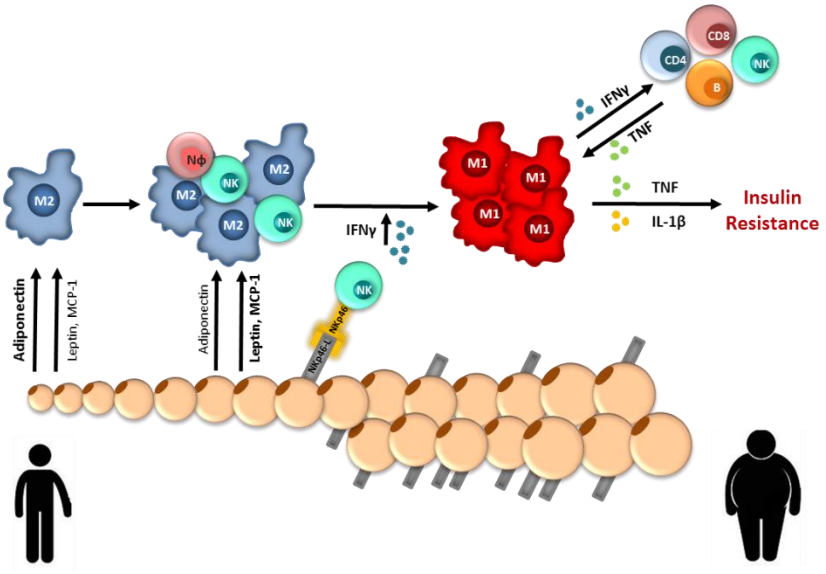


Figure 4: Model of adipose tissue inflammation in obesity. In response to HFD, adipocytes become hypertrophic and later hyperplastic. This is due to a shift in adipokine production from adiponectin to leptin/MCP- 1 and an increase in the number of ATMs, neutrophils, and NK cells in visceral fat. As obesity persists, adipocyte stress drives NK cell activation through NKp46, resulting in local production of IFNγ. This production of IFNγ licenses ATMs toward a proinflammatory M1 state. As a result, ATMs produce proinflammatory cytokines and recruit more proinflammatory cells, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells, into the VAT to amplify the immune response. The chronic systemic presence of pro- inflammatory cytokines ultimately contributes to the development of IR. (Modified from (Wensveen et al., 2015))

The later phases of VAT inflammation are well investigated (Donath and Shoelson, 2011; Johnson et al., 2012). Advanced obesity induces necrosis of adipocytes due to micro-hypoxia (Ye et al., 2007). As a consequence of necrotic cell death, danger-associated molecular patterns (DAMPs) are released in the form of double-stranded DNA (dsDNA) or heat shock proteins (HSP) that drive activation of macrophages and further production of proinflammatory cytokines (Vandanmagsar et al., 2011). The ATMs activation and the resulting proinflammatory environment subsequently leads to recruitment of other proinflammatory cells mainly with a Th1 signature. Th1 effector T cells CD4 and CD8, together with B cells most

greatly increase in later stages of VAT inflammation (Donath and Shoelson, 2011). These cells then become the main producers of IFNγ and they further stimulate ATM polarisation. Additionally, they play a part in development of chronic systemic inflammation by releasing cytokines like TNF. Eventually, the chronic release of these cytokines in the blood stream drives a development of systemic IR through dampening the signalling capacity of the insulin receptor in various different tissues (Rui et al., 2002).

In summary, one of the first steps in VAT inflammation is the expansion in cell numbers of VAT macrophages in response to HFD feeding. To obtain their pro-inflammatory potential, adipose tissue macrophages need to be licensed with the help of IFNγ that is derived from NK cells. This polarisation of VAT macrophages and the propagation of inflammatory environment drives the chronic systemic inflammation and increases the risk for the development of systemic insulin resistance. Nonetheless, whereas IFNγ deficiency and depletion of NK cells in VAT delays M1 ATM polarisation and insulin resistance development in response to HFD feeding, it does not prevent it altogether (Rocha et al., 2008; Wensveen et al., 2015). This data suggests that IFNγ is not the only cytokine involved in M1 polarisation and VAT inflammation, and that there must be an additional cell subset present in VAT that translates adipose tissue stress into an immunological signal towards macrophages.

Non-alcoholic fatty liver disease (NAFLD)

With an increasingly obese population, non-alcoholic fatty liver disease (NAFLD) has become the main cause of chronic liver disease in developed countries (Younossi et al., 2016). It is an important cause of morbidity and mortality and it is considered as a hepatic manifestation of MetS. NAFLD represents a broad spectrum of symptoms, starting from non-alcoholic fatty liver (NAFL) marked by simple steatosis to non-alcoholic steatohepatitis (NASH) without alcohol consumption, or other viral or genetic cause of liver inflammation. Whereas NAFL is usually only associated with excess accumulation of fat (triglycerides) in liver, NASH is marked by hepatocyte injury and cell death together with lobular and portal inflammation that can advance to fibrosis. With progressive deposition of collagen, one third of patients suffering from NASH will develop liver cirrhosis within five to ten years after diagnosis and 25 % of them will develop liver portal hypertension and hepatocellular carcinoma (HCC) (Chalasani, 2012; Younossi, 2015). Even though NAFL, without vascular complications, has a relatively benign prognosis, somewhere around 20 % of individuals with NAFL eventually develop non-alcoholic steatohepatitis (Figure 5).

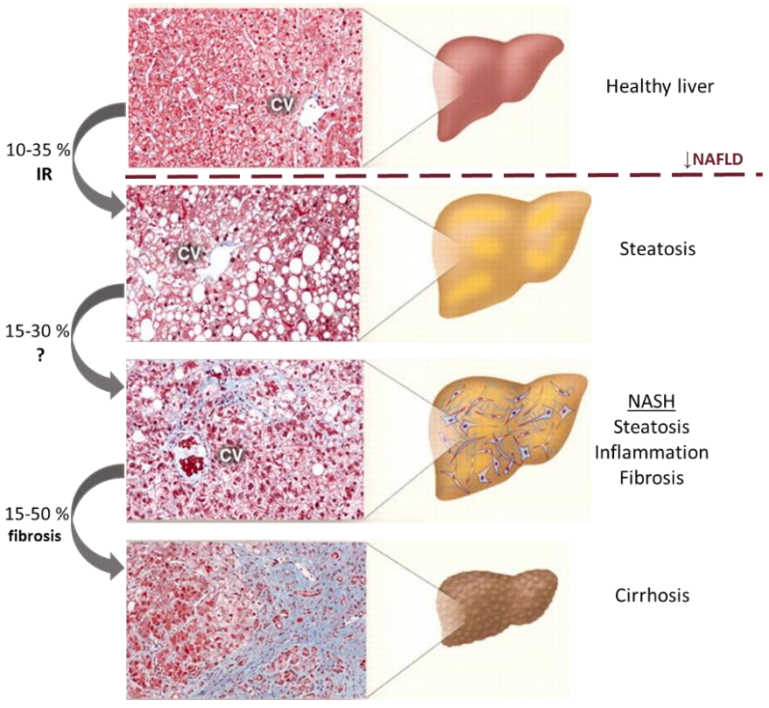


Figure 5: Stages of liver damage in NAFLD. Histological sections illustrating normal liver, steatosis, NASH, and cirrhosis. The accumulation of triglycerides within hepatocytes causes steatosis. Steatosis accompanied by inflammation, apoptosis, and fibrosis is referred to as NASH. NASH can further progress to cirrhosis. Individuals with cirrhosis have an increased risk of hepatocellular carcinoma. (Modified from (Kwanten et al., 2014)).

The term NASH was first used in the 1980s to describe liver disease that looked exactly like alcoholic steatohepatitis but in patients without alcohol consumption. There are several histological features of NASH that are same as in alcoholic steatohepatitis (ASH), including hepatocyte ballooning, steatosis, inflammation and lobular fibrosis. However, in contrast to the increased alcohol consumption that starts and drives the progression of ASH, patients with NASH are usually obese, they have IR and do not abuse alcohol (Ludwig et al., 1980).

NAFLD is currently one of the leading causes of liver transplantation, and its prevalence is between 20 % and 33 % in the general population. An alarming fact is that NAFLD prevalence doubled in the past 20 years while the predominance of other chronic liver diseases stayed unchanged or diminished. Despite the rise in prevalence, NAFLD still remains insufficiently diagnosed and treated in routine medicine practice (Ahmed, 2015; Singh et al., 2015). The large number of NAFLD patients do not have any clinical symptoms and are diagnosed during systematic examinations or visits to a doctor for some other reason. One of the first signs of NAFLD in patients are asymptomatic elevation of one or more liver biochemical tests (AST, ALT, GGT or AF) and hepatomegaly. The occurrence of splenomegaly is the first sign of portal hypertension and advanced liver disease (Milic and Stimac, 2012). The aetiology of the disease is still not clear, but some well-known risk factors are visceral obesity, DM2 and insulin resistance and age above 45 years (Ahmed, 2015).

Research into NAFLD has been hampered by the availability of suitable preclinical models. Several genetic models are available, but their relevance for human disease has been questioned openly (Nowicka et al., 2017; Takahashi et al., 2012). The most widely used model for induction of insulin resistance in mice is administration of a diet containing a high content of saturated fat. This HFD model is able to induce severe steatosis after long-term feeding. Yet, HFD-feeding only causes a very mild inflammatory environment in the liver and no fibrosis. Others have used diets lacking choline or choline and methionine (MCD). Methionine and choline are essential for hepatic β-oxidation and production of VLDL (Anstee and Goldin, 2006). As a result, triglycerides accumulate in liver cells, causing steatosis, steatohepatitis and fibrosis within weeks. However, the MCD model does not cause metabolic syndrome but rather a decrease in body weight. Others have therefore combined the MCD and HFD models. Whereas MCD-HFD fed animals do develop both obesity and liver fibrosis, these two elements of metabolic disease are here mechanistically disconnected and are therefore of questionable relevance. Much about the molecular mechanism underlying liver fibrosis has been learned from administration of hepatotoxic compounds such as CCl_4. Apart from fibrosis, this model

does not share any other aspects of NAFLD. Finally, *Amylin liver* dietary model of NASH containing 40 % fat, and 2 % cholesterol in the food together with 22 % fructose in drinking water, that imitates cafeteria diet, induced development of several NAFLD stages (NAFL, NASH and cirrhosis) confirmed by histology and biochemical methods (Clapper et al., 2013). However, the role of immune cells on liver inflammation was not investigated in this model.

The distinction between various models of liver fibrosis seems trivial, especially regarding aspects that are not directly related to liver damage. Indeed, all models for liver fibrosis share central aspects such as hepatic stellate cells (HSC) activation. However, from a clinical aspect it is crucial to understand the differences between these models, especially regarding the initiation of inflammation and the cellular composition of the inflammatory infiltrate.

2.2.3 Development of NAFLD

Until recently, NAFL was thought to be an inconsequential diagnosis since it was considered a benign change of liver parenchyma with negligible risk for progression to liver fibrosis or liver failure. Newer studies and meta-analysis showed that individuals with NAFL also have higher risk of progression to NASH and development of liver cirrhosis and HCC (Singh et al., 2015; Streba et al., 2015). The mechanisms involved in NAFLD pathogenesis are not completely understood. “The theory of the two hits” is one of the main hypotheses currently accepted. The first stage in the NAFLD development is evolution of liver steatosis and the second one is subsequent apoptosis of hepatocytes leading to hepatic inflammation and fibrosis (Sanches et al., 2015). More precisely, for NAFLD establishment, the “first hit” is lipid accumulation in hepatocytes, mainly in form of triglycerides. This is due to imbalance between two metabolic pathways; first one that promotes synthesis and the uptake of fatty acids by hepatocytes versus the one that promotes oxidation and export of fatty acids. It has been proposed that the most important cause for accumulation of lipids and development of hepatic steatosis is insulin resistance. Additionally, IR leads to peripheral lipolysis (especially in adipocytes due to hormone-sensitive lipase) and hyperinsulinemia. Lipolysis increases circulating free fatty acids and hepatic uptake of FFAs. Hyperinsulinemia intensifies hepatic fatty acids synthesis by inducing glycolysis and it favours the triglycerides accumulation inside the hepatocytes by reducing the ability of the liver to re-esterify and export triglycerides (Mehta, 2002). With the lipids accumulation, liposomes in hepatocytes increase in size and shape large vacuoles that are characteristic for liver steatosis. As a result of steatosis, hepatocytes develop vulnerability to oxidative stress.

Fatty acid oxidation happening in mitochondria leads to production of reactive oxygen species (ROS) and induces the state of oxidative stress. In hepatic steatosis, inability of antioxidant reserves to eliminate accumulated ROS and further increased production of free radicals, leads to oxidative damage of DNA, lipids and proteins, as well as consequent apoptosis (Amano et al., 2014; Subramanian and Chait, 2012). ROS can also cause apoptosis of hepatocytes through lipid peroxidation of the cell membrane and induced expression of the Fas ligand. Since Fas receptor is usually expressed on normal hepatocytes, Fas ligands from one hepatocyte may interact with Fas ligand on another inducing hepatocyte apoptosis. Additionally, ROS can have a part in the gene regulation by encoding proinflammatory and pro-fibrogenic cytokines like TNF, transforming growth factor beta (TGF-β) or IL-8. Therefore, oxidative stress has been suggested to be the "second hit" (Rolo, 2012) responsible for the progression from NAFL to steatosis marked with liver inflammation and fibrosis (Figure 6).

However, as in adipocytes, accumulation of lipids and oxidative stress in hepatocytes might induce activation of local immune cells and results in an inflammation that is a hallmark of NASH.

2.2.4 Role of liver cells in the NASH development

It is now commonly accepted that the local activation of the immune system in the liver is an important component of NASH. Fatty acids and DAMPs, released by apoptotic hepatocytes, can trigger inflammation in fatty livers through activation of inflammasomes (Mehal, 2014) and Toll-like receptors (TLRs) (Roh and Seki, 2013) and thereby stimulate the release of proinflammatory cytokines (Leroux et al., 2012; Tosello-Trampont et al., 2012). It is considered that recruited macrophages and tissue resident Kupffer cells are important effectors in the hepatic inflammation (Wehr et al., 2014) (Figure 6). Additionally, it was recently published that NKT cells and $CD8^+$ T cells have an important part in macrophage recruitment and are emerging as contributors to progressive liver disease (Syn et al., 2010; Wolf et al., 2014). However, the central event in NASH is the progress to hepatic fibrosis, through activation of hepatic stellate cells (HSCs), as it predicts a poor outcome (Angulo et al., 2015; Heymann and Tacke, 2016).

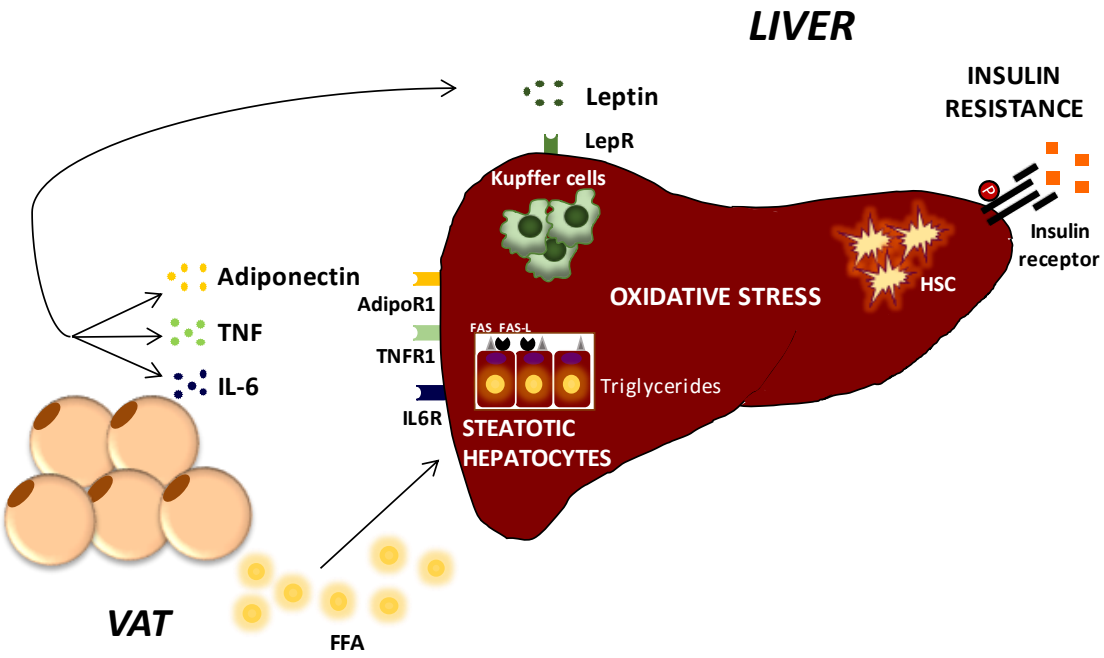


Figure 6: Development of NAFLD and liver inflammation in obesity. Increased adiposity and VAT inflammation induce the release of FFAs and adipokines into circulation. On one hand they promote hepatic steatosis and on other they induce Kupffer cell stimulation. Cytokine release by Kupffer cells induce hepatic stellate cell activation which then in turn produce collagen fibers and induce fibrosis.

Below, I will briefly outline liver cell populations that play a part in development of inflammation and fibrosis.

Hepatic Stellate Cells

Hepatic stellate cells (or Ito`s cells) are located in the space between parenchymal cells and liver sinusoides. In physiological conditions, they comprise around thirty percent of non-parenchymal liver cells and constitute the largest vitamin A deposit in the body. Their activation, proliferation and differentiation due to liver damage and inflammation is the direct cause of fibrosis. Activated HSCs express α-Smooth Muscle Actin (α-SMA) and produce collagen IαI and matrix metalloprotease inhibitors. Thus, at the same time they deposit the intercellular matrix and prevent its dissolution (Wallace et al., 2015). Platelet deriver growth factor (PDGF) and TGF-β derived from M2-activated Kupffer's cells and recruited monocytes are considered the key signals in HSC activation (Seki et al., 2007). Other activation signals include dying hepatocytes, paracrine and endocrine factors such as "hedgehog" ligand (derived from hepatocytes damaged by lipotoxicity), TNF-α, angiotensin II, IL-1, leptin, as well as metabolic factors like endoplasmic reticulum stress and exposure to ROS (Jiang et al., 2013). Inhibition of HSC activation can be induced by various immunological mediators such as IFN-α / β, adiponectin, IL-10 but also IFNγ.

Kupffer cells

Kupffer cells (KC), resident liver macrophages, make up to 15% of the weight of healthy liver and are considered to be the main cell subset of the innate immune system responsible for progression of NAFL to NASH (Heymann and Tacke, 2016). According to classical interpretation, Kupffer's cells, like all macrophages, exhibit two major subsets: proinflammatory M1 and immunomodulatory M2 that induces healing and fibrosis. These two polarized states are classically generated through differentiation in the presence of defined stimuli and it is now known that there is a significant phenotypic overlap between these two states. Polarisation towards an M1 phenotype is the early feature of NAFLD and it is characterized by high expression of Ly6C surface markers. Polarisation is triggered by DAMPs arriving from apoptotic hepatocytes or through IFNγ originating from NK cells. Activated KCs produce proinflammatory cytokines IL-1β, TNF, and IL-12 together with the chemokines CCL2 and CCL5 (Dixon et al., 2013). M1-polarized KCs recruit $CD11b^{+}$ $Ly6C^{hi}$ monocytes from peripheral blood through production of TNF-α and MCP-1, which further increases hepatic

levels of TNF-α, thereby initiating and amplifying inflammation in NASH. (Dixon et al., 2013; Meli et al., 2014).

In later stages of the disease, polarisation of macrophages goes towards an M2, Ly6C^{low} phenotype. For the progression of NASH to fibrosis, TGF-β produced by M2-polarized macrophages, is the key factor because it acts on HSC to produce collagen. Necrotic hepatocytes, along with other Th2 cytokine sources, stimulate the excretion of TGF-β by KCs. The effect of M2-polarized macrophages is seen through function of scar-associated macrophages (SAM), which, besides production of pro-fibrogene mediators PDGF and TGF-β, can also support scar resolution by production of matrix metalloproteases (Dixon et al., 2013; Tsuchida and Friedman, 2017).

T cells

As mentioned in the beginning, T cells are also involved in the progression of NASH. It was found that mice subjected to a high-fat high-carbohydrate diet model had increased infiltration of NKT and CD8$^+$ T cells in the liver. Furthermore, depletion of these cells resulted in slower progression of NASH (Bhattacharjee et al., 2017). Flavell and his group then further showed that NKT cells mainly have a role in development of steatosis, while both CD8$^+$ T cells and NKT cells mutually cause liver damage and promote NASH and HCC (Wolf et al., 2014).

During NASH, hepatic CD4$^+$ T cells predominantly show Th1 and Th17 phenotypes. Presence of IL-12 can induce and activate Th1 CD4$^+$ cells and once activated, they can produce TNF and IFNγ. However, a number of studies showed that there is a reduction in liver CD4$^+$ T cells during diet-induced NAFLD and it is considered that this loss promotes the development of hepatocellular carcinoma (Rau et al., 2016; Weiskirchen and Tacke, 2016).

On the other hand, Th17 CD4$^+$ cells are thought to promote NASH by numerous mechanisms, such as neutrophil activation and direct induction of pro-fibrogenic genes of HSC. During NASH, Th17 CD4$^+$ cells are induced predominantly by IL-6, IL-21, IL-23 and TGF-β. They are characterized by the transcription factors RORγ and STAT3 and by production of IL-17A, IL-21 and IL-22 (Tan et al., 2013). There have been several studies suggesting that the increase in the number of Th17 CD4$^+$ cells in the liver reflects the progress of NAFL to NASH. Additionally, it has been shown that the production of IL-17A by Th17 CD4$^+$ cells can induce expression of TGF-β, α-SMA, and collagen mRNA in HSCs, alluding that NASH is an IL-17A-driven fibrotic process (Chackelevicius et al., 2016; Gomes et al., 2016; Rau et al., 2016).

NK cells

In the context of NAFLD, NK cells appear to inhibit formation of fibrotic lesions through direct interactions with HSCs. Early HSC activation is marked by loss of MHC-I expression and conversion of stored retinol to retinoic acid, which drives expression of retinoic acid early inducible-1 (RAE-1) on the cell surface. RAE-1 is a ligand for an activating NK cell receptor natural killer group 2D (NKG2D). NK cells, activated by the NKG2D receptor, induce apoptosis of HSC through TNF-related apoptosis-inducing ligand (TRAIL) (Suh and Jeong, 2011).

Apart from NKG2D, the NKp46 / NCR receptor has also been found to have a role in prevention of fibrosis development. It was shown that $DX5^{+}NKp46^{+}$ NK cells are increased during disease and that they promote macrophage polarisation to an M1-like phenotype, causing reduced activation of HSC (Tosello-Trampont et al., 2016). Furthermore, IFNγ production by activated NK cells can induce apoptosis and arrest cell cycle of HSC (Fasbender et al., 2016).

Even though in majority of cases a fatty liver remains free of inflammation, around 20 % of the patients who have NAFL develop inflammation and fibrosis. Liver inflammation and apoptosis are distinctive features of NASH, the progressive form of NAFLD. Although the pathophysiology and diagnosis of NASH have been thoroughly studied, much of the work has focused on immune cell mediated activation of hepatic stellate cells and how this promotes or impairs progression of fibrosis.

However, a major question is how immune cell activation is triggered in response to early NAFL and amplified in early stages of liver inflammation in NAFLD. Therefore, we believe that identification of the underlying mechanisms by which immune cells in the liver recognize cell damage signals and lead to their activation are crucial questions which still must be answered.

2.3 Immune sensing of cellular stress and initiation of inflammation

2.3.1 Sterile inflammation in obesity

There are different ways how cells respond to stress. Based on the nature and duration of the stress, cells can either activate survival pathways or initiate cell death that eventually eliminates stressed cells. Usually, the first answer to a stressful situation is regulated towards cell defence and recovery. However, if the stimulus remains unresolved, cells may activate death-signalling pathways. A third option is to signal stress to immune cells and let these highly specialized cells determine how the stress should be resolved. Other than its part in protection of cells, cell stress has a crucial role in host protection against various pathogens by induction of inflammation (Fulda et al., 2010). Apoptosis may be triggered directly by various stress factors, or indirectly through signalling stress to the immune system (Fulda et al., 2010).

Maybe one of the most difficult questions to answer is why obesity causes an inflammatory response. The capacity to store excess energy has been sustained through the course of evolution. Responding to nutrient storage through induction of inflammation is harmful to itself. It is hypothesized that this reaction occurs because the stress that is caused by obesity is similar to the stress caused by an infection. As a result, the body has the same reaction to the obesity as it would to an infection (Manna and Jain, 2015). For instance, it has been noticed that FFA and oxidative stress can induce activation of stress pathways JNK and IKK–NF-κB that have been known to be activated in infection. Additionally, during excessive fat storage in VAT or overloading of lipids in the liver, cells increase membrane lipid peroxidation or they trigger cell death. The outcome of these processes ends in the release of DAMPs and triggering the sterile inflammation (Furukawa et al., 2004). Also, in response to obesity, gut microbiota appears to provide microbial activators that belong to pathogen-associated molecular patterns (PAMPs) (Fleshner, 2013; Tilg and Kaser, 2011). Additionally, the perseverance of these signals may lead to a full inflammatory response that will induce tissue inflammation (Arrese et al., 2016). Therefore, much research in the last few decades was done on identification of innate immune receptors and intracellular signalling pathways that take part in initiation of inflammatory responses in obesity.

2.3.1.1 **Damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and their receptors**

DAMPs are endogenous molecules that are normally found in cells, but under homeostatic conditions they are not exposed to immune cells. Also known as danger signals or alarmins, they are typically nuclear or cytosolic molecules that include heat-shock proteins, proteins derived from the injured tissue, proteins from high mobility group box-1 (HMGB1) but also DNA fragments, cytokine IL-1α and ATP. They are released during necrosis, and can be recognized by immune cells that will initiate strong responses. Therefore, they are important for the start and progression of inflammation in response to infection, but also for sterile inflammation (Matzinger, 2007). In contrast, PAMPs are pathogen-derived and like DAMPs, structurally diverse. PAMPs include bacterial and viral components, like peptidoglycan parts of cell wall, nucleic acids or LPS (Seong and Matzinger, 2004). Both PAMPs and DAMPs are recognized by pattern-recognition receptors (PRRs) that can be found on the surface of immune cells (Miyake, 2007) (Tsan and Gao, 2004).

Innate and innate-like immune cells like NK cells, γδ T cells, macrophages and dendritic cells express on their cell surface PRRs, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) [134, 135]. Upon recognition of PAMPs or DAMPs by PRRs, particular signalling pathways are activated, that lead to production and secretion of cytokines and chemokines that are essential for host defence [137]. Some of these include MAPK, NF-κB or type I interferon pathways. These pathways initiate the release of IL-6, IL-8, IFNγ, TNF and other proinflammatory cytokines like IL-17A that ultimately activate the immune system and attract immune cells to the site of damage (Chen and Nunez, 2010). Since accumulation of fat in hepatocytes can induce necrotic cell death and release of DAMPs, and it is known that in obesity there is a gut-derived LPS present, this might be one of the ways how innate immune cells can be activated.

2.3.1.2 **Induction of stress ligands**

Induction of stress ligands is another way how cells can alert the immune system to danger. These proteins are not usually expressed in high levels on normal cells, however they can be upregulated on unhealthy cells due to malignancy, infection or other types of cellular stress (Shifrin et al., 2014). Accordingly, the recognition of induced self ligands ("induced self") is essential for surveillance of stressed cells that will results in their elimination (Medzhitov and Janeway, 2002). Activated NK cells or γδ T cells can recognize these induced stress ligands by some of the activating receptors and eliminate the target cells (Born et al., 2013; Medzhitov and Janeway, 2002). Probably the most studied receptor involved in recognition of induced-self ligands is NKG2D receptor.

NKG2D is a C-type lectin-like receptor that can be found on surface of NK cells, γδ T cells, activated $CD8^+$ T cells or NKT cells. NKG2D recognizes ligands from MIC and ULBP / RAE1 family in humans and their orthologous subfamiles Rae-1, H60, and MULT-1 in mice. These ligands are induced during cellular stress, malignant transformation, senescence or infection (Jamieson et al., 2002) (Carapito and Bahram, 2015)(Figure 7).

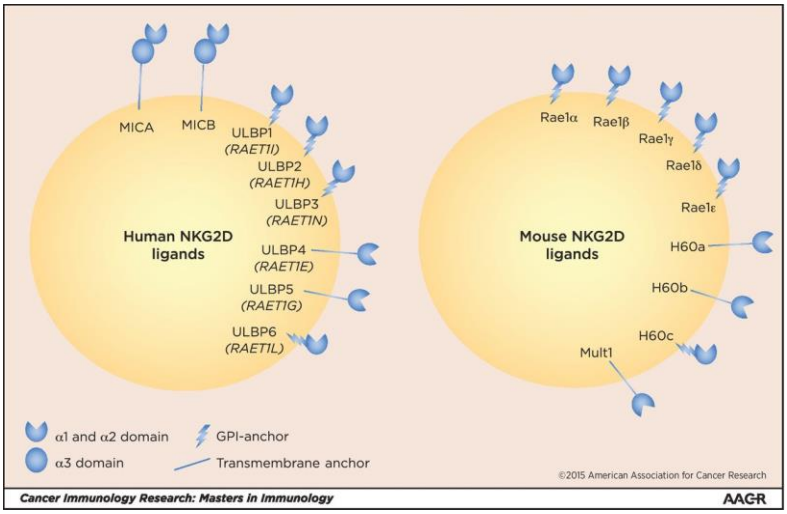


Figure 7: Human and murine NKG2D ligands present on cell surface. Scheme showing all human and mouse NKG2D ligands identified to date. MICA/B and ULBPs are two genetic families of ligands for human NKG2D and Rae1, H60 and Mult1for murine NKG2D receptor. (Taken from Lanier, 2015)

In the context of diabetes, NKG2D has mostly been described in DM1 where it was shown that blocking of NKG2D prevented DM1 in NOD mice. In this research it was shown that RAE-1 ligands were present on pancreas islets and were destroyed by autoreactive $CD8^+$

T cells (Ogasawara et al., 2004). One research dealt with the role NKG2D during the start of DIO, where they saw higher expression of RAE-1ε ligand in VAT of obese mice. However, despite increased ligand expression, experiments done in mice deficient for NKG2D showed no difference in either IR or VAT inflammation compared to C57BL/6 (B6) wild type mice (Chung et al., 2014).

In the context of liver fibrosis, NKG2D has been shown to both promote and inhibit formation of lesions. On one hand, it was found that the level of MICA / B expression on hepatocytes positively correlates with activity of the Death receptor 5 (TRAIL-DR5) system, hepatocyte apoptosis and the degree of fibrosis in NASH patients (Fasbender et al., 2016). On the other hand, it was found that early-activated HSCs express RAE-1 which promotes NK cell activity in eliminating HSC, dependent on NKG2D and TRAIL, thereby limiting fibrosis (Gao, 2010; Radaeva et al., 2006). The differences are likely the result of different models use.

In addition to NKG2D ligands, many other stress ligands have been identified that interact with receptors on various immune cells. Of particular interest are ligands of the butyrophilin superfamily. Members of the Butyrophilin (Btn) and Btn-like (Btnl) family are relatively newly discovered immune regulators. The BTN and BTNL are structurally related to the family of B7 co-stimulatory molecules and several of human and murine BTN and BTNL family members have been reported to dampen or increase αβ and γδ T cell responses. Moreover, in humans it was shown that BTN3A1 has a role in the activation of Vγ9Vδ2 T cells through presentation of phosphoantigens, implying that BTNL molecules are involved in phosphoantigen sensing and have the capacity to regulate T cell-mediated immune responses (Bas et al., 2011; Chapoval et al., 2013; Nguyen et al., 2006; Vavassori et al., 2013). Additionally, few papers showed connection between BTNL family and inflammatory disorders (Fitzgerald et al., 2013; Mitsunaga et al., 2013; Pathan et al., 2009). It was also demonstrated that Btnl1 has a role in regulation of intraepithelial lymphocyte – epithelial cell interactions in the murine small intestinal mucosa and that in the complex with Btnl 6 selectively promotes increase in numbers of intraepithelial Vγ7Vδ4 T cells in the absence of exogenous activation (Lebrero-Fernandez et al., 2016).

Since it has been shown that stress ligands can be induced in response to DIO, they might be important for early recruitment of cells and induction of inflammation in the context of NAFLD.

2.3.2 Immune sensing of metabolic stress

2.3.2.1 **NK cells**

NK cells are important sentinels of the body that can detect infected, transformed or otherwise 'stressed' cells. They have many different activating and inhibitory receptors that help them in detection of different signs of cytopathology while ensuring self-tolerance (Vivier E, 2011). As described previously in the text, our group recently discovered that NK cells also play a role in sensing of metabolic stress in adipose tissue (Wensveen, 2015). Importantly, depletion of NK cells in VAT delays M1 ATM polarisation and development of insulin resistance but does not prevent it altogether.

In addition to NK cells, there are several innate and innate-like immune cells present in VAT and liver that have the ability to detect cellular stress.

2.3.2.2 **γδ T cells**

γδ lymphocytes represent a group of T cells with characteristics of both innate and adaptive immunity. γδ lymphocytes express a specific, but limited, type of T cell receptor (containing γ and δ chains instead of the usual α and β chains) (Figure 8.). In addition, they express innate immune receptors, such as TLR and NKG2D. γδ T cells show a limited, but tissue specific repertoire based on Vγ and Vδ chain usage. While some γδ T cells go to the LNs, the majority migrates to peripheral tissues. Once activated, they produce various proinflammatory cytokines. In mice, Vγ1 cells predominantly produce IFNγ, Vγ4 and Vγ6 cells produce IL-17, IL-21, IL-22 and TNF while Vγ5 cells produce IFNγ, TNF or IL-22.

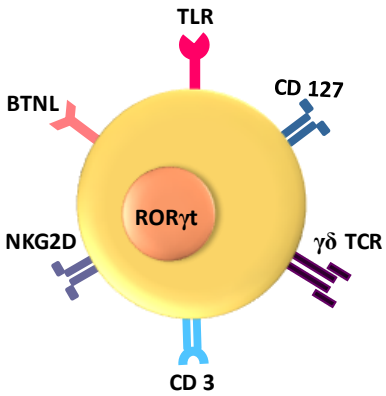


Figure 8. γδ17 T cell. Several surface receptors have been identified as being associated with IL-17-producing γδ T cells.

In the context of obesity and DM2 development, it has been shown that obese TCRδ$^{-/-}$ mice have significantly improved systemic insulin sensing and reduction in inflammation after 24 weeks of HFD feeding in comparison to wildtype (WT) animals (Mehta et al., 2015). However, since γδ T cells are considered to be the first line of defence, it would be important to see what happens with them at the start of DIO.

Importance of hepatic γδ T cells in the initiation of inflammation has also been recognized (Hammerich and Tacke, 2014). One study showed that liver γδ T cells can be activated through lipid antigens bound to CD1d and that this activation can induce the production of IL-17A (Li et al., 2017). Additionally, in a dietary model rich in carbohydrates and fats it was noticed that TCRδ$^{-/-}$ mice have increased body mass but significantly less inflammation when compared to the WT animals fed with the same diet. However, the possible mechanism of activation was not found (Hammerich and Tacke, 2014).

In summary, VAT and liver contain relatively large populations of innate and innate-like lymphocytes. Whereas their presence and sometimes role in development of inflammation has been acknowledged, their activating ligands remain elusive. Importantly, a primary way to identify immune cells that take part in the inflammation has been through measuring their numerical increase in response to DIO. However, tissue-resident immune cells that mediate early stress responses generally do not greatly expand, but rather recruit other immune cells through excretion of cytokines (Walker JA, 2013). Therefore, their role is easily overlooked when measuring cell numbers rather than effector molecules.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratoratory mice

All animal experiments were undertaken in full compliance with Croatian Animal Ethical regulations ("Ordinance on the protection of animals used for scientific purposes", NN 55/2013, which is in accordance with Directive 2010/63/EU of the European Parliament and of The European Council and "Law on animal protection" (NN 135/06, 37/13 and 102/17)) and under a project license to professor Bojan Polić.

The experimental mice used in this study were bred in conditions without the presence of specific pathogens (SPF) in the Laboratory of Mouse Engineering and Breeding Facility of the Faculty of Medicine, University of Rijeka (LAMRI). During the experiments, the mice were kept in the Experimental facility in individually ventilated cageing system (IVC system). All mouse strains used in this study were obtained by genetic modification of C57BL/6J mice. Experiments included 8-12 week-old (as indicated) mice and were age and gender matches within experiments.

The list of mouse strains used is shown in Table 1. Wildtype B6 mice were obtained from the Jackson Laboratory. B6 Klrk1$^{-/-}$, B6 Klrk1 flox (Lenartic et al., 2017; Zafirova et al., 2009) lines were generated and maintained at the Department of Histology and Embryology resource facilities. B6 LysM Cre (004781) were obtained from the Jackson Laboratory. B6 TCRdelta (TCR$\delta^{-/-}$) (002119) mice were kindly provided by professor Adrian Hayday (Itohara et al., 1993). B6 NCRCre-tg mice were kindly provided by professor Veronika Sexl (Vienna, Austria) (Eckelhart et al., 2011). B6 IL-17RA flox mice were kindly provided by professor Ari Waisman (Kumar et al., 2016). B6 Deleter Cre mouse was obtained from professor Rajewsky (Schwenk et al., 1995).

Table 1. Mouse strains used in experiments

1	C57BL/6J	7	LysM ^{Cre}
2	TCR$\delta^{-/-}$	8	Del^{Cre}
3	Klrk1$^{-/-}$ (NKG2D $^{-/-}$)	9	IL-17RA$^{\Delta/\Delta}$
4	Klrk1$^{flox/flox}$	10	LysM $^{Cre/+}$IL17RA$^{flox/flox}$
5	NCR1^{Cre}	11	NCR1$^{Cre/+}$ Klrk1$^{flox/flox}$
6	IL-17RA$^{flox/flox}$		

3.1.2 Buffers and cell culture mediums

Dulbecco's modified eagle medium- DMEM

DMEM medium (Pan Biotech, GmbH, Aidenbach, Germany), 3-10 % Fetal Calf Serum (FCS) (Pan Biotech), 10 mM HEPES (pH 7.2) (Pan Biotech), 2 mM L-glutamin, 10^5 U / l Penicillin (Pan Biotech), 0.1 g / l Streptomycin (Pan Biotech)

Roswell park memorial institute medium - RPMI

RPMI medium (Pan Biotech), 3-10 % FCS (Pan Biotech), 10 mM HEPES (pH 7.2) (Pan Biotech), 2 mM L-glutamin (Pan Biotech), 10^5 U / l Penicillin (Pan Biotech), 0.1 g / l Streptomycin (Pan Biotech), β2- mercaptoethanol (Sigma-Aldrich Corporation, St. Louis, Missouri, USA)

Cell culture freezing medium

70 % RPMI medium (Pan Biotech), 20 % FCS (Pan Biotech), 10 % dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corporation)

Flow cytometry medium (FACS medium)

PBS, 1 % Bovine serum albumin (BSA) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.1 % sodium azide (NaN_3) (Sigma-Aldrich Corporation), 1 mM Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich Corporation)

Red blood cell lysis buffer (10 x)

140 mM ammonium chloride (NH_4Cl) (Sigma-Aldrich Corporation), 2.7 mM potassium chloride (KCl) (Sigma-Aldrich Corporation), 1.5 mM monopotassium phosphate (KH_2PO_4) (Sigma-Aldrich Corporation), 6.5 mM disodium phosphate (Na_2HPO_4) (Sigma-Aldrich Corporation), 0.7 mM Calcium chloride ($CaCl_2$) (Sigma-Aldrich Corporation). For working solution (1X) dilute buffer with H_2O.

10 x PCR reaction buffer

200 mM Tris / HCl (pH 8.4) (Promega, Madison, Wisconsin, USA), 500 mM KCl (Sigma-Aldrich Corporation)

Percoll stock solution

54 ml Percoll (GE Healthcare, Little Chalfont, UK), 6 ml 10X phosphate buffered saline (PBS) (Lonza Group, Basel, Switzerland)

Percoll 80%

40 ml Percoll stock solution, 10 ml 1 x PBS (Lonza Group,)

Percoll 40%

20 ml Percoll stock solution, 30 ml 1 x PBS (Lonza Group)

Fluorescence-activated cell sorting (FACS) buffer

PBS (Lonza Group), 1 % FCS (Pan Biotech), 2 mM EDTA (Sigma-Aldrich Corporation).

Tail lysis buffer

100 mM Tris / HCl (pH 7.5) (Promega), 5 mM EDTA (Sigma-Aldrich Corporation), 0.2 % (w / v) SDS (Sigma-Aldrich Corporation), 200 mM NaCl (Sigma-Aldrich Corporation). At the time of use, add 100 µg / ml Proteinase K (Sigma-Aldrich Corporation) to 300 µL of tail buffer.

TE buffer (Tris-EDTA buffer)

Melt 294.5 g sodium acetate in 500 ml H_2O, 1 mM EDTA (Sigma-Aldrich Corporation), 10 mM Tris/HCl pH 8.0. Adjust pH with NaOH on 5.5 and add H_2O up to 1 L.

TAE buffer (Tris-acetat-EDTA pufer, 50x)

57.1 ml glacial acetic acid (Sigma-Aldrich Corporation), 242 g Tris base (Sigma-Aldrich Corporation), 100 ml 0.5 M EDTA (pH 8.0) (Sigma-Aldrich Corporation), fill with qH_2O up to 100 ml.

Sodium Citrate Buffer

10 mM sodium citrate (Sigma-Aldrich Corporation), 0.05% Tween 20 (Sigma-Aldrich Corporation), pH 6.0

1 % BSA - TBS Buffer

Tris-buffered saline (TBS) (Sigma-Aldrich Corporation) with 1 % BSA (Thermo Fisher Scientific) for blocking

3.1.3 Antibodies

Flow cytometry was performed using the following antibodies, coupled to the indicated fluorochromes. Fluorophore-conjugated antibodies for mouse: CD3 FITC (OKT3); CD3 PerCP-Cyanine5.5 (SK7); TCR gamma/delta (eBioGL3 (GL-3)) Biotin; TCR gamma/delta (eBioGL3 (GL-3)) APC; TCRβ PE (H57-597); TCRβ Biotin (H57-597); CD4 FITC (SK3); CD4 PerCP-Cyanine5.5 (RPA-T4); CD4 PE-Cyanine7 (GK1.5); CD8b FITC (53-6.7); CD8b PE (53-6.7); CD8b APC (53-6.7); CD8b Alexa Fluor 780 (53-6.7); CD8b PE-Cyanine5.5 (53-6.7); CD8b PE-Cyanine7 (53-6.7); CD19 APC-eFluor 780 (HIB19); CD19 PerCP-Cyanine5.5 (SJ25C1); FOXP3 FITC (PCH101), CD86 FITC (GL1); IL-17A FITC (eBio17B7), IL-17A PE-Cyanine7 (eBio17B7); IL-17F Alexa Fluor 488 (eBio18F10); IFNG FITC (XMG1.2); IFNG PE (XMG1.2); IFNG APC (XMG1.2); TNFA PE-Cyanine7 (MP6-XT22); TNFA Alexa Fluor 488 (MP6-XT22); iNOS PE (CXNFT); LAP (Latency Associated peptide) PE-Cyanine7 (FNLAP); IL-10 APC (JES5-16E3); IL-4 PE-Cyanine7 (11B11); CD49b (Integrin alpha 2) FITC (eBioY418 (Y418)); CD27 PE (LG.7F9); CD27 PE-Cyanine7 (O323); KLRG1 APC (2F1); CD11b PE-Cyanine7 (M1/70); NK1.1 APC-eFluor 780 (PK136); CD68 PE (FA-11); CD11c APC (N418); Ly-6G PE (RB6-8C5); Ly-6G Alexa Fluor 780 (RB6-8C5); Ly-6G Biotin (RB6-8C5); Ly-6G PE-Cyanine7 (RB6-8C5); Ly-6C PerCP-Cyanine5.5 (HK1.4); F4/80 PE (BM8); F4/80 APC-eFluor 780 (BM8); CD206 FITC (MR5D3); CD314 (NKG2D) PE-Cyanine7 (1D11); CD335 (NKp46) FITC (29A1.4); CD335 (NKp46) APC (9E2); CD196 (CCR6) APC (R6H1); ROR gamma (t) PE (B2D); T-bet PE-Cyanine7 (eBio4B10 (4B10)); EOMES FITC (WD1928); TCR Vδ4 FITC (GL-2); TCR Vδ4 PE (GL-2); TCRVγ7; TCRVγ1 FITC (2.11); TCRVγ1 APC (2.11); TCRVγ2 APC; TCRVγ3 PE; TCRVγ4 APC (UC3-10A6); CD122 FITC (TM-b1); CD44 APC-eFluor 780 (IM7), BrdU Staining Kit for Flow Cytometry FITC; CD25 PE (CD25-3G10); CD69 PE (H1.2F3); CD62L (L-Selectin) PE-Cyanine7 (MEL-14); CD127 APC-eFluor 780 (A7R34); CD127 PE (eBioRDR5); CD45R (B220) APC-eFluor 780 (RA3-6B2); CD45.2 PerCP-Cyanine5.5 (104); Mouse IgM PE (II/41); Ki-67 PE (SolA15); Streptavidin, (R-PE); Streptavidin APC-eFluor™ 780; Fixable Viability Dye eFluor™ 780; Brefeldin A Solution (1000X); MO/RT IL-17A Biotin (EBIO17B7); anti-MO IL17A purified (EBIO17CK15A5); Smooth Muscle Actin (αSMA) Purified (1A4 (asm-1)).

Commercial antibodies were purchased from eBioscience™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) apart from CD206 FITC which was bought from Invitrogen™ (Thermo Fisher Scientific) and BrdU kit which was obtained from Becton Dickinson (BD, Franklin Lakes, New Jersey, USA). All cell surface and intracellular stains were performed in

96-well U-bottom plates. For intracellular staining, the cells were first fixed with Cytofix / Cytoperm buffer (BD) and then resuspended in permeabilization buffer Perm / Wash (BD).

3.1.4 Stimulating reagents

PMA and ionomycin were purchased from Sigma-Aldrich Corporation and used as stimulating reagents for immune cells. They were diluted with PBS and stocks were stored at −20 °C.

3.2 Methods

3.2.1 Laboratory mice genotyping

3.2.1.1 **DNA isolation from murine tail**

Freshly cut or frozen biopsy of ~ 5 mm murine tale was placed in 1.5 ml Eppendorf tubes and incubated overnight in a thermoblock at 56 °C with 0,5 ml of Tail lysis buffer. Next, the tubes were centrifuged in a small table centrifuge (5 min / 12000 rpm) to pellet cellular debris. The supernatant was transferred into a clean, labeled tube. DNA was precipitated by addition of 0.5 ml of isopropanol and mixed by slight rotation of the tube. The samples were then centrifuged (5 min / 12000 rpm) and supernatant was removed. The precipitate was incubated 1 hour with 0.8 ml of 70 % EtOH at + 4 ºC to dissolve the residues of precipitated salts. Samples were then centrifuged (5 min / 12000 rpm), dried and DNA was dissolved in 200 µL TE buffer and incubated for 15 min at 56 °C. Genomic DNA was stored at +4 ºC.

3.2.1.2 **Polymerase chain reaction (PCR)**

Polymerase Chain Reaction (PCR) was used to amplify a particular part of the DNA molecule *in vitro.* PCR reaction mix had 5 µl of 10 x reaction buffer, 3 µl $MgCl_2$ (50 mM) (NEB, Ipswich, Massachusetts, USA), 1 µl dNTP (5 mM) (Metabion, Munich, Germany), 2 µl forward primer (Metabion), 2 µl reverse primer (Metabion), 0.5 µl Taq polimerase (0.025 U / µl) (NEB) and 36.5 µl qH2O, making a total volume of 50 µL. In each Eppendorf tube with PCR reaction mix, 1 µl of template DNA was added. PCR reaction was conducted in PCR thermoblock (Bio-Rad, Hercules, California, USA). This technique was used to check the genotype of the animals used in the experiments and based on the genotype, different sets of primers were used (Table 2).

Table 2. List of primers used for PCR

Klrk1$^{-/-}$	NKGFPS.a2 (a2S): 5'-CAT AAA GTC CGC TTT GAT GTT A-3' NKGFPS.b2 (b2S): 5'-GCA GAT TCC CAA AAT TCT TG-3' NKGFPS.c2 (c2M): 5'-CTG AAC TTG TGG CCG TTT-3'
IL17RA$^{flox/flox}$ *IL17RA*$^{\Delta/\Delta}$	IL17RAF (For1): 5'-GGC AGC CTT TGG GAT CCC AAC-3' IL17RAR (Rev1): 5'-CTA CTC TTC TCA CCA GCG CGC-3' 5`for 6 (Del1): 5'-GTG CCC ACA GAG TGT CTT CTG T-3'
Klrk1$^{flox/flox}$	NKFS.a2: 5'-ACA AGG CCC ACA TTT TCT AG-3' NKFA.b2: 5'-GGC CTA TTA TTT CAG CAA CA-3'
NCR1^{Cre}	NCRCre F: 5'-ATG CGG TGG GCT CTA TGG CTT CTG-3' NCRCre R: 5'- GAC CAT GAT GCT GGG TTT GGC CCA GAT G-3'
Del^{Cre}	SC-1: 5'-GTC CAA TTT ACT GAC CGT ACA-3' SC-3: 5'-CTG TCA CTT GGT CGT GGC AGC-3'
TCR$\delta^{-/-}$	BWF: 5'-TGT CTG AAG GGC AAT GAC TG-3' BWR: 5'-GCT GAT CCG TGG CAT CTA TT-3' DWF: 5'-CCT GAC CTG TGA CTA GCT CC-3' DELTA WT/NEO R: 5'-GAA AGA GGG AGC GGT GGT AT-3'
LysM^{Cre}	Lyz-Cre Mut: 5`-CCC AGA AAT GCC AGA TTA CG-3' Lyz-Cre Wt: 5`-TTA CAG TCG GCC AGG CTG AC-3' Lyz-Cre Com: 5`-CTT GGG CTG CCA GAA TTT CTC-3'

3.2.1.3 **Agarose gel electrophoresis of PCR products**

Horizontal agarose gel electrophoresis was used to determine the size of the DNA fragments. The 1.5 % agarose gel was made by dissolving the agarose in a boiling TAE buffer. 1 μL of Ethidium-bromide (EtBr) (Sigma-Aldrich Corporation) per 60 ml of cooled (~ 45 °C) gel was added and poured into a gel mould. In the still liquid gel, combs were immersed to enable the formation of the wells after gel polymerization. Molecular-weight size marker was put to the first well to determine the size of the individual DNA fragments. DNA bands were visualised with Alliance Imaging System from UVITEC Limited, Cambridge, UK.

3.2.2 Dietary models

Dietary models were used to induce insulin resistance and NAFLD: 8 - 12 week old male C57BL/6 and genetically modified mice were fed with a HFD as a model for type 2 diabetes and with SSD to induce NAFLD.

3.2.1.1 **High fat diet (HFD)**

In HFD (Bregi, Rijeka, Croatia) 50 - 60 % of calories is derived from animal fat (40 – 45 % fat content). Feeding typically resulted in glucose intolerance and insulin resistance within 10-12 weeks. Mice were fed with high fat diet 2 - 4 weeks. Control animals were fed with NCD containing 5 – 6 % fat (SSNIFF, Spezialdiaten, GmbH, Soest, Germany).

3.2.1.2 **Steatosis-Steatohepatitis diet (SSD)**

To induce NAFLD, a modified Amylin Liver Nash Model (AMLN) (Clapper et al., 2013) dietary model was used, termed the steatosis-steatohepatitis diet. SSD contains 22 % of fructose, 2 % of cholesterol and 40 % of fat (Clapper et al., 2013). This diet induced NASH within 14 - 18 weeks. Control animals were fed with a normal chow diet (NCD) containing 5 – 6 % fat (SSNIFF).

3.2.3 Immune cell isolation protocols

3.2.3.1 **Isolation of visceral adipose tissue lymphocytes**

Visceral adipose tissue lymphocytes were isolated from the two lobes of visceral adipose tissue as described (Valentić et al., 2015). In brief, adipose tissue was cut into small parts with scissors and placed in a 50 ml tube, containing 5 ml of freshly prepared 3% RPMI with 1 mg/ml Collagenase D (F. Hoffmann-La Roche, Basel, Switzerland). Tissue was incubated in a thermostatic shaker for 1 h at 37 °C, shaking at 270 rpm. Afterwards, the tube was vortexed and 5 ml of fresh, cold 3% RPMI was added. The tube was centrifuged at 1500 rpm for 5 min and remaining supernatant was removed by inverting the tube. The pellet was resuspended in 1 ml of hypotonic solution (Erylysis buffer), vortexed and the suspension was run over a 70 µm cell strainer placed on a 2 ml Eppendorf tube containing 500 µl of cold 3 % RPMI. The tube was centrifuged in a table centrifuge at 4000 rpm for 5 min at room temperature. Next, the supernatant was removed and the pellet was resuspended in 250 µl 3% RPMI.

3.2.3.2 **Isolation of hepatic lymphocytes**

Perfusion

Animals were euthanatized by CO_2 / O_2 intoxication, followed by CO_2 suffocation. The left ventricle and the right atrium of the heart were identified. The heart was held upright and using a blunt tipped scissors, the right atrium was cut. A perfusion needle was inserted perpendicularly through the apex of the left ventricule. The perfusion rate was slow and steady and 8-10 ml of PBS was used per mouse.

Isolation

After perfusion, the entire liver, except the gall bladder, was isolated. The liver was homogenized through a 70 μm cell strainer that was placed on a 50 ml tube. 5 – 10 ml of 3 % RPMI was used for each liver to get a single-cell suspension. The cell suspension was centrifuged at 1700 rpm. Supernatant was discarded and the pellet was resuspended in a 40 % Percoll solution. The resuspended pellet was loaded on a layer of 80 % Percoll solution and then centrifuged at 2100 rpm for 30 min at room temperature. The ring of leukocytes was harvested from the Percoll and washed with 8 ml of 3 % RPMI. The tube was centrifuged at 1700 rpm for 5 min. The pellet was resuspended in 1ml of Erylysis buffer and transferred to a 2 ml Eppendorf tube. After 3 minutes, 500 μl of cold 3 % RPMI was added. The tube was centrifuged in a table centrifuge at 4000 rpm for 5 min at room temperature. Next, the supernatant was removed and the pellet was resuspended in 350 μl 3% RPMI.

3.2.3.3 **Isolation of splenic lymphocytes**

Spleen was harvested and homogenized through a 100 μm cell strainer using 3 % DMEM. The homogenate was then centrifuged at 1500 rpm for 4 min. The precipitate was resuspended in 3 ml of Erylysis buffer. After incubation for 5 min on ice, 7 ml of 3 % DMEM was added. The suspension was centrifuged for 5 min 1500 rpm, after which the supernatant was discarded and the pellet resuspended in 5 ml of 3 % RPMI.

3.2.4 Cell counting

The number of cells per milliliter of the isolation suspension was determined by counting in Neubauer's chamber. Only live cells were counted with the use of trypan blue, vital stain that selectively colours dead cells blue. 25 μl of cell suspension was mixed with 200 μl trypan blue (1: 9) and applied to the chamber. The total number of cells per milliliter of the

suspension was obtained by multiplying the mean number of cells per square (1 x 1 mm) with a correction factor of 90 000 to correct for chamber volume (0.1 x 1 x 1 mm) and suspension dilution factor (1 : 9).

3.2.5 Flow cytometry

3.2.5.1 **Cell surface stain**

Phenotypic lymphocyte analysis was performed by fluorophore-labeled antibodies that are specific to certain cellular markers. For flow cytometry, the single cell suspension of liver, VAT and spleen was prepared according to the protocols described above. For the analysis of surface markers, labeled antibodies were diluted in FACS medium (40 µl) containing specific antibodies, as well as 2.4G2 antibody that blocks FcRII / III (CD16/32) to prevent nonspecific binding of labeled antibodies. After 30 min of incubation at + 4 °C, the cells were washed with FACS medium, centrifuged for 4 min at 300 g and resuspended in 120 µl of FACS medium.

Samples were analysed on the same day as staining. The results of phenotypic cell assays were obtained by flow cytometry FACSVerse (BD) and analysed using FlowJo (FlowJo LLC, Ashland, Oregon, USA).

3.2.5.2 **Intracellular cell stain**

For intracellular staining, kit for fixation and permeabilization of cells was used according to the manufacturer's instructions (BD Biosciences, San Jose, USA). After cell surface staining, the cells were first fixed for 20 min at room temperature with Cytofix / Cytoperm (BD) buffer and then washed in permeabilization buffer Perm / Wash (BD). After centrifugation, cells were incubated in 40 µl of antibody mix for 30 minutes at +4 °C. Cells were then washed, centrifuged and resuspended in FACS buffer.

Samples were either analysed on the same day as staining, or stored overnight at +4 °C and analysed the following day. The results of phenotypic cell assays were obtained by flow cytometry FACSVerse (BD) and analyzed using FlowJo (FlowJo LLC).

3.2.5.3 **Intracellular staining of Ki67 and transcription factors**

Ki-67 and transcription factors (TF) staining was performed after cell surface staining using the Fixation / Permeabilisation kit for intranuclear staining corespondingly with the manufacturer’s instructions (eBioscience). Briefly, cells were stained for surface markers, washed, centrifuged and re-suspended in Fixation / Permebilization solution at RT. After 20

minutes, cells were washed in permeabilization buffer and resuspended in 40 µl of Ki67 or TF antibody mix (1 : 100 in 1 x permeabilization buffer). Cells were incubated for 30 minutes at +4 °C, washed in buffer, centrifuged and then re-suspended in 120 µl of FACS medium.

The results of phenotypic cell assays were obtained by flow cytometry FACSVerse (BD) and analysed using FlowJo (FlowJo LLC).

3.2.5.4 **Viability dye**

Viability dye was used at a dilution factor of 1:1000 in FACS medium together with cell surface staining antibodies. Cells were stained for 30 minutes at +4 °C. eBioscience™ Fixable Viability Dye eFluor™ 780 was used for all stainings.

3.2.6 Stimulation assays

3.2.6.1 **PMA / ionomycin stimulation**

A total of 100 µl (10^6) of cells in 10 % RPMI were added to a 96-well U-bottom plate containing 50 µl of PMA (1:25 000) / ionomycin (1:500) and 50 µl of Brefeldin A (1:250). Cells were maintained in incubator at 37 ºC, 5 % CO_2 for 4 hours. After incubation, the cells were centrifuged and stained for cell surface and intracellular markers as described above and then subjected to flow cytometric analysis.

3.2.7 Quantitative RT–PCR

3.2.7.1 **RNA isolation**

A total of 100 µl (10^6 cells) of liver homogenate was added to 175 µl of RNA Lysis Buffer (SV Total RNA Isolation System, Promega) to isolate RNA. The cells were lysed by mixing well, vortexing and pipetting. 350 µl of RNA Dilution Buffer was added, mixed by inverting and placed into heat block at 70 °C for 3 minutes. Samples were centrifuged at 12,000 × g for 10 minutes at RT. Cleared lysate was transferred into new tube and 200 µl of 95 % ethanol was added. This mixture was transfer to the Spin Column Assembly and centrifuged at 12 000 × g for 1 minute at RT. Flow-through was discarded and 600 µl of Wash Solution was added. Samples were centrifuged again at 12 000 × g for 2 minutes at RT. Flow-through was discarded.

Volume of 50 µl of DNase incubation mix was added and incubated for 15 minutes at 20 – 25 °C. After incubation, 200 µl of DNase Stop Solution was added, samples were centrifuged and 600 µL of Wash Solution was added. Samples were centrifuged again at 12 000

× g for 1 minute at RT. Flow-through was discarded and 250 µl of Wash Solution was added. Samples were centrifuged at high speed for 2 minutes. Spin Basket was transferred from the Collection Tube to the Elution Tube. 100 µl Nuclease-Free Water was added to the membrane, centrifuged at 14 000 × g for 1 minute and the Elution Tube containing the purified RNA was stored at –80 °C.

3.2.7.2 **cDNA synthesis**

cDNA was synthesized using Reverse transcriptase Core kit 500 (Eurogentec, Seraing, Belgium). In brief, 200 ng or RNA was reversly transcribed to cDNA using random nonamers, according to manufacturer instructions.

3.2.7.3 **qPCR**

qPCR was performed on a 7500 Fast qPCR machine (Applied Biosystem, Thermo Fisher Scientific, Waltham, USA) by using SYBR™ Green dye (MESA green, Eurogentec).

To determine the expression of *H60, MULT-1, Rae1*ε and *Rae1*δ qPCR SYBR™ Green dye (MESA green, Eurogentec, Seraing, Belgium) was used according to the manufacturer's instructions. SYBR Green detects PCR product as they accumulate during PCR. Per sample, mastermix had 10 µl 2 x SYBR™ Green dye buffer, 2 µl of forward and 2 µl of reverse cDNA primers (*H60, MULT-1, Rae1*ε*, Rae1*δ and *Hprt*), 2 µl qH2O and 4 µl of 5 x diluted cDNA template, making a total volume of 20 ul. Relative expression of mRNA was normalized by determining the expression of *Hprt* gene.

Table 3. List of primers used for PCR

RAE-1δ	For	5′-CAACTTGACCATCAAGGCTCCTA-3′
	Rev	5′-GATAAGTATTTCACCCACGAAGCA-3′
Rae-1ε	For	5′-CAGGTGACCCAGGGAAGATG-3′
	Rev	5′-CTCAACTCCTGGCACAAATCG-3′
H60	For	5′-GAGCCACCAGCAAGAGCAA-3′
	Rev	5′-CCAGTATGGTCCCCAGATAGCT-3′
Mult1	For	5′-CTCATAGGAACAGCATGA-3′
	Rev	5′-TCCTGTGAAATGTTTGTC-3′
Hprt	For	5'-CACAGGACTAGAACACCTGC-3'
	Rev	5'-GCTGGTGAAAAGGACCTCT-3'

3.2.8 Histology

3.2.8.1 **Tissue fixation and paraffin embedding**

Liver was perfused with cold PBS and harvested. Two small parts of different liver lobes were collected. Liver tissue was incubated in 4 % PFA fixative for a minimum of 48 hrs at room temperature. Samples were transferred into histokinette (TP1020, Leica, Wetzlar, Germany) with increasing series of alcohol (50 – 100 %) and three series of xylene, at room temperature. 2 μm thick sample sections were made using a microtome (Microtome HM340E, Microm, Thermo Fisher Scientific). For each sample, a minimum of 4 cuts were made. Sections were mounted on silanized slides. After overnight drying, they were either histochemicaly or immunohistochemicaly stained.

3.2.8.2 **Histochemical staining**

Hematoxylin and Eosin (H&E) Staining

Paraffin sections were de-waxed and hydrated in distilled water. Nuclei were stained with the alum haematoxylin for 5 minutes. Slides were rinsed in running tap water for 10 minutes and differentiate by quickly dipping in and out of 0.3 % acid alcohol. They were washed in running tap water for 5 minutes. Sections were stained with eosin for 1 - 2 minutes, washed once in tap water and once in distilled water for 15 seconds. Next, slides were dehydrated in three changes of 100 % ethanol, cleared in xylene and mounted in Entellan (Merck Millipore, Burlington, Massachusetts, USA).

Sirius Red Staining

Paraffin sections were de-waxed and hydrated. Nuclei were stained with Weigert's haematoxylin for 20 minutes, and slides were washed for 10 minutes in running tap water. Slides were stained in picro-sirius red for one hour and afterwards washed in two changes of acidified water. Water was removed from the slides by vigorous shaking. Next, slides were dehydrated in three changes of 100 % ethanol, cleared in xylene and mounted in Entellan (Merck Millipore).

3.2.8.3 **Immunohistochemical staining**

Antigen retrieval

Sections were de-paraffinised and hydrated in distilled water. Slides were placed in Sodium Citrate Buffer (pH 6.0) and heated in a microwave oven for 3 min on 700 W and 15 min at 150 W. After boiling, the slides were allowed to cool for 20 minutes in solution. Sections were rinsed 3 times for 2 minutes with demi-water and 3 times for 5 minutes with PBS.

Immunohistochemistry protocol

After washing in PBS endogenous peroxidase activity was blocked using 0.3 % hydrogen peroxide in PBS. Slides were incubated for 10 minutes in the dark. Next, slides were washed 3 x 5 min in PBS. Prior to incubation with primary antibody goat - anti - mouse (aSMA), slides were incubated for 1 h with goat serum or 3 % BSA. Slides were left with primary antibody diluted in 1 % BSA - TBS, at 4 °C. overnight. Next, slides were washed 3 x 5 min with PBS

Secondary antibody, rat–anti–mouse-POD was diluted in 1 % BSA - TBS, was incubated for 45 min at + 4 °C. Sections were washed 3 x 5 min in PBS and staining was visualised with Dako liquid DAB+ substrate chromogen (Agilent Technologies, Santa Clara, California, USA). Slides were counterstained with haematoxylin for 5 minutes and washed in tap water for 10 min. Afterwards they were dehydrated, cleared and mounted in Entellan (Merck Millipore).

3.2.9 Excision of visceral adipose tissue from live mice (VATectomy)

Surgical removal of the perigonadal fat pads (VATectomy) has been previously described (Šestan et al., 2015). In brief, mice were injected intraperitoneally with sedative, 30 min before the start of the surgical procedure. They were placed in the anesthetic chamber, and after they were anesthetized, hair from the abdomen was removed. Mice were placed with its back on the operation table and inhalation anesthesia was applied. Skin was disinfected and incision in the skin was made with scalpel. Next, a small cut (0.5 cm) into the muscle and peritoneum was made. Two perigonadal adipose tissues were pulled out one by one with tweezers and an incision was made at the basis of adipose tissue to completely remove the tissue. All three layers (peritoneum, muscle and skin) were then sewed together. Ten days after surgery, sewing material was removed and fourteen days after surgery, experiments were started.

3.2.10 Image J analysis

Steatosis and fibrosis analysis on slide sections was carried out using ImageJ (NIH, Bethesda, Maryland, USA). Calculator Plus was used to correct the background. Treshold adjustments were made to recognize and determine areas of interest. Analysis tools were used to quantify red fibrotic or white steatotis areas.

3.2.11 Data analysis and statistics

Data analysis was done using the GraphPad Prism 5 program (GraphPad Software, Inc, San Diego, California). The choice of the appropriate test was dependent on the number and distribution of the examinees. Mann-Whitney test or Student's t-test was used when two groups of animals were compared. When comparing more than two groups, ANOVA test with Bonferroni correction was used.

4. RESULTS

4.1 Role of γδ T cells in VAT inflammation

4.1.1 Characterization of γδ T cells in VAT

In response to stress factors, γδ T cells are one of the first cellular populations to secrete proinflammatory cytokines in order to recruit and activate other immune cell populations against an imminent threat (Sutton et al., 2012). γδ T cells may therefore play an important role in obesity-induced inflammation. Therefore, I characterized γδ T cells in VAT under homeostatic conditions. In general, γδ T cells are highly prevalent in epithelial and mucosal tissues. However, the quantification of VAT leukocytes showed that γδ T cells are also present in relatively high numbers in VAT, comprising ~ 6 % of total immune cells (Figure 9a). In comparison to splenic γδ T cells, most of VAT γδ T cells were $CD27^-$ and expressed the markers CD44 and CD69. Compared with splenic γδ T cells, VAT γδ T cells had lower expression of CCR6 (Figure 9a).

There are several different γδ subsets characterized by expression of specific Vγ and Vδ gene segments. As mentioned in the introduction, these subsets are functionally distinct and mainly tissue specific. For this reason, we decided to identify the subsets of γδ T cells present in VAT with commercially available antibodies. With my analysis I identified Vγ1, -4, -5, -6 and -7 (Tonegawa`s nomenclature (Heilig and Tonegawa, 1986) (Figure 9b). However, the cumulative frequency of these subsets remained below 30 %, suggesting that an as of yet unidentified γδ T cell subset dominates the pool in VAT. Expression of Ki67 was higher in VAT than in splenic γδ T cells (Figure 9c), suggesting a higher turn-over rate in this tissue. Next, I analysed the ability of VAT γδ T cells to produce cytokines. *In vitro* stimulation for 4 hours with phorbol 12-myristate-13-acetate (PMA) and ionomycin showed that VAT γδ T cells had a lower capacity to produce IFNγ and TNF, but increased capacity to produce IL-17A in comparison to splenic γδ T cells (Figure 9d).

In summary, γδ T cells are present at relatively high percentages in VAT. The activation status of γδ T cells in VAT showed that majority of cells are $CD69^{hi}$. Approximately 75 % of cells are $CD27^-$ suggesting that they are poised to production of IL-17A. This was further confirmed through *in vitro* stimulation assay where indeed the main cytokine produced by γδ T cells was IL-17A.

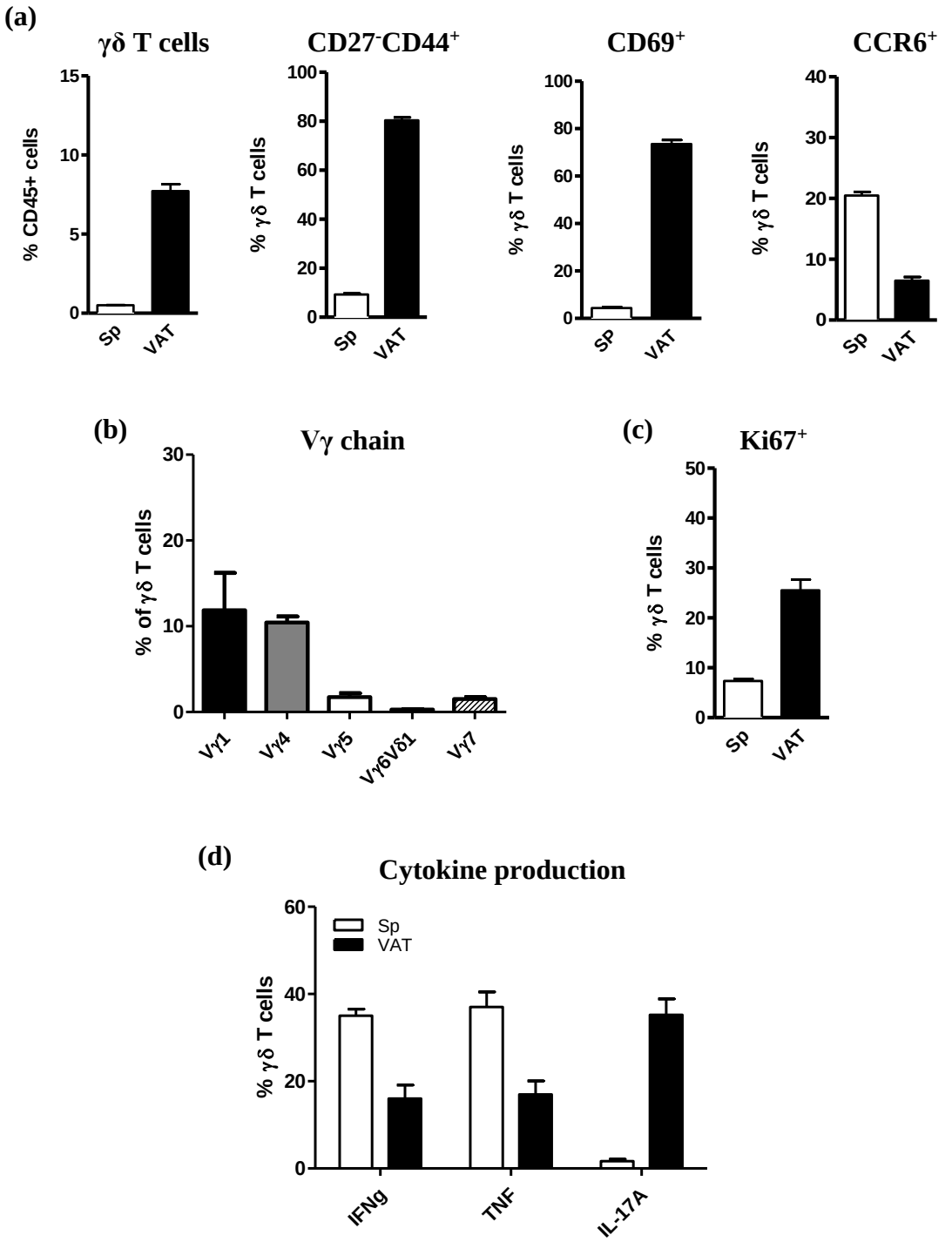


Figure 9. γδ T cell phenotype in VAT (a) Splenic and VAT-resident γδ T cells were isolated, and the percentage of cells expressing the indicated markers was determined by flow cytometry. (b) Flow cytometry analysis of γδ T cells subsets based on Vγ chain was made. (c) Ki67 expression was determined in splenic and VAT-resident γδ T cells by flow cytometry (d) Splenic and VAT derived γδ T cells were stimulated with PMA and ionomycin and the expression of IFNγ, TNF and IL-17A was determined by flow cytometry. Cells were gated on $CD3\varepsilon^{+}TCR\delta^{+}$ population (n = 5).

4.1.2 High-fat diet increases production of IL-17A in VAT

Emerging evidence has demonstrated the importance of innate immune cells in sensing metabolic stress (Donath and Shoelson, 2011; O'Sullivan et al., 2016; Wensveen et al., 2015). However, there are still some links missing to fully explain early events leading to obesity-induced inflammation. It is well known that the levels of proinflammatory cytokines are increased in obesity and that they contribute to insulin resistance and low-grade systemic inflammation. IL-17A can regulate adipogenesis and glucose metabolism in VAT (Zuniga et al., 2010). Because I found that VAT γδ T cells produce IL-17A, I wanted to address whether γδ T cells contribute to VAT inflammation during initial stages of obesity.

I analyzed adipose tissue γδ T cells after three weeks of HFD feeding. Results showed that even though there was no difference in the percentage, there was an increase in absolute numbers of VAT γδ T cells in animals fed with an HFD (Figure 10a) in comparison to mice fed an NCD. This difference was not due to proliferation of resident cells as Ki67 showed no differences between NCD and HFD fed animals (Figure 10b). This suggests an increased influx of cells in obese VAT. Phenotypic analysis revealed that they remained $CD27^{-}CD44^{+}$ and there was no difference between the groups in the expression of CD69 and CCR6. However, γδ T cells in obese VAT acquired a more activated ($CD127^{+}$) phenotype upon HFD feeding (Figure 10c). In response to HFD there was also an increase in the Vγ1 subset of γδ T cells (Figure 10d), however, it was not statistically significant. Although there was no difference in the percentage of the cells (Figure 10e) the number of IL-17A producing cells was almost doubled in HFD fed animals (Figure 10f).

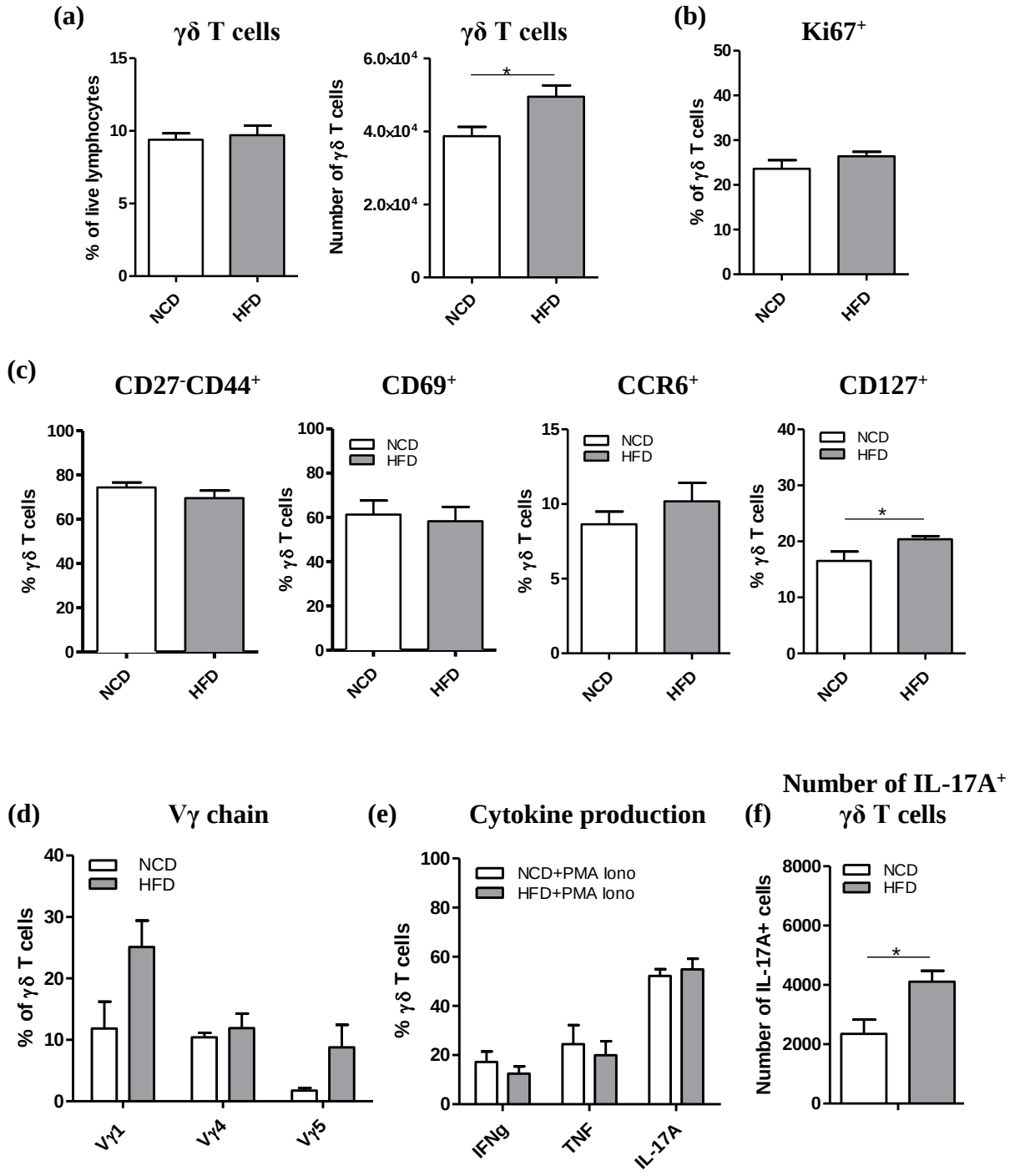


Figure 10. γδ T cells increase and obtain more activating phenotype during HFD feeding
(a) Percentage and number of VAT-resident γδ T cells and the percentage of cells expressing (b) Ki67, (c) indicated markers as well as (d) analysis of γδ T cells subsets based on Vγ chain determined by flow cytometry after 3 weeks of NCD or HFD feeding. (e) VAT resident γδ T cells were stimulated with PMA and ionomycin and the expression of IL-17A, IFNγ and TNF (f) as well as quantification of IL-17A$^+$ γδ T cells was determined by flow cytometry (n = 10) after 3 weeks of NCD or HFD feeding. Gated for CD3ε$^+$TCRδ$^+$. *P < 0.05 (Mann-Whitney u-test).

To determine the role of γδ T cells in VAT in response to HFD feeding, I used mice that are deficient in all γδ T cells (TCRδ$^{-/-}$ knock out mice) (Itohara et al., 1993). Eight to ten week old TCRδ$^{-/-}$ and WT male mice were fed an NCD or HFD and their weights were measured over a period of three weeks. There was no difference between the NCD groups, but in HFD fed groups of mice, TCRδ$^{-/-}$ knock out mice gained more weight (Figure 11a) and there was a significant increase in VAT fat pad weight (Figure 11b) in comparison to WT mice.

Although γδ –deficient mice had greater VAT mass than WT mice following HFD-feeding, they did not differ from WT mice in either the number of NK cells (Figure 11c) or M1 macrophages (Figure 11d) in visceral adipose tissue. Therefore, γδ T cell may reflect low-level inflammatory changes associated with adipocyte stress. It still needs to be elucidated if they orchestrate the progression of inflammation through activation of other cells.

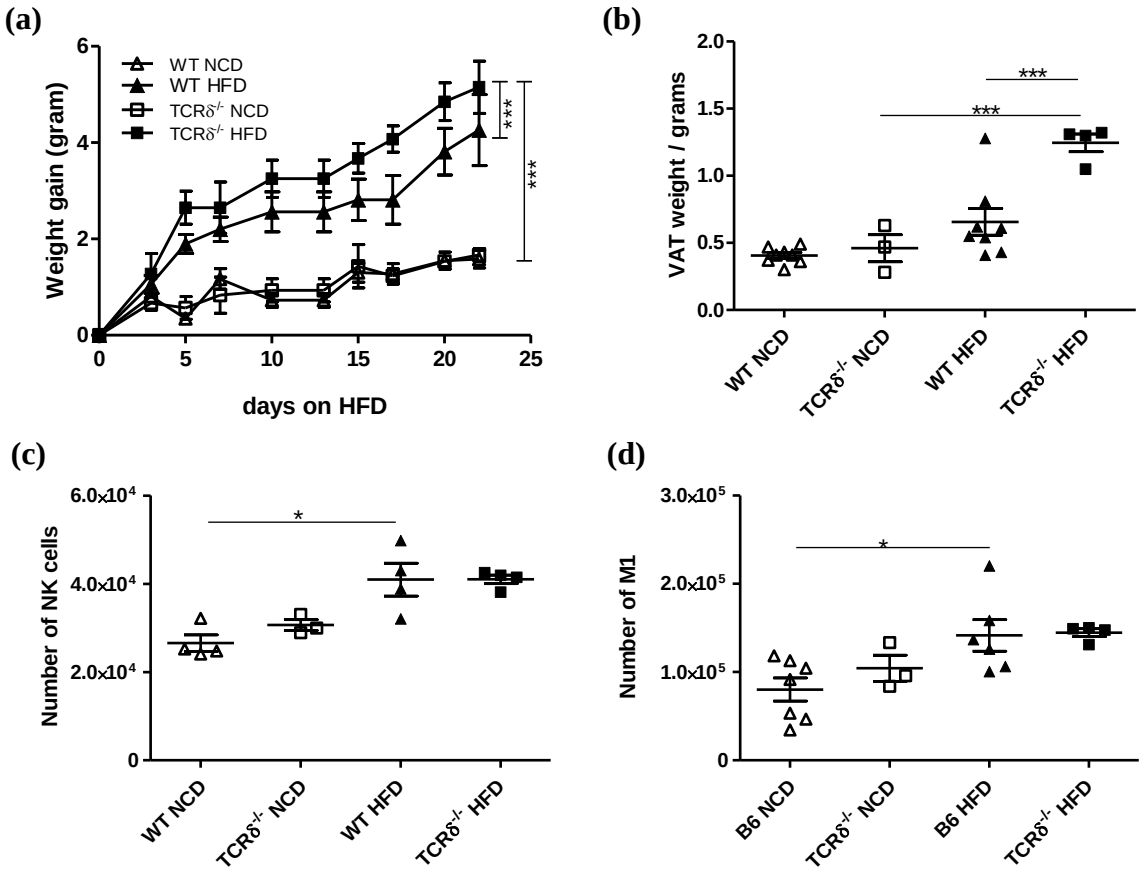
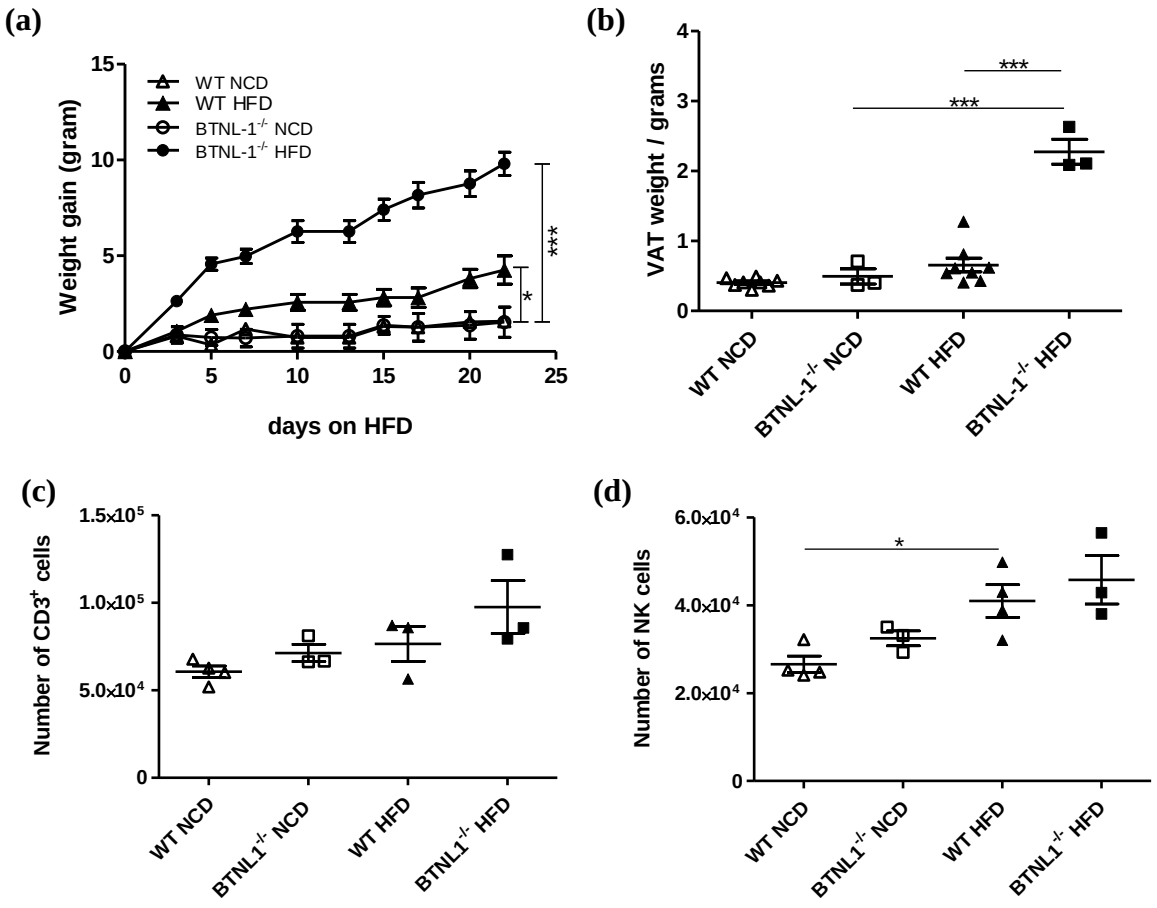


Figure 11. **Lack of γδ T cells increases weight gain but has no effect on number of NK cells and M1 macrophages** TCRδ$^{-/-}$ and WT mice were placed on HFD for 3 weeks. (a) Weight gain was measured over 3 weeks' time after an NCD or HFD feeding (n = 3-8). (b) Fat pad weight and (c) number of NK cells ($CD45^{+}NK1.1^{+}CD3\varepsilon^{-}$) and (d) M1 macrophages ($CD19^{-}CD11b^{+}F4/80^{+}GR1^{Dim/-}CD11c^{+}CD86^{Dim}$) was determined after 3 weeks of an NCD or HFD feeding *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

γδ T cells acquire the capacity to produce IL-17 independently of TCR signalling during thymic differentiation (Haas et al., 2012). Murine Butyrophilin-like (BTNL) molecules can promote maturation and expansion of tissue-specific γδ T cells. Btnl 1 molecule is primarily located in intestinal epithelium where it critically regulates the intraepithelial Vγ7 T cells, however not much is known about its presence and role in VAT. Therefore, I wanted to investigate if BTNL-1 has an effect on homeostasis or inflammation in VAT in the context of DIO. BTNL1-/- and WT male mice were fed an NCD or HFD and their weights were measured over a period of three weeks. Similar to TCR$\delta^{-/-}$ knock out mice, there was no difference between the NCD groups, but in HFD groups of mice, BTNL1$^{-/-}$ knock out mice gained much more weight (Figure 12a) and there was almost a four- fold increase their VAT fat pad weight (Figure 12b) in comparison to WT mice. Although BTNL1 –deficient mice accumulated a greater VAT mass than WT mice in response to HFD, they did not differ from WT mice in the number of either CD3^{+} cells (Figure 12c), NK cells (Figure 12d) or M1 macrophages (Figure 12e) in visceral adipose tissue. These findings could indicate that BTNL1 molecule in VAT might play a role in homeostasis of VAT rather than inflammation.



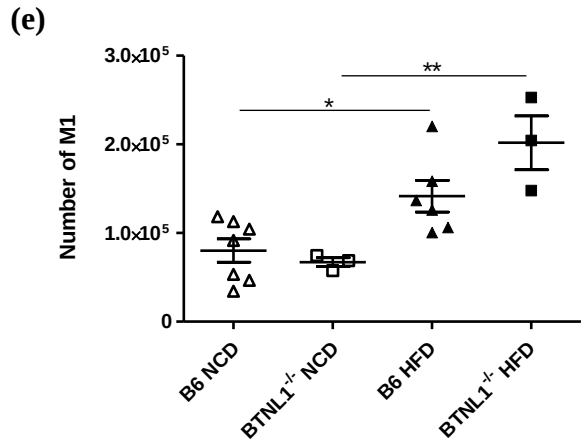


Figure 12. BTNL1$^{-/-}$ mice have increased weight gain BTNL1-/- and WT mice were placed on HFD for 3 weeks. (a) Weight gain was measured over 3 weeks' time after an NCD or HFD feeding (n = 3-8). (b) Fat pad weight and number of (c) CD3ε+, (d) NK cells ($CD45^+NK1.1^+CD3\varepsilon^-$) and (e) M1 macrophages ($CD19^-CD11b^+F4/80^+GR1^{Dim/-}CD11c^+ CD86^{Dim}$) was determined after 3 weeks of an NCD or HFD feeding*P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

In conclusion, γδ T cells are enriched in visceral adipose tissue and even under normal conditions they are already in the activated state ($CD44^{hi}$ $CD69^+$) and can produce proinflammatory cytokines. In response to HFD, number of γδ T cells that produce IL-17A increases. However, it is not clear if they play a role in establishing inflammation in our mouse model of DM2. The use of TCRδ$^{-/-}$ mice showed that in the absence of γδ T cells, I do not see any differences in either NK cells or macrophages, but I do see increase in weight gain and fat pad weight suggesting that they might be more important for homeostasis than inflammation of VAT.

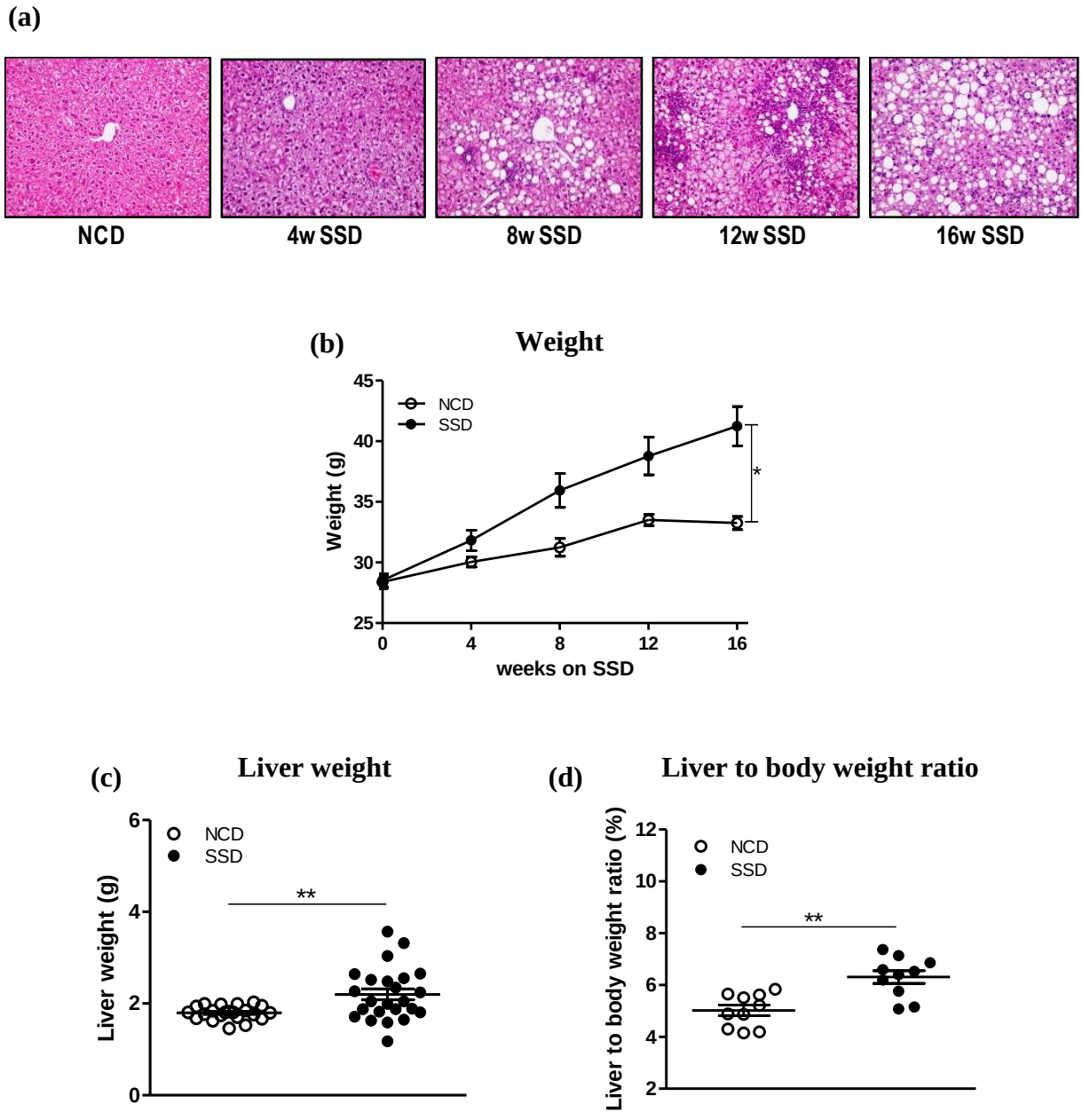
4.2 Role of γδ T cells in liver inflammation

4.2.1 SSD model of NAFLD reflects clinical disease progression

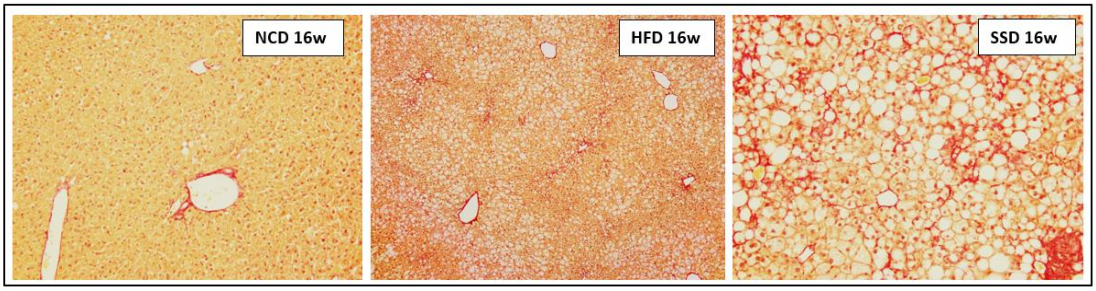
Mouse models of NAFLD and NASH usually involve genetic disruption or overexpression of genes involved in hepatic function. Alternatively, liver fibrosis is induced through administration of hepatotoxic compounds (e.g., CCl_4) or by nutrient deficient diets (methionine and choline deficiency). These models readily induce liver fibrosis, but lack metabolic dysfunction such as obesity and IR that usually accompanies NAFLD (Machado et al., 2015). To reproduce both the liver disease phenotype and its aetiology, in my research I used a modified *Amylin liver NASH model* called Steatosis-steatohepatitis diet (SSD). SSD contains 22 % of fructose, 2 % of cholesterol and 40 % of fat (Clapper et al., 2013). With the help of histological H&E staining, I confirmed that SSD fed mice develop all stages of NAFLD within 16 weeks starting with mild accumulation of fat that causes "ballooning" of hepatocytes (4 weeks SSD). Steatosis develops around week 8 and it is either macrovesicular, with a single large fat droplet or microvesicular with several smaller well-defined droplets. In an addition to steatosis, inflammatory foci, as a sign of steatohepatitis, appear around week 12 of SSD feeding. Around week 16, features of hepatocellular injury and fibrosis can be seen, indicating progression of NASH (Figure 13a). Development of fibrosis was further confirmed by Sirius red staining which also showed a characteristic "chicken wire" pattern due to deposition of collagen along the sinusoids and around the hepatocytes (Figure 13e). Obesity is the most common cause of NAFLD and hepatomegaly is one of the first clinical signs of NAFLD. Therefore, I measured body weight, liver weight and liver weight to body weight ratio in WT male mice that were on an NCD or SSD diet for 16 weeks. As expected, the SSD diet promoted obesity with substantial weight gain as compared to an NCD diet group (Figure 13b). The SSD group also had enlarged livers, increased liver weight (Figure 13c) as well as an increased liver to body weight ratio (Figure 13d) in comparison to the NCD group.

Since HFD is an established model for obesity and IR, I wanted to see how this model compares to the SSD diet with regards to development of fibrosis and steatosis. It is known that IR increases FFA influx and increases triglyceride synthesis and storage, leading to steatosis, oxidative stress and fibrosis. Histological results showed that mice on HFD for 16 weeks indeed develop low levels steatosis; however, there was no sign of fibrosis (Figure 13e and 13f) suggesting that SSD was a much better model for NAFLD. Therefore, I decided to continue my research on NASH with an SSD model.

In summary, SSD diet displays all features of NAFLD, including weight gain, hepatomegaly, progressive advancement of the disease from ballooning to NASH marked with inflammation and fibrosis.



(e)



(f)

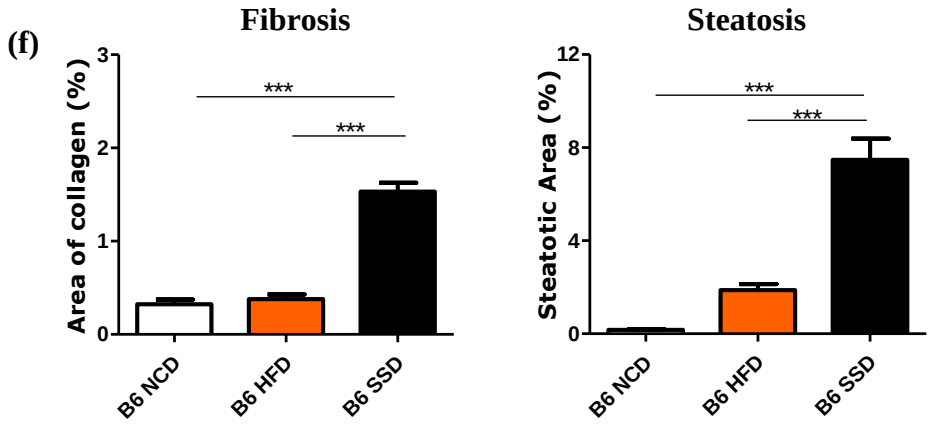


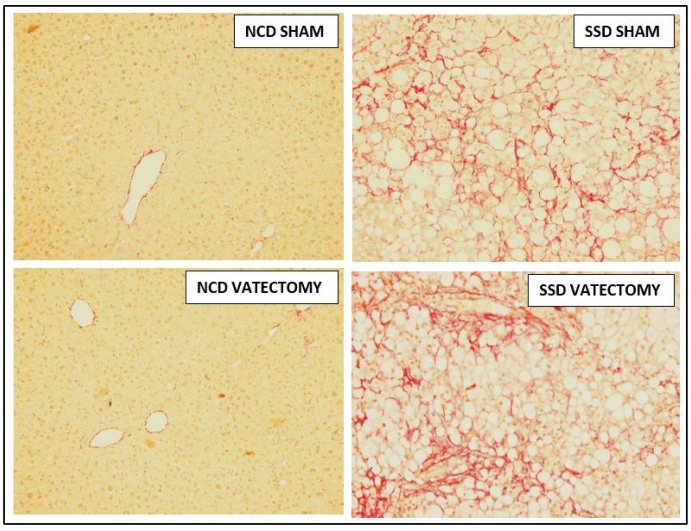
Figure 13. SSD diet-fed mice develop progressive NAFLD/NASH. (a) Representative H&E sections from mice on an NCD and SSD diet for 4, 8, 12 and 16 weeks. (b) Body weight over period of 16 weeks in an NCD and SSD group of mice (n = 5-10). (c) The liver weight and (d) the ratio of liver weight to body weight after 16 weeks on an NCD and SSD (n = 10-25). (e) Representative slides and (f) quantitive assessment for comparison of NCD, HFD and SSD in induction of fibrosis (stained for collagen fibers (red) with Sirius red) after 16 weeks on different diets. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney test or ANOVA).

4.2.2 Hepatic inflammation is a trigger for development of NASH in SSD model

Excess in free fatty acids and chronic low-grade inflammation originating from VAT are considered two of the most important factors contributing to progression from simple steatosis to steatohepatitis (Berlanga et al., 2014). Therefore, I wanted to see if in our SSD model VAT inflammation is essential for the development of fibrosis. We performed VATectomy where we removed visceral adipose tissue and after 2 weeks placed animals on an NCD or SSD diet. Results showed that VATectomized mice that were placed for 16 weeks on an SSD diet had significantly higher hepatic steatosis and fibrosis in comparison to animals that were only sham operated (Figure 14a and 14b). Thus, in our model local VAT inflammation is not essential for the development of fibrosis most likely due to dietary cholesterol that enables VLDL accumulation in liver from food itself.

Since hepatic inflammation is a necessary component for the development of NASH (Koyama and Brenner, 2017; Tilg and Moschen, 2010), I quantified the major immune cell populations over time in liver following SSD feeding. Analysis showed that within the first 8 weeks of feeding, mainly innate lymphocytes (cNK, γδ T cells) increase in numbers (Figure 14c). On week 12, CD8 T cells (Figure 14d) and cells of the myeloid linage (neutrophils, eosinophils, and proinflammatory Gr-1^{+} macrophages) dominated the response (Figure 14e). Histological results on week 12 also correspond with the onset of inflammatory foci and fibrosis (Figure 13a).

(a)



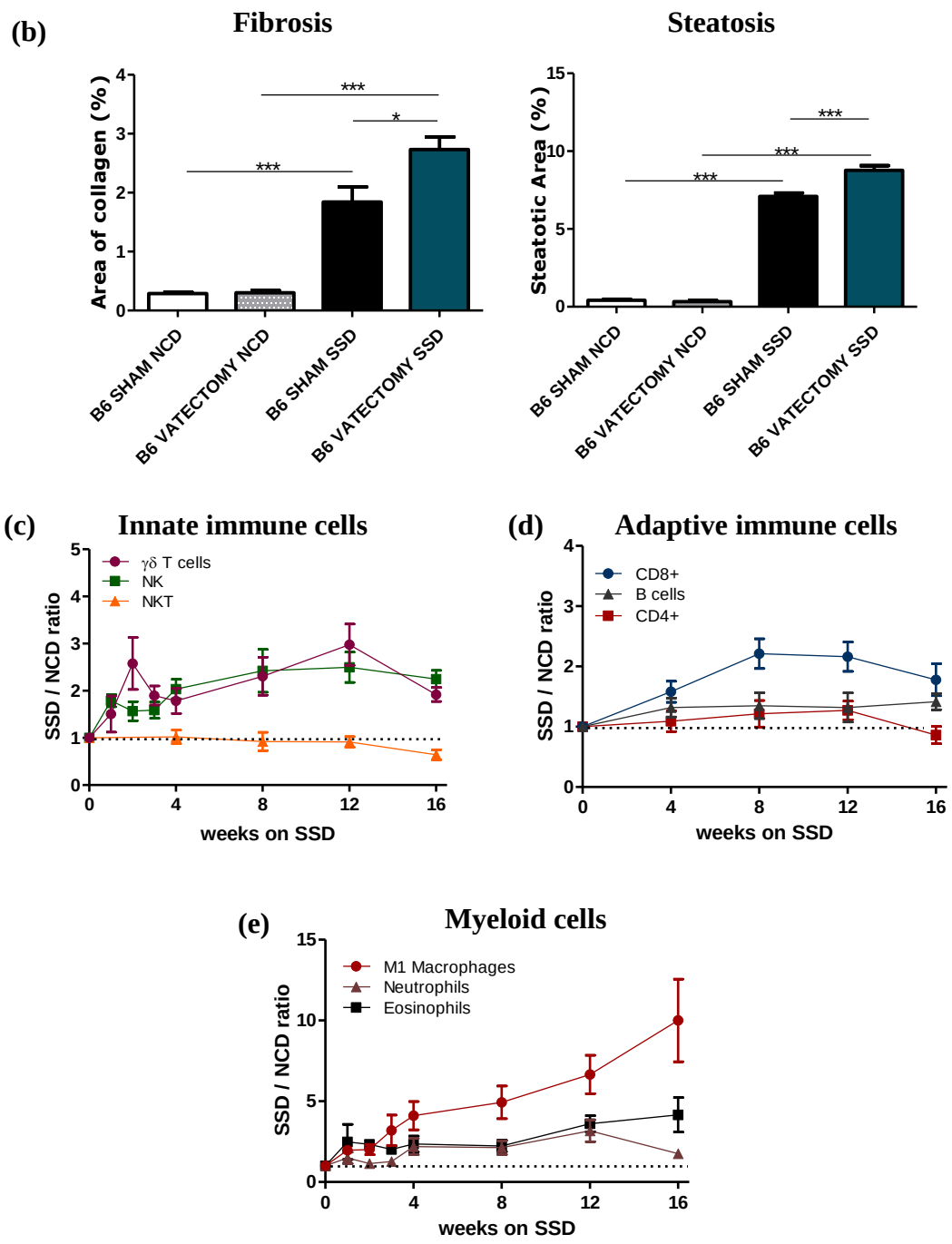


Figure 14. Development of liver steatosis and fibrosis in SSD model. (a-b) Mice were subjected to either VATectomy or sham operation and were let to recover for 2 weeks. After recovery, they were fed an SSD or an NCD diet for 16 weeks. (a) Representative slides and (b) quantitive assessment of steatosis and fibrosis (stained for collagen fibers (red) with Sirius red) (c-e) Liver immune cells were isolated from the liver to follow the cell kinetics in the response to SSD diet over a 16-week period. (c) Innate immune cells (NK cells were gated based on $NK1.1^{+}CD3\varepsilon^{-}CD49b^{+}$, NKT cells based on $NK1.1^{+}CD3\varepsilon^{+}$, γδ T cells on $CD3\varepsilon^{+}TCR\delta^{+}$ population). (d) Adaptive immune cells (CD8 and CD4 T cells were gated based on $CD3\varepsilon^{+}$ and $CD8^{+}$ or $CD4^{+}$ population, respectively; B cells were gated based on $CD3\varepsilon^{-}CD19^{+}$). (e) Cells of myeloid linage (Neutrophils were gated based on $CD19^{-}CD11b^{+}F4/80^{-}GR1^{hi}$ gating, eosinophils $CD19^{-}CD11b^{+}F4/80^{+}GR1^{Dim/-}$ and macrophages $CD19^{-}CD11b^{+}F4/80^{+}GR1^{in}$).

4.2.3 γδ T cells produce IL-17A in liver as an early response to SSD

Non-alcoholic steatohepatitis is closely associated with chronic liver inflammation and cytokine production. It was reported that NASH patients have increased levels of proinflammatory cytokines in serum (Stojsavljevic et al., 2014). Since in the first few weeks of SSD feeding there is an increase in innate cells, I decided to isolate, purify and stimulate lymphocytes to look at the production of cytokines. I focused on IFNγ, TNF and IL-17 because those are the main cytokines that are being produced by innate and innate-like cells.

Although there is a mild increase in the accumulation of innate lymphocytes during initial stages of SSD, I noticed that there is a dramatic increase in production of the proinflammatory cytokine IL-17A in response to SSD within the first 2 weeks of SSD feeding. This difference was not observed in production of either TNF or IFNγ (Figure 15a). Interestingly, in the SSD-fed group, IL-17A was produced *ex vivo*, without any stimulation, and its production was further increased after stimulation with PMA and Ionomycin (Figure 15b). This is an indication that these cells were stimulated recently *in situ*. IL-17A was produced almost exclusively by $CD3^+$ cells. The increase in IL-17A production in response to SSD feeding appears specific for liver, as virtually no IL-17A producing cells were observed in spleen (Figure 15c).

Further analysis of IL-17A producing $CD3^+$ cells revealed that in our model γδ T cells are the primary source of IL-17A during first two weeks of SSD feeding (Figure 15d).

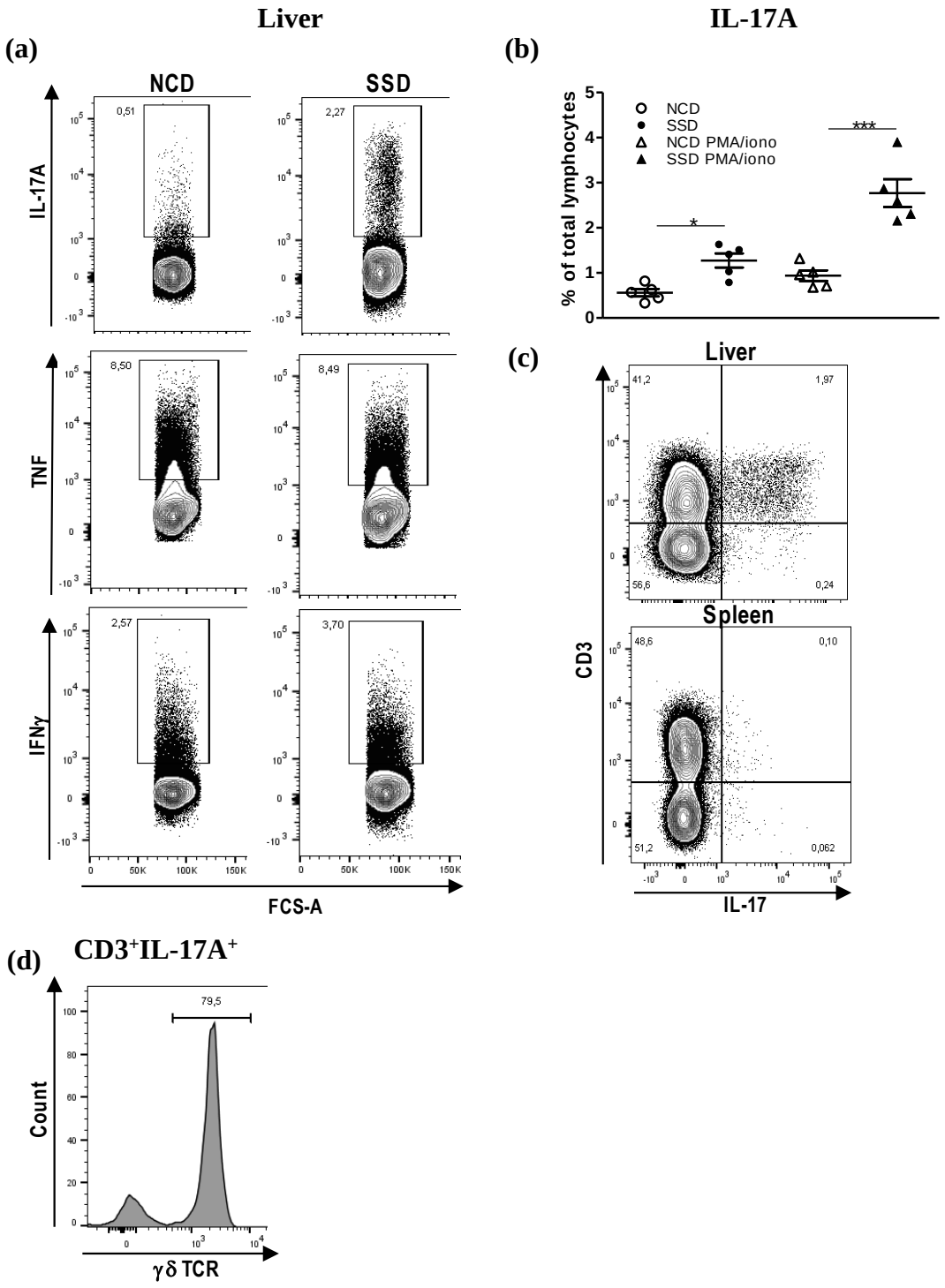


Figure 15. Cytokine production in liver within first 2 weeks of SSD. (a-d) Mice were fed an SSD or NCD diet for 2 weeks. (a) Flow cytometric analysis of total IL-17A, TNF and IFNγ in liver. Cells were gated for live and $CD45^+$. Numbers outside the quadrants (left) indicate the percentage of cells in each. (b) Results of intracellular flow cytometric analysis of IL-17A production by lymphocytes *ex vivo* and upon *in vitro* stimulation with PMA and ionomycin (n=5). (c) Flow cytometry analysis of IL-17A production in liver and spleen in $CD3\varepsilon^+$ cell fraction after *in vitro* stimulation with the PMA and ionomycin. (d) Flow cytometry of $CD3\varepsilon^+IL17A^+$ cells expressing $TCR\delta^+$ marker. Number above the line indicates the percentage of cells that are $CD3\varepsilon^+IL17A^+TCR\delta^+$. *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

Since my results showed that γδ T cells are one of the first cell subsets that are activated within the first two weeks of SSD feeding, I wanted to further investigate their phenotype and ability to excrete cytokines. The first thing I did was quantification of γδ T cells after 2 weeks of an NCD and SSD feeding. The analysis showed a statistically significant increase in both relative and absolute numbers of cells in mice fed with SSD (Figure 16a). This increase was at least partially due to proliferation (Figure 16b) as measured by expression of the Ki-67, a protein which is expressed during the G_1, S and G_2 phases of cell cycle, but absent in quiescent (G_0) cells. I next measured IL-17A production by these cells with and without PMA / ionomycin stimulation. FACS analysis showed that SSD causes spontaneous IL-17A production by γδ T cells without any stimulation. The increase in production of IL-17A was even more dramatic after stimulation with PMA and ionomycin (Figure 16c). The IL-17 cytokine family is composed of six members. Since IL-17F also belongs in the same group of proinflammatory cytokines, and has the greatest similarity to IL-17A, I also looked for the production of this cytokine. However, γδ T cells appeared to produce very little IL-17F. Moreover, SSD feeding did not result in an increase of IL-17F production, suggesting that this cytokine is not involved in SSD-induced liver pathology (Figure 16d). γδ T cells are able to produce high amounts of IFNγ and TNF. In order to see if they produce these cytokines in the context of NAFLD, I stimulated liver γδ T cells with PMA and ionomycin and checked for production of IFNγ and TNF. The analysis showed that they can produce IFNγ and TNF, but there was no difference in their production between the NCD and SSD diet fed groups (Figure 16e).

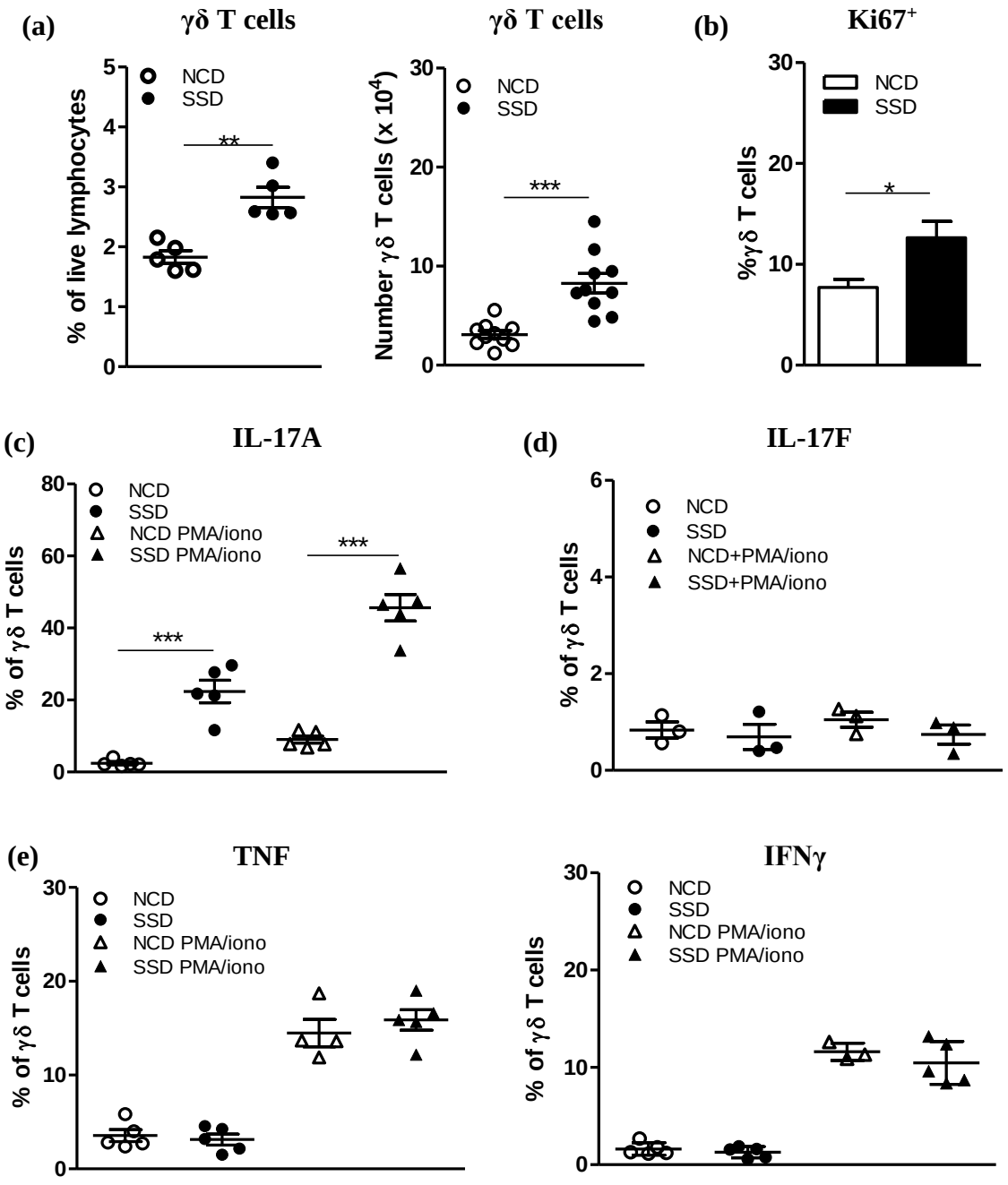


Figure 16. Liver γδ T cells within first 2 weeks of SSD. (a-d) Mice were fed an SSD or an NCD diet for 2 weeks. (a) Percentage and number of γδ T cells in the liver in an NCD and SSD fed mice. (b) Hepatic γδ T cells were isolated and expression of Ki67 was determined. Results of intracellular flow cytometry analysis of (c) IL-17A, (d) IL-17F, (e) TNF and IFNγ production by γδ T cells ex vivo and upon *in vitro* stimulation with PMA and ionomycin (n = 5) Gated for $CD3\varepsilon^{+}TCR\delta^{+}$ cells. *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

γδ T cells that produce IL-17A typically express RORγt and lack surface expression of CD27 (Ivanov et al., 2006). In contrast, IFNγ production of these cells is associated with expression of both CD27 and T-bet. Both subsets tend to express CD44. Phenotypic analysis showed that the liver γδ T cells were either $CD27^{-}CD44^{+}$ or $CD27^{+}CD44^{+}$ and notably, when mice were placed on an SSD, I observed a significant increase in the fraction of $CD27^{-}CD44^{+}$ γδ T cells (Figure 17a). It has been shown in the literature that IL-7 promotes survival, activation and triggers the production of IL-17A in innate-like γδT-IL17 cells while γδT-IFNγ cells instead depend heavily on IL-15. Upon examination of the receptors for IL-7 (CD127) and IL-15 (CD122, Rβ), I found that majority of γδ T cells expressed CD127 marker and this expression was further increased with SSD feeding (Figure 17b). Majority of liver γδ T cells in normal conditions expressed transcription factor T-bet. In response to SSD, there was a strong increase in the number of $Ror\gamma t^{+}$ cells, whereas I saw a relative reduction in the number of γδ T cells expressing T-bet (Figure 17b).

Unlike αβ T cells, T cell receptor chain usage is linked to functionality of γδ T cells. In that regard, $V\gamma1^{+}$ γδ T cells are a major source of IFNγ (Ribot et al., 2009), whereas $V\gamma4^{+}$, $V\gamma5^{+}$ and $V\gamma6^{+}$ cells are considered major producers of IL-17A (Papotto et al., 2017). Whereas $V\gamma1^{+}$ is mainly located in peripheral lymphoid tissues, $V\gamma4^{+}$ populates lung, $V\gamma5^{+}$ skin and $V\gamma6^{+}$ reproductive mucosa. Under lean conditions, I observed that hepatic γδ T cells exhibited a mixed chain usage, which was distinct from γδ T cells in other organs (Garman et al., 1986; Jameson and Havran, 2007). In lean animals, the main subpopulation was Vγ1. In response to SSD their relative contribution decreased in favour of Vγ6 (Figure 17d). Indeed, I could show that Vγ6 cells were the main subpopulation producing IL-17A (Figure 17e).

It is known that engagement of NKG2D can activate cytolytic responses of γδ T cells (Nielsen et al., 2015). Therefore, I decided to see if hepatic γδ T cells express this receptor. The analysis showed that hepatic γδ T cells express NKG2D molecule under lean conditions. Importantly, its expression is increased in response to an SSD diet (Figure 17f). Interestingly, the majority of IL-17A was produced by $NKG2D^{+}$ cells (Figure 17g).

In summary, γδ T cells in livers of SSD-fed mice show all characteristics typically associated with the production of IL-17A by these cells. In addition, I see a notable increase of NKG2D expression, which may be of importance for the activation of these cells.

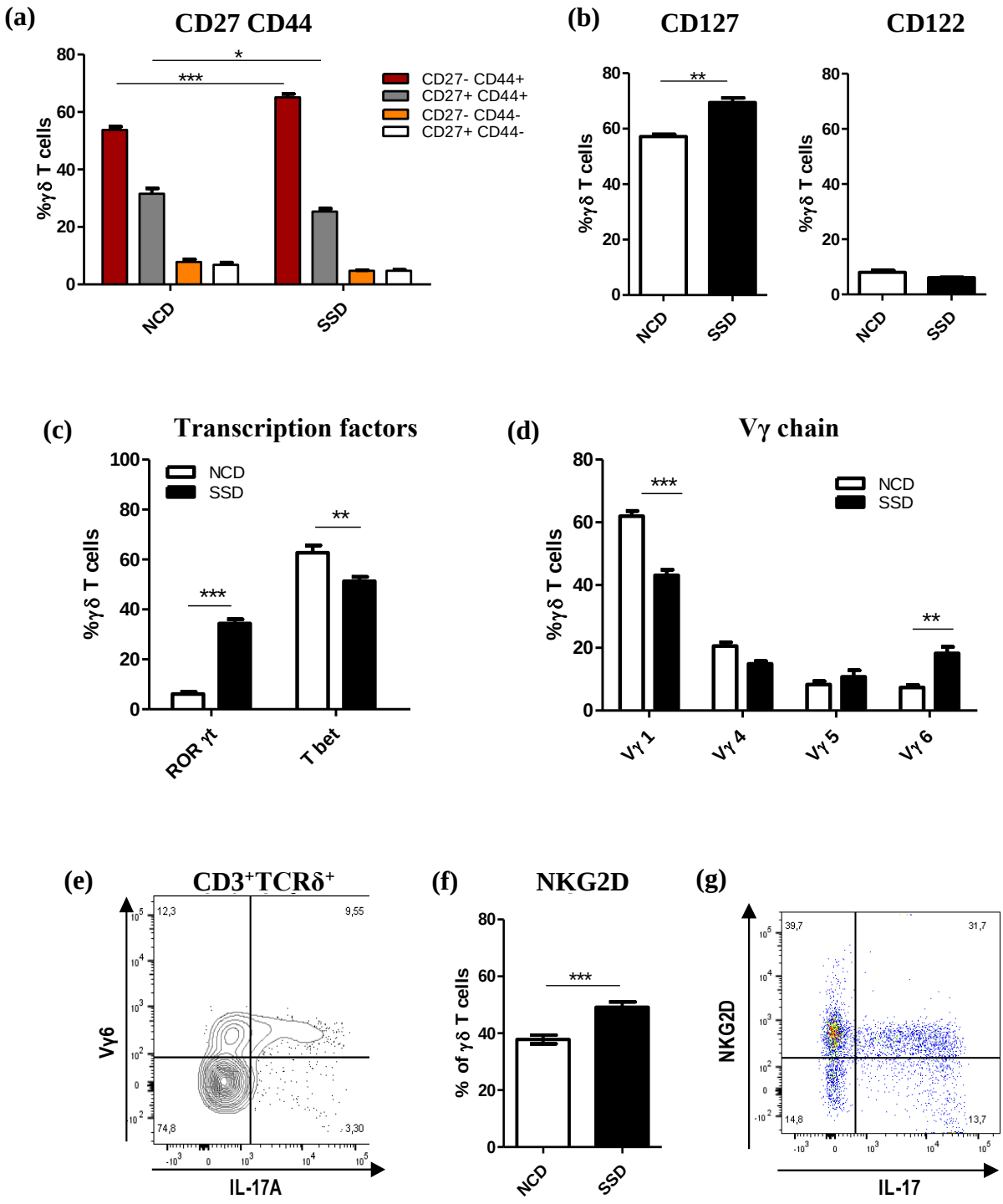
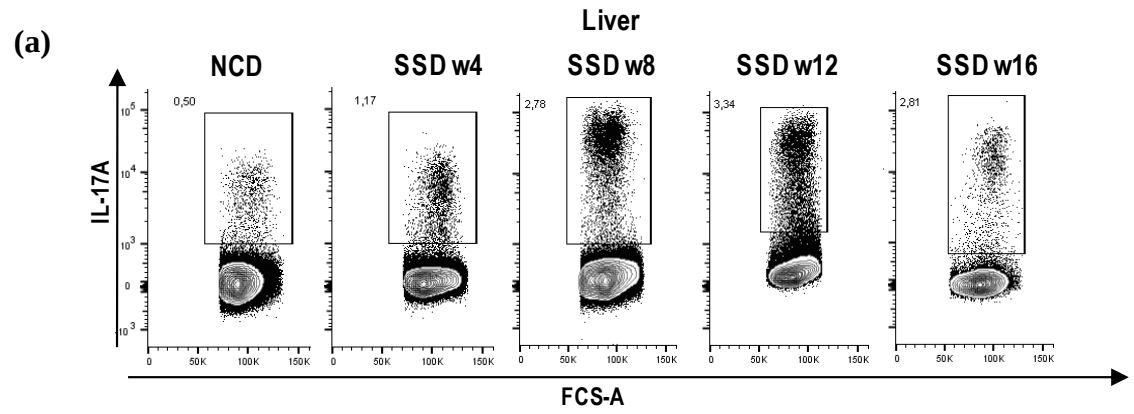


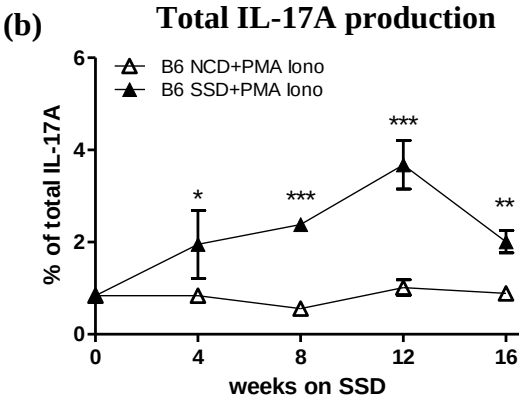
Figure 17. Liver γδ T cells phenotype within first 2 weeks of SSD. (a-g) Mice were fed an SSD or an NCD diet for 2 weeks. (a) Percentage of hepatic γδ T cells expressing CD27 and CD44, (b) transcription factors and (c) CD127 and CD122 determined by flow cytometry. (d) FACS analysis of Vγ chain usage and (e) intracellular IL-17A expression by Vγ6 chain. (f) Expression of NKG2D molecule in hepatic γδ T cells determined by flow cytometry (g) Results of intracellular flow cytometry analysis of IL-17A production by NKG2D$^+$ cell on an SSD diet. Numbers in corners indicate the percentage of cells (n = 5) Gated for CD3ε$^+$TCRδ$^+$ cells. *P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney u-test or ANOVA).

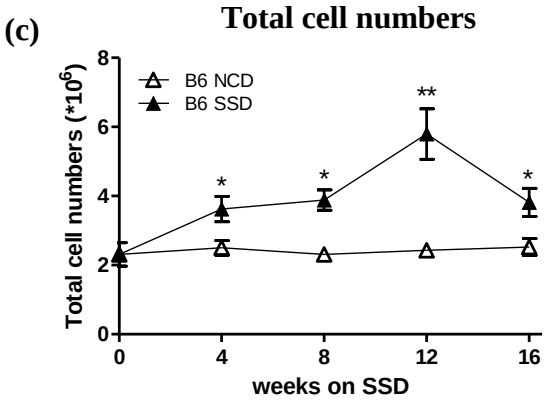
4.2.4 IL-17A plays a critical role in the pathogenesis of liver fibrosis

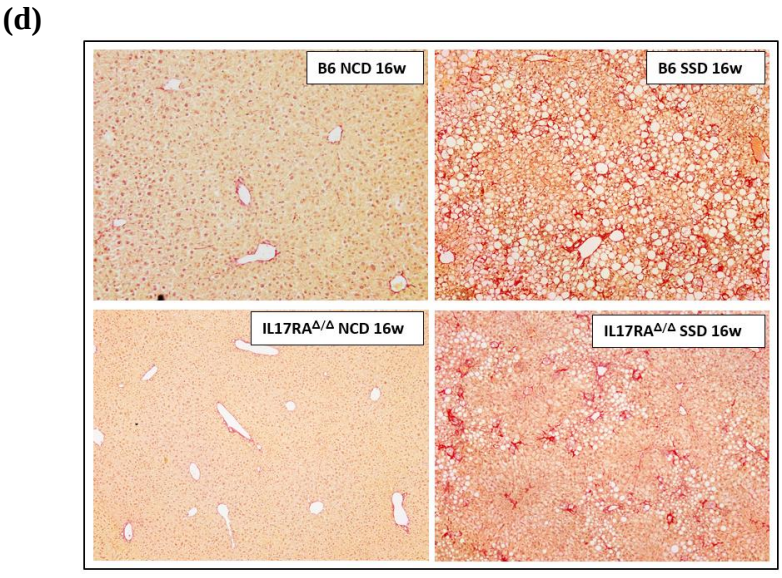
Liver fibrosis is a common outcome of NAFLD and it is a significant cause of liver failure, as well as the principal cause of morbidity and mortality (Friedman, 2003). Hepatic stellate cells (HSCs) are the main matrix-producing cells in the process of liver fibrosis (Moreira, 2007). Numerous mechanisms contribute to the activation of HSCs and lead to the switch from quiescent phenotype to the "myofibroblast-like" phenotype that leads to the production of collagen. IL-17A has been connected with HSC activation marked by an increased expression of IL-6, a-SMA and collagen1α1 in CCl_4–induced liver injury (Tan et al., 2013). Therefore, I wanted to investigate if in our NAFLD model, IL-17A is involved in driving the fibrotic process. Considering that IL-17A is produced very early after initiation of SSD feeding I wondered whether its effect is only important during the initial stages of disease or continuously. I followed its production through 16 weeks of SSD. Results showed that there is a continuous increase in its production (Figure 18a and 18b) up until week 12 of feeding (Figure 18b). I noticed that on week 16, when fibrosis develops, there is a decrease in the number of IL-17A producing cells (Figure 18b), which corresponded wit a minor decrease in the total number of leukocytes in liver (Figure 18c).

IL-17A signals through a heterodimeric receptor complex of IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC). To demonstrate the functional role of IL-17A, I used IL-17RA$^{\Delta/\Delta}$ mice that lack a functional IL-17A receptor (Ye et al., 2001). Mice were placed on an SSD or NCD for 16 weeks. From the literature, it is known that IL-17A deficient mice had greater weight than WT mice over time in both NCD and HFD conditions (Zuniga et al., 2010). However, in our model I did not observe increased body weight of IL-17RA$^{\Delta/\Delta}$ mice over WT controls (data not shown). Based on histological features, IL-17RA$^{\Delta/\Delta}$ mice had normal liver when compared to B6 animals on an NCD diet. However, when they were placed on an SSD diet for 16 weeks, IL-17RA deficient animals had significantly less fibrosis in comparison to B6 animals on the same diet (Figure 18d and 18e). They did not show any decrease in the steatosis, but this is partially expected because it is known that IL-17 deficiency can even enhance adipose tissue accumulation and accelerate hepatic triglyceride accumulation whilst reducing hepatocellular damage in mice (Harley et al., 2014; Zuniga et al., 2010).


(a)
Liver
NCD
SSD w4
SSD w8
SSD w12
SSD w16
IL-17A
FCS-A
0,50
1,17
2,78
3,34
2,81


(b)
Total IL-17A production
B6 NCD+PMA Iono
B6 SSD+PMA Iono
% of total IL-17A
weeks on SSD


(c)
Total cell numbers
B6 NCD
B6 SSD
Total cell numbers (*10^6)
weeks on SSD


(d)
B6 NCD 16w
B6 SSD 16w
IL17RA^Δ/Δ NCD 16w
IL17RA^Δ/Δ SSD 16w

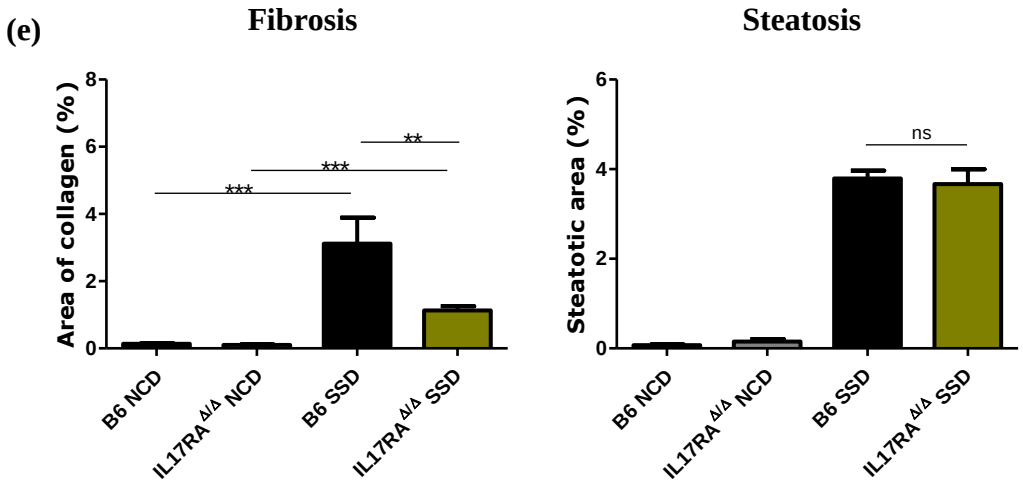


Figure 18. IL-17A production and its role in SSD model of NAFLD. (a) Representative FACS plots and (b) result of quantification of intracellular flow cytometry analysis of IL-17A production by lymphocytes upon *in vitro* stimulation with PMA and ionomycin on an NCD and after 4, 8, 12 and 16 weeks on an SSD diet. (c) Total immune cell numbers on an NCD and after 4, 8, 12 and 16 weeks on an SSD. (d) Representative microscopic sections and (e) quantitive assessment of liver steatosis and fibrosis (stained for collagen fibers (red) with Sirius red) in B6 and IL17RA$^{\Delta/\Delta}$ after 16 weeks on and NCD or SSD diet (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

IL-17A has been shown to be able to directly activate HSC [121, 123]. However, many hepatic cell subsets, including parenchymal, non-parenchymal cells and resident immune cells, express the IL-17 receptor and IL-17A may therefore mediate indirect activation of HSC through macrophages. To test this, I made use of IL-17RA-flox mice, which allow cell-specific elimination of the IL-17RA (Ye et al., 2001). It has been described that IL-17A has a critical role in neutrophil recruitment and induction of macrophage-mediated inflammation (Tan et al., 2013). Therefore, I crossed IL-17RA-flox mice on LysM^{cre} animals. These mice lack IL-17RA on monocytes, mature macrophages and granulocytes. Genetic ablation of IL-17RA in myeloid cells showed a trend towards reduced fibrosis, even though this did not reach statistical significance (Figure 19a and 19b). Surprisingly, unlike IL17R$^{\Delta/\Delta}$ mice, Lys^{Cre}IL17RA$^{flox/flox}$ mice had a small but significant reduction in the level of steatosis, indicating that macrophages might play a role in fat accumulation in liver cells. However, based on cell numbers, there was no difference in accumulation of either monocytes (Figure 19c) or neutrophils (Figure 19d).

Taken together, these findings indicate that in our model IL-17A does not have a role in accumulation of neutrophils, monocytes and KC but it might directly act on HSC and induce their activation.

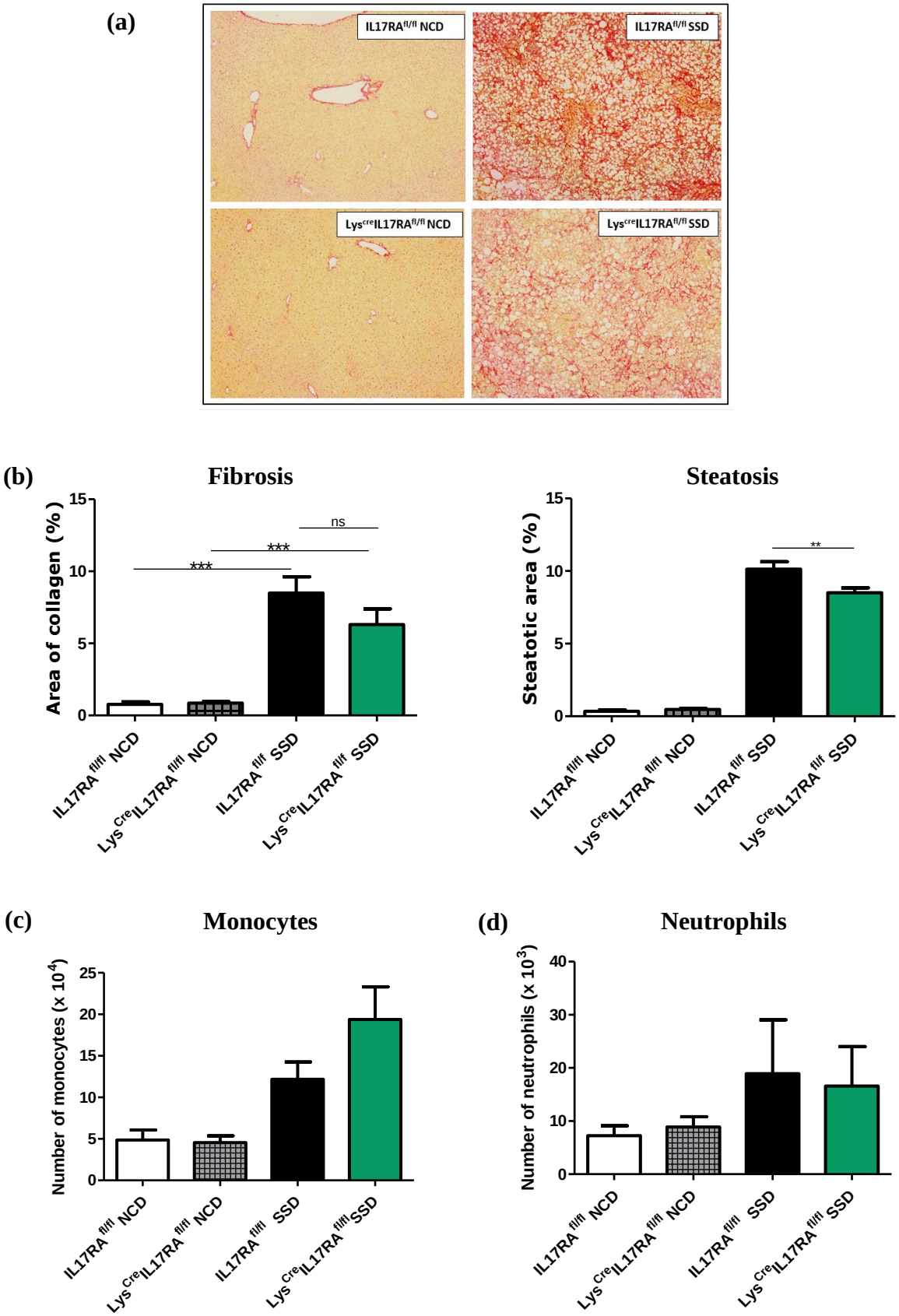
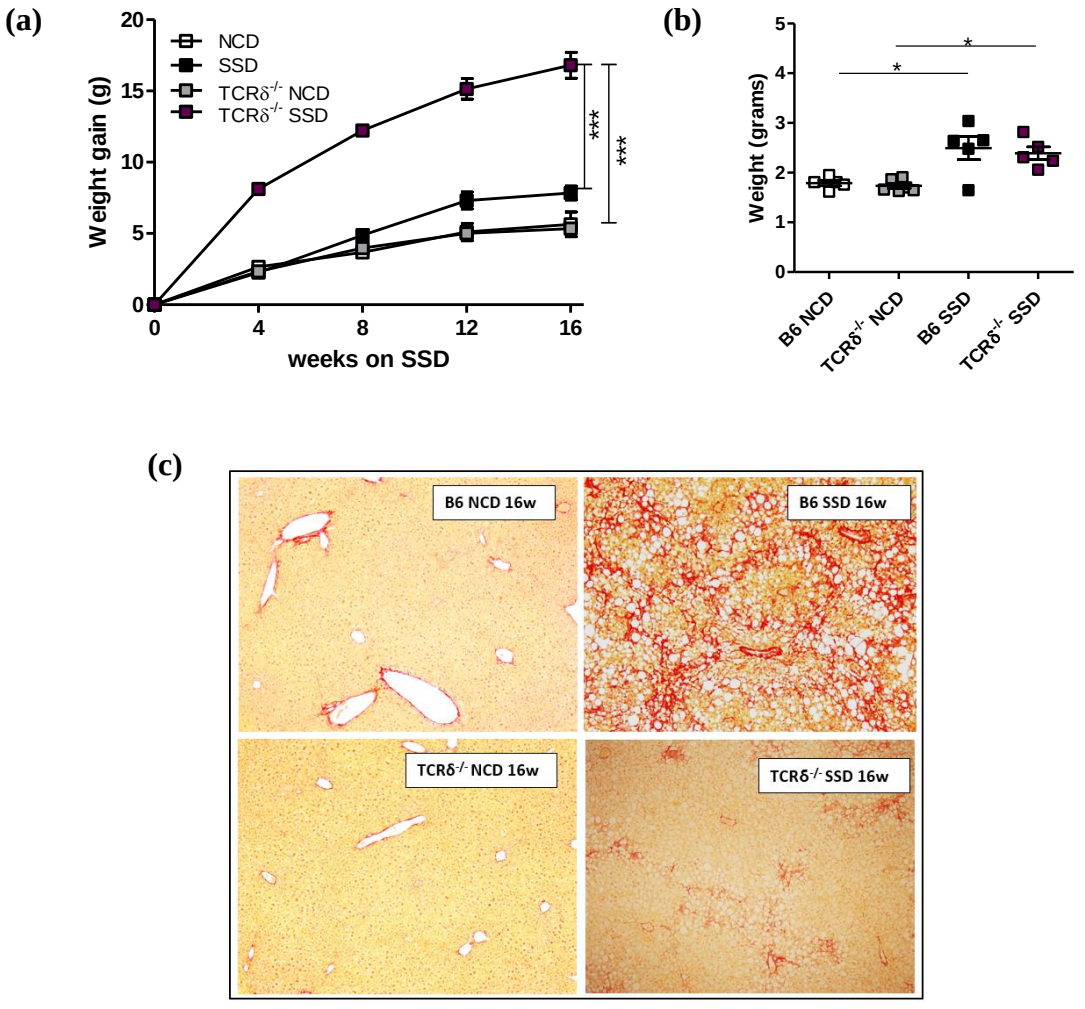


Figure 19. Lack of IL-17RA on myeloid cells does not affect the level of fibrosis (a-d) Mice were fed an SSD or an NCD diet for 16 weeks. (a) Representative microscopic sections and (b) quantitive assessment of liver steatosis and fibrosis (stained for collagen fibers (red) with Sirius

red) in B6 and Lys^{cre}IL17RA$^{flox/flox}$ mice. (c) Number of monocytes and (d) neutrophils in liver after 16 weeks of an NCD and SSD in B6 and Alb^{cre}IL17RA$^{flox/flox}$ mice (n = 4 - 5). *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

4.2.5 γδ T cells and NKG2D promote liver fibrosis

Considering that IL-17A plays a significant role in development of the fibrosis in our model and that the γδ T cells are a major producer of IL-17A from the start of an SSD diet, I hypothesized that γδ T cells might have a role in the development of fibrosis. To confirm this model, WT B6 and TCRδ$^{-/-}$ mice were fed with an NCD or SSD diet for a period of 16 weeks. TCRδ$^{-/-}$ mice showed increased weight gain in comparison to B6 mice (Figure 20a). However, there was no difference in liver weight between SSD groups after 16w of SSD (Figure 20b). Despite having increased weight gain, I observed that TCRδ$^{-/-}$ mice had significant reduction in fibrosis in comparison to B6 mice. In contrast, there was no reduction in the level of hepatic steatosis (Figure 20c and 20d).



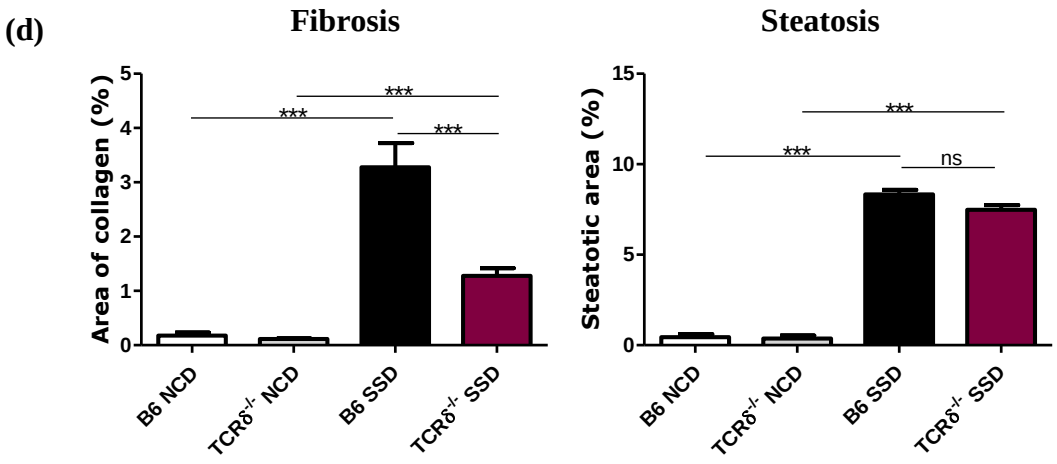
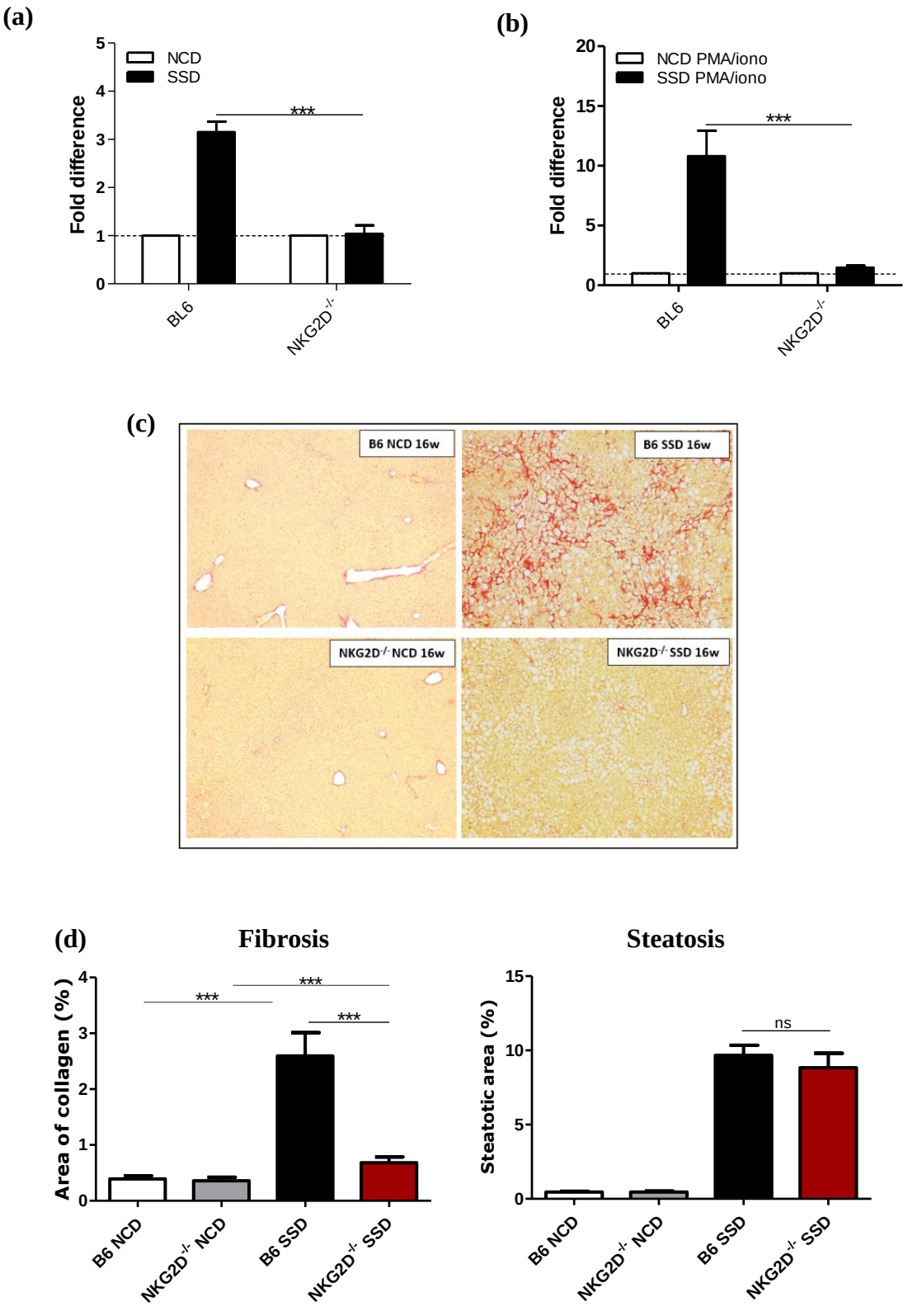


Figure 20. SSD-fed TCRδ$^{-/-}$ mice have reduction in fibrosis. (a) Weight gain over period of 16 weeks in an NCD and SSD group of B6 and TCRδ$^{-/-}$ mice (n = 5). (b) The liver weight after 16 weeks on an NCD and SSD group of B6 and TCRδ$^{-/-}$ mice. (c) Representative microscopic sections and (d) quantitive assessment of liver steatosis and fibrosis (stained for collagen fibers (red) with Sirius red) in B6 and TCRδ$^{-/-}$ mice after 16 weeks on and NCD or SSD diet (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

I next wanted to elucidate how γδ T cells in liver are activated in response to SSD. As shown in Figure 17f, in response to an SSD diet, there was an increase in expression of the activating receptor NKG2D on γδ T cells. It as been shown in several models that NKG2D engagement on γδ T cells can elicit cytokine production (Strid et al., 2011), such as in HCMV-specific γδ T cells (Nielsen et al., 2015). I showed in Figure 17g that after 2 weeks of SSD diet almost all IL-17 produced by γδ T cells came from the NKG2D^{+} subset. Thus, I wanted to see how the lack of NKG2D receptor will affect γδ T cells and their production of IL-17 cytokine. I placed B6 and NKG2D$^{-/-}$ animals on an SSD or NCD for 2 weeks and quantified the fold increase in number of γδ T cells and the production of proinflammatory cytokine IL-17A. Results showed that the increase in γδ T cells, as well as the increase in IL-17A production were completely prevented by NKG2D-deficiency (Figure 21a and 21b). To see if NKG2D has a role in promotion of fibrosis, NKG2D$^{-/-}$ were placed on an SSD diet and liver fibrosis was analysed after 16 weeks. Histological staining with Sirius red showed a remarkably strong reduction of fibrosis in NKG2D$^{-/-}$ mice, whereas steatosis was the same as in B6 mice (Figure 21c and 21d).

Alpha-smooth muscle actin (αSMA) is present in smooth muscle of the vasculature, perisinusoidal cells (HSC), and myofibroblasts derived from perisinusoidal cells. HSC are known to express αSMA prior to secreting collagen and its expression is therefore closely

associated with liver fibrosis. To see if NKG2D has an effect on activation of HSCs, the relative abundance of activated HSCs was assessed by expression of the HSC marker αSMA. The histological analysis showed that there were less αSMA positive cells in NKG2D$^{-/-}$ (Figure 21e and 21f).



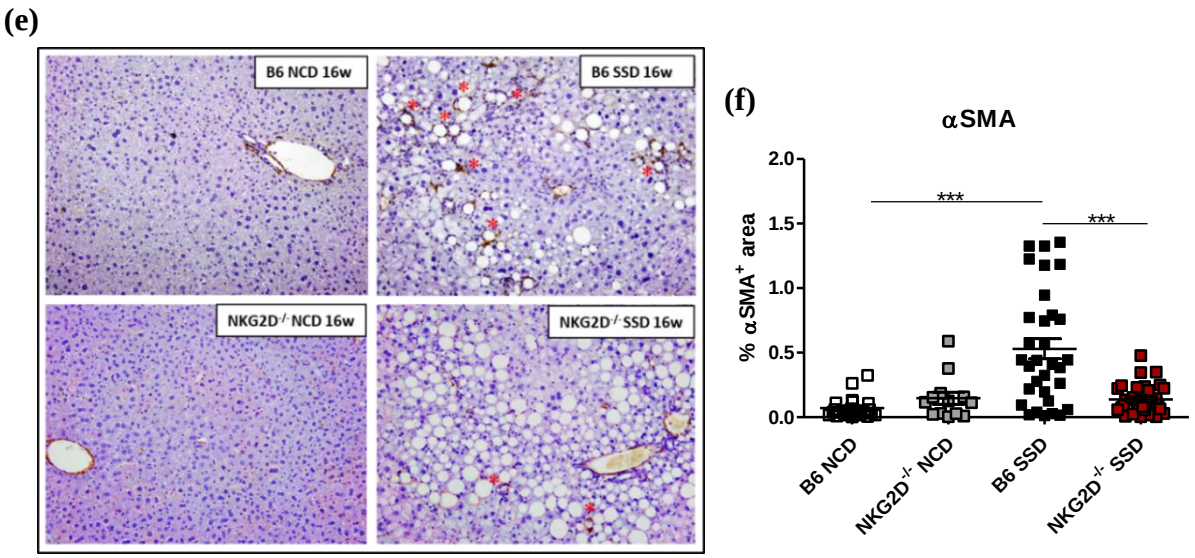


Figure 21. SSD-fed NKG2D$^{-/-}$ mice have reduction in fibrosis. Fold increase in number of (a) γδ T cells and (b) IL-17A production after 2 weeks of an NCD or SSD. (c) Representative microscopic sections and (d) quantitive assessment of liver steatosis and fibrosis (stained for collagen fibers (red) with Sirius red) in B6 and NKG2D$^{-/-}$ mice after 16 weeks on and NCD or SSD diet (n = 6). (e) Representative slides and (f) quantification of αSMA area in B6 and NKG2D$^{-/-}$ mice after 16 weeks on and NCD or SSD diet (n = 6) (* indicate αSMA positive cells) *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

In addition to γδ T cells, NKG2D is particularly potent in the activation of NK cells. Since I also observed an early increase in hepatic NK cells in response to SSD, I wanted to investigate if the phenotype I see in NKG2D$^{-/-}$ is the result of NKG2D activation on NK cells. To do so, I made use of NKG2D flox mice, which were backcrossed on NCR1cre mice to specifically eliminate NKG2D on NK cells. NCR1^{cre}NKG2D$^{flox/flox}$ mice were placed on an SSD for 16 weeks. Analysis of liver slides showed that B6, NKG2D$^{flox/flox}$ littermates and NCR1^{cre}NKG2D$^{flox/flox}$ mice had all similar levels of fibrosis and it was significantly higher than in total NKG2D knock out mice (Figure 22a and 22b). In contrast, steatosis was the same in all four groups.

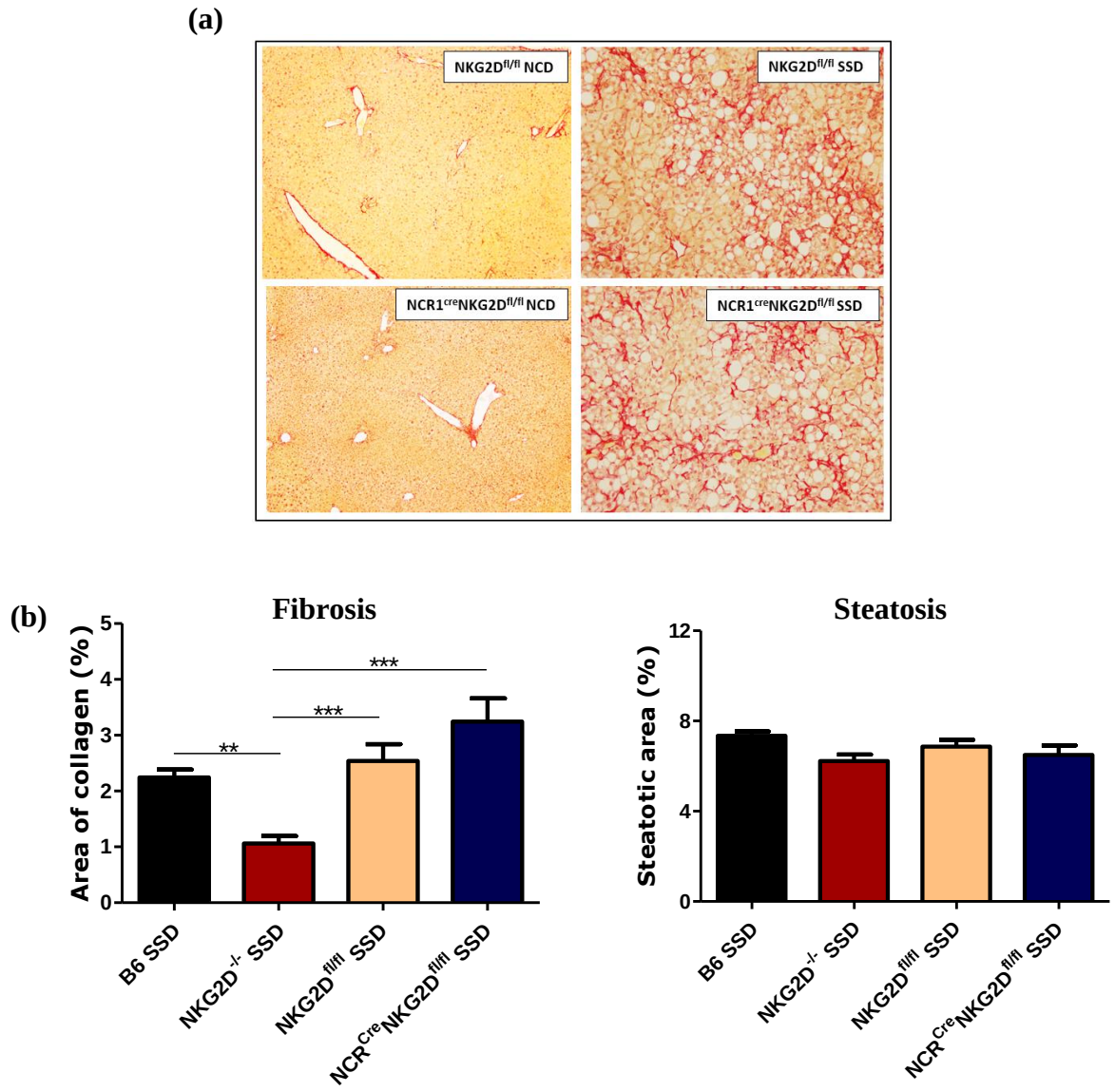


Figure 22. NKG2D on NK cells is not responsible for the reduced levels of fibrosis. (a) Representative microscopic sections and (b) quantitive assessment of liver steatosis and fibrosis (stained for collagen fibers (red) with Sirius red) in B6, NKG2D$^{-/-}$, NKG2D$^{flox/flox}$ and NCR1^{cre}NKG2D$^{flox/flox}$ mice after 16 weeks on NCD or SSD diet (n = 5 - 6). *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA).

Finally, I wanted to see whether induction of NAFLD is associated with upregulation of its ligands in the liver. Normal mouse hepatocytes can express low levels of mRNA for NKG2D ligands (Gao, 2010; Spear et al., 2013); however, several NKG2D ligands can be detected at high levels on hepatocytes in case of induced liver injury (Cheng et al., 2009; Kahraman et al., 2012). I analyzed expression of all NKG2D ligands expressed in B6 mice by qPCR. My analysis showed that only in a case of H60 there was a trend in increase of ligand

expression (Figure 23a). Notably, expression of H60 positively correlated with production of IL-17A by γδ T cells (p=0,0537; R^2=0,4338). These findings indicate that γδ T cells in metabolically stressed hepatic tissue are specifically activated through their NKG2D receptor by H60 (Figure 23b).

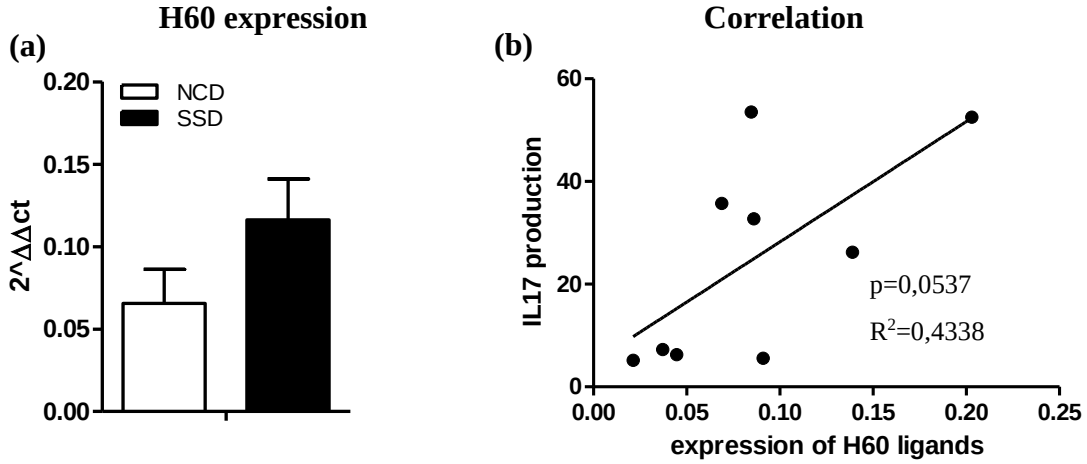


Figure 23. Expression of H60 ligand correlates with IL-17A production (a) Results of quantitative PCR analysis for H60 ligand and (b) correlation between IL-17A production and transcript expression of H60 in liver homogenates of mice fed an NCD or an SSD for 2 weeks

5. DISCUSSION

In this thesis, I managed to show that SSD diet is a good model for NAFLD because it resembles human disease course starting from simple steatosis to steatohepatitis with fibrosis. I also showed that in response to nutrient excess one of the initiating events in NAFLD is the mobilisation of γδ cells, through an increase of the NKG2D ligand H60. NKG2D engagement induces an increase in IL-17A production, which is important for the development of liver pathology in our model of NASH. Therefore, my data indicate that activation of the NKG2D receptor on γδ T cells, resulting in their production of IL-17A is crucial in the development of liver fibrosis in the SSD model of NAFLD.

The role of the immune system has been recognized in the pathogenesis of various obesity-related co-morbidities, such as IR and DM2. However, the specific role of immune cells in the development of NAFLD has not yet been completely understood. There are several studies that are suggesting that inflammation might precede steatosis in some cases of NASH (Wolf et al., 2014; Zhao et al., 2015) and even more importantly, it has been shown that inflammation is a main factor that predicts the long-term prognosis of NAFLD (Tilg and Moschen, 2010). However, in both DM2 and NAFLD the main unanswered question is how immune cells are activated in the first place. Therefore, the main aim of this thesis was to determine how, in the context of two different dietary models of metabolic disease, the immune system initiates inflammatory processes in VAT and liver.

Previous studies showed that the excess of macronutrients in both adipocytes and hepatocytes leads to hypertrophy and cellular stress (Sethi and Vidal-Puig, 2007). The physiological response to biological or physical stress factors is inflammation, with the purpose to resolve the stress factor and restore tissue homeostasis. In case of metabolic disease, cellular stress is not resolved by the immune system resulting in a chronic inflammatory response that may ultimately lead to insulin resistance, DM2 and NAFLD. The immune response to cellular stress may comprise many cell types, but it primarily involves cells of the innate and innate-like immune system. To investigate connections between obesity-induced stress and initiation of inflammation in VAT and liver, I used diet induced obesity models which enabled me to follow the development of DM2 and NAFLD from early stages.

As mentioned previously, high-fat diet in mice is an established model for impaired glucose tolerance and DM2. In this model, start of diet-induced obesity is marked by an increase in proinflammatory NK cells, M1-macrophages and neutrophils that later, through the production of cytokines and chemokines, attract other cells like B cells, CD4, CD8, Th17 and

γδ T cells (Chatzigeorgiou et al., 2012; Chawla et al., 2011). Because the role of γδ T cells in VAT inflammation has been poorly defined, I decided to focus on them in my research. γδ T cells are innate-like cells that have a role in tissue homeostasis (reviewed in (Nielsen et al., 2017)) and surveillance (Vantourout and Hayday, 2013) but are also able to provide rapid response against pathogens (Zheng et al., 2013) or tumour cells (Zou et al., 2017). Because of their T-cell receptor, composed of γ and δ chains, they are considered "unconventional" and even though they represent relatively small population of T cells in lymphoid organs, they are present at relatively high numbers in VAT. Previous studies have shown that adipose tissue γδ T cells are the main producers of IL-17A and that this IL-17A acts on (pre)adipocytes rather than on immune cells. γδ T cell derived IL-17A regulates lipid uptake and insulin sensitivity in fat cells under lean conditions (Zuniga et al., 2010). However, an interesting phenomenon was observed in one study where VAT γδ T cells after prolonged (5 month) HFD feeding spontaneously produced excessive amount of IL-17A without any stimulation (Zuniga et al., 2010). γδ T cells have been recognized as crucial immune cells for initiation of inflammation through IL-17A production in models of infection and autoimmune disease (Jensen et al., 2008; Roark et al., 2008). In line with these observations, it was shown in one study that TCR$\delta^{-/-}$ mice have reduced macrophage accumulation even after 10 weeks of HFD, suggesting a role of γδ T cells in progression and development of VAT inflammation (Mehta et al., 2015). In contrast, my data indicates that the absence of γδ T cells within first few weeks does not have a role in progression of inflammation, but rather on homeostasis of adipose tissue which was observed by increased weight gain and fat pad weight of animals lacking γδ T cells. A possible explanation is that IL-17A has a dual effect - one in homeostasis of adipose tissue and other in adipose tissue dysfunction in obesity. Something similar can also be seen in the case of IL-6 cytokine. IL-6 has been recognized as an initiator of insulin resistance, however, in last few years it has been suggested that it is also a homeostatic regulator of energy and glucose metabolism (Timper et al., 2017). Since IL-17A is a cytokine that has a potential of tissue damage (Jin and Dong, 2013), it is possible that its production is tightly regulated. In normal conditions, low levels might be important for tissue homeostasis and in obesity, increase of IL-17A and inhibition of adipogenesis might serve as an attempt to increase insulin production and improve glucose tolerance to overcome obesity-associated insulin resistance.

In addition to adipose tissue, with obesity progression several other organs that contribute to systemic insulin resistance, like skeletal muscle and liver, exhibit signs of inflammation (Lumeng and Saltiel, 2011). In the case of liver, this inflammation is mainly induced due to hepatic lipid overload that leads to oxidative stress and apoptosis – two

underlying causes of NASH (Sanches et al., 2015). There are several animal models currently used to study NAFLD. However, most of these are a poor model of human disease, as they mirror either metabolic characteristics of the disease or development of liver pathology, but not both. Since obesity is associated with NAFLD one of the first models used was the HFD model. HFD mimics the initial stages of human fatty liver disease including weight gain, IR and severe liver steatosis, however it is not able to induce liver inflammation and fibrosis. This lead us to the conclusion that the HFD model is good for studying DM2 and NAFL but not for elucidating the transition from NAFL to NASH (Ito et al., 2007). The MCD diet model is one of the most frequently used models to study NASH because it induces in relatively short time (2-6 weeks) all features of NASH including steatosis, oxidative stress, apoptosis, liver inflammation and fibrosis. A major flaw of the MCD model is that the metabolic profile of NASH is missing. Indeed, it even induces weight loss and cachexia, and causes in general low levels of glucose, insulin and triglycerides and does not lead to IR (Itagaki et al., 2013). We therefore consider it highly unlikely that the underlying cause of liver inflammation in the MCD model is representative of what happens early in human NAFLD. Humans consume quite a lot of fructose-rich foods and drinks, and this has been correlated with the development of obesity and NASH (Longato, 2013). We therefore developed an SSD diet, which is based on high levels of fructose, cholesterol and fat. Indeed, this diet induced all stages of non-alcoholic fatty liver disease as well as the main metabolic dysfunctions associated with NAFLD, such as weight gain and hepatomegaly. Therefore, with this diet we got a unique new model to study NAFLD but also the opportunity to translate our research to the clinic.

Liver inflammation in the context of NASH is mainly accompanied by a mononuclear infiltrate (KCs, monocytes) however, other cells may also be encountered. One of those cell subsets are γδ T cells, especially the Vγ1 subset, that are found abundantly in the liver and change in number and function with ongoing liver inflammation of bacterial or viral origin (Carding and Egan, 2002). It has been known for over two decades that γδ T cells are implicated in human liver diseases but the exact contribution of these cells to liver immunopathology in NAFLD/NASH remained elusive. In the context of liver autoimmune diseases, it was noticed that patients with autoimmune hepatitis have increased numbers of γδ T cells. Using a mouse model of concanavalin A (ConA)-induced hepatitis it has been shown that γδ T cells can have a protective role against liver inflammation (Martins et al., 1996; Zhao et al., 2011). In contrast, several studies showed that the increase in γδ T cell numbers in livers of hepatitis C virus (HCV) patients correlated with worse liver immunopathology (Agrati et al., 2001; Nuti et al., 1998). Additionally, it was shown that γδ T cells isolated from livers of HCV patients are cytotoxic

against primary human hepatocytes in culture (Tseng et al., 2001). However, the possible role of γδ T cells in development of liver fibrosis has been poorly studied. By using a short term SSD, I was able to identify that in response diet-induced stress, indeed γδ T cells were one of first cells that accumulated and produced proinflammatory IL-17A cytokine. A similar increase in γδ T cells was found in hepatitis B infected human fibrotic livers but also in several mouse models upon various inflammatory conditions and hepatic fibrosis (Hammerich et al., 2014; Tan et al., 2013). One study that used two different experimental liver injury models, CCl_4 and MCD diet, described a role of γδ T cells in prevention of fibrosis progression. Interestingly, this prevention of fibrosis was not mediated through cytokine production but through induction of apoptosis in HSC via Fas/Fas-ligand (FasL) interactions (Hammerich et al., 2014). Another research that used the CCl_4 model suggested that production of interleukin-17A by γδ T cells was induced through contact with activated HSCs and that this IL-17A then further exacerbated progression of liver fibrosis (Seo et al., 2016). Clearly, the context of immune cell activation is important for the role of γδ T cells and γδ T cell derived IL-17A in the development of liver pathology. We show in our model of fatty liver disease that γδ T cells are activated in early NAFLD and that IL-17A produced by these cells is important for the development of liver inflammation and fibrosis.

An increase in IL-17A production was described for several human liver diseases including acute liver injury (Tan et al., 2013), primary biliary cirrhosis (Lan et al., 2009) and hepatocellular carcinoma (Zhang et al., 2009). The importance of IL-17A in the induction and pathogenesis of liver diseases was further confirmed in multiple mouse models of liver injury (Meng et al., 2012; Yan et al., 2012). Recent findings have also suggested that IL-17A might play a role in regulation of obesity and NAFLD (Zuniga et al., 2010). Human studies have shown increased levels of IL-17A in obese patients and individuals that have NAFLD (Ahmed and Gaffen, 2010; Harley et al., 2014; Winer et al., 2009). However, the main cellular source IL-17A remained elusive and its precise mechanistic role in development of disease is still not clear. According to several studies, IL-17A aggravates hepatic inflammation (Giles et al., 2015; Tang et al., 2011) by inducing leukocytes infiltration (Ouyang et al., 2008) and it promotes liver fibrosis through upregulation of the TGF-β receptor on HSC (Fabre et al., 2014). In our model, IL-17A is also important for the development of NAFLD. This could be seen in a long-term model of SSD where in the absence of IL-17RA on all cells there was a significant reduction in fibrosis in comparison to controls. However, despite the fact that fibrosis was reduced, there were no differences in liver steatosis. This observation was partially expected because it is known that IL-17A deficiency can enhance liver triglyceride accumulation, which lead to

steatosis, but also reduce hepatocellular damage in mice that can cause fibrosis (Harley et al., 2014; Zuniga et al., 2010).

Even though in humans γδ T cells can contribute to IL-17A production, in the context of fibrosis IL-17 is mainly considered to be produced by Th17 $CD4^+$ T cells and neutrophils (Hammerich et al., 2011). In the MCD mouse model it was shown that infiltration of Th17 cells in the liver is a crucial step for initiation of liver inflammation and development of fibrosis (Rolla et al., 2016). Similar observation was made in an HFD model where it was shown that the number of Th17 cells positively correlated with liver pathology of NASH (Vonghia et al., 2015). While Th17 cells are the most-studied sources of IL-17A it has become clear that during the early phases of inflammation, IL-17A is mostly produced by innate and innate-like cells (Vonghia et al., 2015) (Sutton et al., 2009). So far, secretion of IL-17A by γδ T cells is mainly mentioned in the context of propagation of the response and subsequent activation of other immune cells. In that role, it was shown that γδ T cell derived IL-17A can attract neutrophils, activates inflammatory monocytes [13] or sustain IL-17A production by CD4+αβ T cells (Sutton et al., 2009). In our model we have shown that γδ T cells are important for progression to NASH. However, the exact functional relationships between them and other sources of IL-17A, should further be investigated. On the other hand, we managed to show that IL-17A is not important for the activation of monocytes and neutrophils as they accumulated equally in the $Lys^{cre}IL17RA^{flox}$ mice. This result might suggest that IL-17A has a role on some other liver cells or it might act directly on HCS. The latter idea is in accordance with two models of liver fibrosis - bile duct ligation (BDL) and carbon tetrachloride (CCL_4) that showed direct effect of IL-17A on increased expression of pro-fibrogenic genes in HSC (Gandhi, 2017; Meng et al., 2012).

It has been shown in infection models with *Mycobacterium tuberculosis* (Lockhart et al., 2006), *E. coli (Shibata et al., 2007)* and *Bordetella pertussis (Misiak et al., 2017)* that γδ T cells can be the main early source of proinflammatory cytokines (Jensen et al., 2008). However, their action is not only limited to infections, but they also play a role in various inflammatory conditions like arthritis, colitis and uveitis (Cui et al., 2009; Roark et al., 2007) where they can contribute to inflammatory pathology. γδ T cells that produce IL-17A mainly acquire their effector potential early during development in the foetal thymus and persist as long-lived cells in thymus or in the periphery. Activation of IL-17A producing cells is thought to be mostly independent of their TCR. Instead, these cells respond to stress signals such as cytokines, TLR ligands and through ligands for activating receptors like NKG2D (Bekiaris et al., 2013; Ribot et al., 2012). NKG2D has been investigated in the liver in the context of fibrosis. However, the

majority of research has been focused on NK cells, IFNγ and their capacity to kill activated HSC (Radaeva et al., 2006). I showed in this thesis that stimulation through NKG2D was a main activating factor that contributed to IL-17A production by hepatic γδ T cells. Importantly, NKG2D on NK cells was not important for development of fibrosis in our model. However, the exact mechanism of how NKG2D induces IL-17A production still needs to be elucidated. Interestingly, NKG2D on γδ T cells, in both humans and mice, has only been described to promote the production of IFNγ (Dandekar et al., 2005; Nedellec et al., 2010; Rincon-Orozco et al., 2005). However, there are a few studies in the context of human disease that link NKG2D to IL-17A production. Three of them showed that $CD4^+$ $NKG2D^+$ T cells from patients with DM2 (Phoksawat et al., 2017), Crohn's disease (Pariente et al., 2011) or *Mycobacterium tuberculosis* infection (Paidipally et al., 2009) produced more IL-17A than the $CD4^+$ $NKG2D^+$ T cells from healthy donors. Even though this phenomenon has been observed, not one study provided a possible mechanism. Based on the literature and the research that has been done on the role of NKG2D on NK cells, it is known that NKG2D has two adaptor molecules - DAP 10 and DAP 12 that can play a role in either the production or the release of the cytokines (Malarkannan et al., 2007; Zhang et al., 2015). The first mechanism starts with phosphorylation of ITAM motifs of DAP-12 by Src family and leads to activation of Syk-Zap70-PLCγ-NFκB signalling pathway and induction of cytokine production through activation of transcription (Vivier et al., 2013). However, since there are no DAP 12 adaptor molecules in T cells, this mechanism is reserved for NK cells. In the DAP-10-mediated signalling pathway, activated Src family phosphorylates motifs of DAP-10, which then recruits PI3K / Akt, or Grb2-Vav-ERK complex that enables cytokine secretion. Additionally, it has been shown that activation of PI3K/Akt pathway results in significant increase in IL-17A production in rheumatoid arthritis (Kim et al., 2005) and that PI3K-Akt-mTORcomplexe has an important role in differentiation and function of Th17 cells and their ability to produce IL-17A (Kurebayashi et al., 2012; Nagai et al., 2013). Therefore, it would be important to see if NKG2D pathway has the same effect on production or secretion of IL-17A by γδ T cells.

Several stress-induced ligands have been connected with activation of liver resident cells in infection or tumour transformation (Gao and Radaeva, 2013). Those are mainly ligands for stimulatory receptors on NK cells like NKp30, NKp44, NKp46 and maybe best-defined - NKG2D (Lanier, 2005). Elevated levels of RAE-1, MULT-1 and H60 ligands present in infection or induced liver injury, trigger the activation of either NK or NKT cells leading hepatocyte death and consequent hepatocellular damage (Cheng et al., 2009). One study showed that NKG2D ligands might also play an important role in NAFLD because their data

revealed that MICA/B mRNAs levels are significantly increased in NAFL and NASH patients when compared to normal healthy control livers. Additionally, expression of mRNAs of NKG2D ligands positively correlated with hepatocyte apoptosis (Kahraman et al., 2010). However, majority of research focused only on NK cells. Our data indicate that the link between obesity induced cellular stress and γδ T cells and IL-17 production is the upregulation of NKG2D H60 ligand on hepatocytes. Nevertheless, when H60 expression was directly compared between NCD and SSD fed groups, this difference was not statistically significant. As mentioned previously, expression of H60 is scarce on normal mouse hepatocytes, however elevated levels are detected in several liver conditions like viral infection or drug-induced liver injury (Cheng et al., 2009; Vilarinho et al., 2007). Since both infection and toxic liver injury are severe acute states, it is expected that they cause a rapid increase in the expression of stress induced ligands. In NAFLD, disease progression is relatively slow and in the initial period of diet-induced obesity only a minority of cells starts to accumulate fat and sense stress. We expected that H60 ligands are present only on a minority of stressed hepatocytes in steatotic lesions. Since we are not able to distinguish normal from stresses cells, this may occlude H60 induction on stressed hepatocytes in the SSD model. Nonetheless, by conducting a correlation study, I was able to see that in samples where I saw increased expression of H60 there was also more IL-17A produced by γδ T cells, indirectly confirming that these two events are connected.

In summary, my study established that γδ T cells are immune cell mediators that link cell induced stress due to nutrients' excess to early development of NAFLD. This process is mediated through NKG2D induced IL-17A production by γδ T cells. Thus, blocking of NKG2D or IL-17A signalling may be of great importance for preventing transition from simple steatosis to NASH and development of fibrosis.

6. CONCLUSIONS

- γδ T cells are enriched in visceral adipose tissue and even under normal conditions they can produce proinflammatory cytokines.

- In response to HFD, number of γδ T cells that produce IL-17A increases; however, it is not clear if they play a role in establishing inflammation in mouse model of DM2.

- SSD diet is a good model for NAFLD since it induced all stages of human disease course starting from simple steatosis to steatohepatitis with fibrosis.

- Initiating event in NAFLD, in response to SSD, is increase in the mRNA expression of NKG2D ligand H60 on hepatocytes.

- Engagement of NKG2D on γδ cells in SSD fed mice induces an increase in IL-17A production.

- IL-17A is an important cytokine for the development of liver pathology in our model of NASH.

- Lack of γδ T cells, NKG2D or IL-17A significantly decreases development of liver fibrosis.

7. LITERATURE

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List of abbreviations

Akt - protein kinase B

ASH - alcoholic steatohepatitis

BMI - body mass index

BSA - bovine serum albumin

Btn - butyrophilin

Btnl - butyrophilin -like

B6- C57BL6/J wild type strain

DAMPs - danger-associated molecular patterns

DIO - diet induced obesity

DM1 - diabetes mellitus type 1

DM2 - diabetes mellitus type 2, šećerna bolest tipa 2

DMEM - Dulbecco's modified eagle medium

DMSO - dimethyl sulfoxide

dsDNA - double-stranded DNA

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

FACS - fluorescence-activated cell sorting

FCS - fetal calf serum

FFA - free fatty acids

FPG - fasting plasma glucose

Grb-2 - growth factor receptor-bound protein 2

HbA1c - glycosylated haemoglobin

HCC - hepatocellular carcinoma

HFD - high fat diet, visoko-kalorijska prehrana bogata mastima

HMGB1 - high mobility group box-1

HSC - hepatic stellate cells

HSP - heat shock proteins

IFN - interferon

IGT - impaired glucose tolerance

IL - interleukin

ILC2- type 2 innate lymphoid cells

iNKT - invariant-chain natural killer T

IR - insulin resistance

IRS1-4 - Insulin receptor substrate 1-4

iNOS- inducible nitric oxide synthases, inducibilna dušik oksid sintetaza

KC - kupffer cells

LAMRI - Laboratory of mouse engineering and breeding facility of the Faculty of medicine, University of Rijeka

LPS - lipopolysaccharide

MCD - methionine choline deficient diet

MCP-1 - monocyte chemoattractant protein

MetS - metabolic syndrome

NAFL - non-alcoholic fatty liver

NAFLD - non-alcoholic fatty liver disease, nealkoholna bolest masne jetre

NASH - non-alcoholic steatohepatitis, nealkoholni steatohepatitis

NK- natural killer

NKG2D - NK cell receptor natural killer group 2D

NLR - NOD-like receptors

OGTT - oral glucose tolerance test

PAMP - pathogen-associated molecular patterns

PDGF - platelet deriver growth factor

PI3K - phosphatidylinositol 3-kinase pathway

PIP2 - phosphatidylinositol bisphosphate

PIP3 - phosphatidylinositol triphosphate

PKB - protein kinase B

PMA - phorbol 12-myristate-13-acetate

PRR - pattern-recognition receptors

RAE-1 - retinoic acid early inducible-1

RAS-MAPK - mitogen-activated protein kinase

RLR - RIG-I-like receptors

ROS - reactive oxygen species

RPMI - Roswell park memorial institute medium

SOCS - suppressor of cytokine signalling

SPF - specific pathogens free

SSD - steatosis-steatohepatitis diet

TGF-β - transforming growth factor beta

Th1 - type 1 T-helper

TLR - toll-like receptor

TNF - tumour necrosis factor

TRAIL - TNF-related apoptosis-inducing ligand

TRAIL-DR5 - death receptor 5

Treg - regulatory T cells

VAT - visceral adipose tissue

VLDL - very low-density lipoprotein

WHO - World Health Organization

WT - wildtype

α-SMA - α-Smooth Muscle Actin

9. CURRICULUM VITAE

Sonja Marinović (née Valentić), PhD student, was born August 9th, 1987. She graduated Molecular Biology at the Department of Biology at the Faculty of Science, University of Zagreb in 2011, with the topic "HER-2 / neu polymorphisms in tumorigenesis of sporadic colon cancer". She enrolled to the graduate studies in Biology – Division of Biology, Faculty of Science, University of Zagreb, in 2013.

Since November 2013 she has been employed as a research fellow – PhD student, at the Department of Histology and Embryology at the Faculty of Medicine, University of Rijeka and working on the UKF project „The role of pathogen-driven inflammation of visceral adipose tissue in the development of Diabetes Mellitus type II“ and HRZZ project "Immune mechanisms in the development of inflammation and metabolic syndrome in obesity" in the group of prof. dr. sc. Bojan Polić, MD. She went on research visit to the London Research Institute / CR-UK, London United Kingdom, from March to May 2015.

She is the co-author on four scientific papers published in journals with an international review indexed in CC. She has participated in several international scientific conferences with oral and poster presentations.

From academic year 2013/2014 she actively participates in teaching at the Department of Histology and Embryology. She teaches practical courses of Histology and Embyology for students of medicine and dental medicine and leads students who are doing laboratory work or graduate theses.

10. PUBLICATIONS

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(# These authors equally contributed to the work)