Technische Universität München

Fakultät für Medizin

Immunomodulation of metabolic tissue homeostasis

Sini Susan Joseph

Vollständiger Abdruck der von der Fakultät für Medizin
der Technischen Universität München zur Erlangung des
akademischen Grades eines Doktors der Naturwissenschaften
genehmigten Dissertation.

Vorsitzender : Prof. Dr. Percy A. Knolle

Prüfende der Dissertation:

1. Prof. Dr. Dr. h. c. Matthias H. Tschöp
2. Prof. Dr. Hannelore Daniel

Die Dissertation wurde am 30.09.2019 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 12.05.2020 angenommen.

Immunomodulation of metabolic tissue homeostasis

Dissertation zur Erlangung des

Doktorgrades der Naturwissenschaften

Sini Susan Joseph

Technische Universität München, Fakultät für Medizin

Helmholtz Zentrum München, Institut für Diabetes und Adipositas

Betreuer: Prof. Dr. Dr. h.c. Matthias H. Tschöp

March 2014 – December 2018

Table of contents

List of Abbreviations

GLP-1	Glucagon like peptide-1
GLP-1R	Glucagon like peptide-1 receptor
GLP-1/Dexa	Glucagon like peptide-1/Dexamethasone
ST2	Suppression of tumorigenicity 2
EGFR	Epidermal growth factor receptor
IL-6	Interleukin-6
NAFLD	Non alcoholic fatty liver disease
TNFα	Tumour necrosis factor alpha
IgM	Immunoglobulin M
IgD	Immunoglobulin D
IL-10	Interleukin 10
TGF-β	Transforming growth factor-beta
HFD	High fat diet
IgG	Immunoglobulin G
Bregs	B regulatory cells
Foxp3	Forkhead box P3
Tregs	T regulatory cells
IL2	Interleukin 2
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome
TCR	T cell receptor
CD	Cluster of differentiation
CD25	High affinity IL2 receptor alpha chain
PPAR- γ	Peroxisome proliferator-activated receptor gamma
IL-33	Interleukin 33
visWAT	visceral white adipose tissue
scWAT	subcutaneous white adipose tissue
BAT	Brown adipose tissue
AREG	Amphiregulin

Satb1	Special AT-rich sequence-binding protein-1
DPP4	Dipeptidyl Peptidase 4
GIP	Gastric inhibitory polypeptide
cAMP	cyclic adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
Ca^{2+}	Calcium
NFAT	Nuclear factor of activated T-cells
IL-1RA	Interleukin-1 receptor antagonist
Gp130	Glycoprotein 130
IL6Rα	Interleukin-6 receptor alpha
BSA	Bovine Serum Albumin
HBSS	Hank's Balanced Salt Solution
Cpt1b	Carnitine palmitoyl transferase 1b
Pdk4	Pyruvate dehydrogenase kinase 4
SNAT1	Sodium coupled neutral amino acid transporter 1
HFHS	High fat high sugar
SD	Standard diet
PBMC	Peripheral blood mononuclear cells
RBC	Red Blood Corpuscles
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
FCS	Fetal Calf Serum
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
PE	Phycoerythrin
FACS	Fluorescence –activated cell sorting
RPMI	Rosewell Park Memorial Institute
cDNA	complementary DNA
qPCR	Quantitative polymerase chain reaction
C5a	Complement component 5a
CXCL10	C-X-C motif chemokine ligand10
SDF-1	Stromal cell-derived factor 1
sICAM-1A	soluble intercellular adhesion molecule1
CXCL1	C-X-C motif chemokine ligand1
TIMP-1	Tissue inhibitor of metalloproteinase 1

MCSF	Macrophage colony-stimulating factor
EDL	Extensor Digitorum Longus
TA	Tibialis anterior
MLC	Myosin light chain
STAT3	Signal transducer and activator of transcription 3

List of figures

List of tables

Summary

Obesity and associated metabolic diseases are characterized by an enhanced inflammatory state that contributes to a plethora of dysfunctions in metabolically relevant organs. Immune cells have detrimental, but also beneficial effects resulting in tissue-specific metabolic perturbations. Disruption of immune tolerance and homeostasis is mainly caused by an imbalance in homeostatic and proinflammatory immune responses. Therefore, modulation of immune cells may be pivotal in restoring the disrupted tissue homeostasis with the goal to prevent or lower these metabolic aberrations. Herein I focused on immune-metabolic interventions utilizing pharmacological and physiological approaches. *Specifically, we addressed how GLP-1 (glucagon like peptide-1) receptor agonist (Quarta et al. 2017) and GLP1/Dexamethasone co-agonist on one side and physical activity on the other side impact tissue-residing immune cells and immune-metabolic crosstalk so as to interfere with these metabolic perturbations with the vision to lower the incidence of metabolic insults.*

GLP-1R agonists are known to have potential effects on glycemic control and weight loss, but its actions on the adaptive immune system remains largely unknown. We thus investigated the impact of GLP-1R agonist (Quarta et al. 2017) and GLP-1/Dexa co-agonist on immune cells in diet-induced obese mice. Our results show that GLP-1R agonist treatment reduced high fat diet induced B cells maturation in spleen that was absent in mice lacking GLP-1R pointing to a possible involvement of GLP-1R signaling in the B cell maturation process. Total T lymphocyte frequencies were also increased in circulation with GLP-1R agonist and in spleen upon treatment with GLP-1/Dexamethasone (GLP-1/Dexa) co-agonist. GLP-1R agonist and to a larger extent GLP-1/Dexa conjugate lowered significantly several inflammatory markers, thus ameliorating the systemic inflammatory state of diet-induced obesity.

Physical inactivity - a major contributing factor to obesity was studied as physiological intervention. The impact of voluntary wheel running on immune cells was investigated by focusing specifically on skeletal muscle-residing regulatory T cells. Regulatory T cells, characterized by the expression of the transcription factor Foxp3, limit inflammation and contribute to immune tolerance and tissue homeostasis. Upon exercise, *ex-vivo* Treg frequencies in skeletal muscles were significantly increased regardless of diet. To understand whether voluntary wheel running has a lasting effect on Treg frequencies we removed running wheels for 4 weeks prior to study end. Treg frequencies were increased to an even larger extend in these pre-exercised mice compared to mice with continuous access to running wheels, highlighting a potential memory effect of exercise on Treg maintenance post exercise. *De-novo* Treg induction capacity was also enhanced in the popliteal lymphnodes of pre-exercised mice. Increased levels of amphiregulin, ST2, EGFR and Ki67 indicated that upregulated *ex-vivo* muscle-residing Tregs in pre-exercised mice may be suppressive and proliferative. We found higher IL6 receptor levels on Tregs in pre-exercised mice and utilizing T cell specific IL6Rα KO mice we revealed that the exercise-induced Treg upregulation is IL6Rα signaling dependent. However, mice with muscle specific deletion of IL-6 demonstrated that exercise induced cytokine IL-6 derived from skeletal muscle is not required for increasing Treg frequencies. Further phenotypic characterization of Tregs from both T cell-specific IL6Rα and skeletal muscle IL6 KO revealed that both IL-6 and IL-6Rα signaling are involved in the upregulation of AREG, EGFR and ST2 levels in muscle-residing Tregs.

Taken together, these data demonstrate the involvement of immune cell modulations in the beneficial metabolic effects of our pharmacological and physical interventions. Moreover, the mechanistic details provide novel insights into the exercise-induced induction of immunomodulatory cells and suggests the potential for interventions aiming at maintaining or restoring tissue homeostasis, thereby resisting/lowering further metabolic insults.

Zusammenfassung

Stoffwechselerkrankungen wie Fettleibigkeit sind mit Entzündungsreaktionen verbunden und tragen zu einer Vielzahl von Fehlfunktionen in metabolisch relevanten Organen bei. Immunzellen können eine schädliche oder nützliche Rolle in diesen gewebespezifischen Stoffwechselstörungen spielen. Beeinträchtigungen der Immuntoleranz und der Homöostase können vor allem durch ein Ungleichgewicht der homöostatischen und proinflammatorischen Immunantwort entstehen. Eine Modulation von Immunzellen kann entscheidend sein um die gestörte Gewebe-Homöostase wiederherzustellen und damit diese Stoffwechselveränderungen zu verhindern oder zu verringern. In dieser Arbeit konzentrierte ich mich auf immunmetabolische Interventionen unter Verwendung pharmakologischer und physiologischer Ansätze.

Mit dem Ziel Adipositas bedingte Stoffwechselstörungen zu verringern haben wir die Effekte von GLP-1 (Glukagon ähnliches Peptid-1)-Rezeptor-Ko-Agonisten und körperlicher Aktivität auf gewebeansässige Immunzellen und auf die Interaktion von Immunzellen und Metabolismus untersucht. GLP-1R-Agonisten werden zur Blutzuckerkontrolle und Gewichtsabnahme eingesetzt. Obwohl deren Rezeptoren auf Immunzellen vorhanden sind, ist die Wirkung auf das adaptive Immunsystem weitgehend unbekannt. Daher haben wir zunächst die Auswirkungen von GLP-1R-Agonisten und Co-Agonisten auf Immunzellen bei Mäusen, die eine kalorienreiche Ernährung erhalten haben, untersucht. Unsere Ergebnisse zeigten, dass reife B-Zellen, die bei einer kalorienreichen Ernährung hochreguliert waren, durch Behandlung mit GLP-1R-Agonisten in der Milz reduziert wurden und dass Mäuse ohne GLP-1R ein erhöhtes Level an reifen B-Zellen aufwiesen. Beide Ergebnisse deuten auf eine mögliche Beteiligung des GLP-1R-Signalweges am B-Zell-Reifungsprozess hin. Außerdem waren die Gesamtfrequenzen der T-Lymphozyten im Blutkreislauf nach Behandlung mit dem GLP-1R-Agonisten sowie in der Milz nach Behandlung mit dem GLP-1-Koagonisten GLP-1/Dexa erhöht. Sowohl GLP-1R-Agonist als auch GLP-1/Dexa-Konjugat verbesserten die durch die

Ernährung induzierte systemische Entzündung, indem sie mehrere Entzündungsmarker, die bei Fettleibigkeit hochreguliert waren, signifikant senkten. Dies trifft vor allem auf das Konjugat zu.

Da körperliche Inaktivität einer der Hauptfaktoren für Fettleibigkeit ist und Skelettmuskeln ein metabolisch relevantes Organ sind, wurde als zweite Intervention die Wirkung von körperlicher Aktivität auf Immunzellen untersucht, wobei der Schwerpunkt auf Skelettmuskelansässigen regulatorischen T-Zellen lag. Regulatorische T-Zellen sind gekennzeichnet durch die Expression des Transkriptionsfaktors Foxp3 und begrenzen Entzündungsreaktion wodurch sie zur Immuntoleranz und Gewebehomöostase beitragen. Nach körperlicher Aktivität waren die ex-vivo Treg-Frequenzen in den Skelettmuskeln unter beiden Ernährungsbedingungen signifikant erhöht. Um zu verstehen, ob die körperliche Aktivität einen nachhaltigen Einfluss auf die Treg-Frequenzen hat, haben wir die Laufräder 4 Wochen vor Studienende entfernt. Die Treg-Frequenzen waren bei diesen Mäusen (pre-exercise) noch stärker erhöht als bei Mäusen mit ständigem Zugang zu Laufrädern, was einen potenziellen Memory-Effekt von sportlicher Bewegung bei der Aufrechterhaltung der Treg-Funktion unterstreicht. Zusätzlich war die De-Novo-Treg-Induktionskapazität in den Popliteal-Lymphknoten von Mäusen nach pre-exercise verbessert. Die hochregulierten ex-vivo muskelansässigen Tregs bei Mäusen mit pre-exercise wiesen erhöhte Amphiregulin, ST2, EGFR und Ki67 Werte auf, was darauf hindeutet, dass sie suppressiv und proliferativ sind. Die Mäuse mit pre-exercise zeigten außerdem höhere Expressionswerte von IL6-Rezeptoren auf Tregs. Unsere Studien mit körperlicher Aktivität in T-Zell-spezifischen IL6R KO-Mäusen belegten, dass die Treg-Hochregulierung IL6Ra-signalabhängig ist. Allerdings deuten Experimente mit Mäusen mit muskelspezifischer Deletion von IL-6 darauf hin, dass das durch Bewegung induzierte Zytokin IL-6 keine Voraussetzung für die erhöhten Treg-Frequenzen ist. Die weitere phänotypische Charakterisierung von Tregs aus beiden KOs ergab, dass sowohl IL-6 als auch IL-6R-Signale an der Hochregulation von AREG-, EGFR- und ST2-Levels in muskelansässigen Tregs beteiligt sind.

Zusammengenommen zeigen die hier präsentierten Daten eine Beteiligung des pharmakologischen also auch des physiologischen Ansatzes an den beobachteten Immunzellveränderungen. Darüber hinaus liefern die mechanistischen Details neue Erkenntnisse über die bewegungsinduzierte Induktion von immunmodulatorischen Zellen und verdeutlichen ihr Potenzial für Interventionen, die darauf abzielen, die Gewebshomöostase aufrechtzuerhalten oder wiederherzustellen um so weitere metabolische Störungen zu verhindern/verringern.

1. Introduction

1.1 Obesity-associated inflammation

Obesity and type 2 diabetes represent one of today's leading health concerns (Yumuk et al. 2015). The prevelance of obesity has increased dramatically over the last decades and based on the estimations by World Health Organization (WHO), more than 1.9 billion adults are over-weight and over 650 million are obese (WHO, Obesity and overweight fact sheet 2016). Obesity is characterized by comorbidities such as type 2 diabetes mellitus (Wilding 2014), Parkinson's disease (Chen et al. 2014), certain types of cancer (Berger 2014), Alzheimer's disease (Naderli et al. 2009), nonalcoholic fatty liver disease (NAFLD) (Koppe 2014), hypertension and cardiovascular diseases (Roche & Silversides 2013, Guo & Garvey 2016, Ortega et al. 2016). An equilibrium between energy intake and expenditure has to be maintained in order to prevent excess body weight gain. Disruption of this energy balance leads often to obesity and type 2 diabetes (Lustig 2006, Spiegelman & Flier 2001). The intake of high calorie diets and lack of physical activity are the major contributing factors (Must & Tybor 2005, Hill et al. 2012). The search for underlying mechanisms behind the pathogenesis of metabolic diseases revealed inflammation as a critical mediator of obesity-induced insulin resistance (Hotamisligil et al.1993, McArdle et al. 2013). Nutrient overload also activates inflammatory responses in metabolically relevant organs like adipose tissue (Xu et al. 2003), skeletal muscle (Hong et al. 2009), brain (De Souza et al. 2005), liver (Cai et al. 2005) and pancreas (Ehses et al. 2007). Chronic inflammation leads to the development of obesity-associated metabolic disorders, angiogenesis, cardiovascular diseases, cancer etc. (Kolb et al. 2018). Several studies highlight the role of pro-inflammatory interleukines and cytokines such as TNFα contributing to obesity-linked insulin resistance and type 2 diabetes (Hotamisligil et al.1993, Perry et al. 2015). Previous reports show that body fat mass in obese subjects strongly associates with increased levels of chronic inflammatory markers (Festa et al. 2001). Various pro-inflammatory and anti-inflammatory signaling pathways have been described at the interface of obesity and inflammation (Lumeng & Saltiel 2011). Therefore,

different therapeutic strategies aiming at the pathogenic processes linking inflammation and obesity would be highly beneficial.

1.2 Immune metabolism crosstalk

Immune cells play a major role in driving obesity-associated inflammation and can contribute to the metabolic dysfunction associated with obesity. Both immune and metabolic regulations are highly integrated and dependent on each other (Hotamisligil 2006). Obesity is associated with the accumulation of immune cells in several tissues (Lumeng & Saltiel 2011). Immune cells initiate a cascade of inflammatory events that contribute to pro-inflammatory cytokine secretion, defective insulin signaling and glucose uptake, resulting in systemic insulin resistance (Hotamisligil et al.1993, Xu et al. 2003). Several studies demonstrated that obesity can alter adipose tissue function, while adipose tissue is infiltrated by mast cells, neutrophils, dendritic cells and macrophages which produce pro-inflammatory cytokines in mice (Weisberg et al. 2003, Han & Levings 2013) as well as in humans (Cancello et al. 2006). These pro-inflammatory molecules like TNFα, a known key mediator of insulin resistance, have a direct effect on cellular metabolism (Hotamisligil et al.1994). Activation of inflammatory pathways has been observed in different tissues (Donath & Shoelson 2011). Murine and human skeletal muscles also show accumulation of inflammatory macrophages in obese conditions (Fink et al. 2014). Obesity can also induce a switch in the macrophage polarization towards an inflammatory phenotype (Lumeng et al. 2007). In addition to the innate immune system, adaptive immune cells are also implicated in the obesity associated tissue inflammation. In obese populations, both innate and adaptive immunity are compromised by several factors which include nutrient overload, oxidative stress and hormonal imbalance (Mishra et al. 2016, Furukawa et al. 2004). Obesity- associated inflammation is not only caused by the infiltration of effector immune cells but also by a reduction or alteration of immunoregulatory cells (Feurer et al. 2009, Nishimura et al. 2013). Several cellular and signaling networks link immune and metabolic systems together (Osborn & Olefsky 2012) and many beneficial immunologic pathways are found to be dysregulated in obesity (Brestoff & Artis 2015). Understanding how different interventions

influence the immune system could help in understanding the pathogenesis of metabolic diseases in detail. New therapeutic interventions based on immunomodulation could thus be advantageous in the management of insulin resistance and its associated co-morbidities.

1.2.1 The adaptive immune system in metabolic disorders

The adaptive immune system comprises T and B lymphocytes which enable immunological memory after the exposure and activation by a specific antigen. T lymphocytes are thymus-derived (Janeway et al. 2001) and express the surface antigens, CD4 (helper T cells) or CD8 (cytotoxic T cells). In a classical view, $CD4^+$ T cells comprise mainly Th1 and Th2 cells which produce pro-inflammatory and anti-inflammatory cytokines respectively (Osborn & Olefsky 2012). $CD8^+$ T cells secrete pro-inflammatory cytokines and contribute to macrophage recruitment and inflammation in obesity (Nishimura et al. 2009). Another subpopulation of $CD4^+$ T cells, known as regulatory T cells (Tregs) are the main mediators of immune suppression and dominant tolerance (Sakaguchi et al. 1995). Previous reports show that obesity- associated insulin resistance could be normalized with the transfer of $CD4^+$T cells in diet-induced obese mice (Winer et al. 2009). High-fat diet fed mice exhibited an increased total T cell number and a reduction in regulatory T cells in the skeletal muscles which correlated with insulin resistance (Khan et al. 2015). Diet-induced obese mice also had reduced Treg numbers in adipose tissue and upregulating Treg frequencies improved insulin sensitivity (Feuerer et al. 2009). In leptin deficient ob/ob mice, the induction of regulatory T cells has been shown to alleviate adipose tissue inflammation and insulin resistance (Ilan et al. 2010).

B lymphocytes are also part of the adaptive immune system. Apart from antibody production, B lymphocytes possess additional functions of secreting cytokines and the ability to act as an antigen presenting cell. They are characterized by the B-lymphocyte antigen, CD19 (Tedder & Issacs 1989), derive from bone-marrow and can either mature there or in the spleen. B cells undergo different transitional stages to become mature B cells and differentiate into marginal zone B cells or follicular B cells. These intermediate populations

can be identified by differential surface expression of immunoglobulins, IgM and IgD. Immature B cells express IgM molecule on the cell surface and mature B cells are marked by an additional appearance of IgD (LeBien & Tedder 2008, Loder et al. 1999, Yuan & Witte 1988, Shaikh et al. 2014, Janeway et al. 2001). Upon activation, mature B cells differentiate into short-lived plasmablasts, long-lived plasma cells secreting antibodies or into memory B cells (Nutt et al. 2015). Another subpopulation of B cells involved in the suppression of immune responses and immunomodulation are regulatory B cells (Bregs) which mediate their regulatory function mainly through the production of the anti-inflammatory cytokines, IL-10 and TGF-β (Mizoguchi & Bhan 2006, Iwata et al. 2011). Apart from conventional B cell markers, mouse Bregs express CD5 and CD1d (Yanaba et al. 2008). B lymphocytes also promote systemic and local inflammation in diet- induced obese mice. B cells accumulate in visceral adipose tissue of HFD-fed mice and exacerbate inflammation. B cells enhance the activation of T cells and promote insulin resistance through the production of pathogenic IgG antibodies (Winer et al. 2011, Shaikh et al. 2014). B cell developmental processes are also altered under high fat diet conditions (Winer et al. 2014). A potential beneficial effect of B cells in obesity was demonstrated by the IL-10 secreting Bregs. Nishimura et al. reported that Breg cell dysfunction enhanced adipose inflammation in DIO mice and adoptive transfer of Bregs ameliorated those effects (Nishimura et al. 2013). Diminished frequencies of Bregs were also observed in the blood samples from obese humans (Garcia-Hernandez et al. 2018). In every adaptive immune response, maintaining a balance between the effector T or B cells and the regulatory cells is critical in controlling the magnitude of immune responses and for establishing tolerance to self and non-self antigens.

1.3 Regulatory T cell - mediators of peripheral tolerance

Recognition of self versus non-self antigens is achieved by central or peripheral tolerance. Central tolerance happens during the T cell development in the thymus. If an auto-reactive T cell escapes negative selection, then another mechanism called peripheral tolerance helps to prevent their activation

(Sakaguchi et al. 1995, Thornton & Shevach 1998). Regulatory T cells (Tregs) play a pivotal role in the maintenance of immune system homeostasis and suppressing the effector responses. Tregs are the main mediators of peripheral immunological tolerance (Bilate & Lafaille 2012). Tregs are produced in the thymus or can also be induced from naive T cells in the periphery. Thymus-derived Tregs are already functionally mature and antigen-primed before encountering peripheral antigens (Sakaguchi et al. 2008). Tregs are characterized by the expression of the transcription factor, Forkhead box protein 3 (Foxp3) which is the main regulator for Treg development and function (Fontenot, Gavin & Rudensky 2003). Tregs also possess high expression of the IL2 receptor, CD25, essential for Treg development (Sakaguchi et al. 2007). In mice, mutations of the Foxp3 gene results in a fatal lymphoproliferative disorder as in scurfy mice, and in humans it leads to the immune regulation disorder, IPEX (Brunkow et al. 2001, Bennett 2001) highlighting the importance of Foxp3 in maintaining the normal immune homeostasis.

Tregs exert their action through several ways: They modulate antigen presenting cell function and suppress effector cells through production of inhibitory cytokines like IL10 and TGFβ (Asseman et al. 1999, Fahlen et al. 2005). They exert direct cytotoxic effects through secretion of granzyme and perforin (Cao et al. 2007) and can also disrupt the metabolic environment of effector T cells (Caridade et al. 2013). As Tregs do not always need direct contact, they can act on cells at their proximity as well, independent of an antigen, termed as bystander suppression (Thornton & Shevach 2000, Schmidt et al. 2012).

Naïve T cells can also acquire Foxp3 expression in the periphery. This process can be exploited to control unwanted immune responses. Previous studies have shown that induction of Tregs from the naïve T cells *in-vitro* is possible in the presence of TGF-β (Chen et al. 2003, Apostolou & von Boehmer 2004), but those Tregs were found to be functionally unstable (Floess et al 2007). Later, other studies demonstrated that Treg induction can be best achieved using stimulation with a strong agonistic ligand provided under sub-immunogenic conditions (Daniel & Boehmer 2011, Kretschmer et al. 2005, Gottschalk &

Corse E Allison 2010). Foxp3^{+}Tregs also express different homing receptors like adhesion molecules and chemokine receptors and these control their migration and localization to the inflamed tissues or sites and tumors (Campbell & Koch 2011). Tregs can also expand clonally *in-vivo* and *in-vitro* due to antigenic stimulation and can retain their suppressive function after expansion. Different stimuli can also lead to the expansion of Tregs *in-vivo* and *in-vitro* via certain molecules that are expressed on them (Sakaguchi et al. 2008).

1.4 Tissue-residing regulatory T cells

Apart from the lymphoid tissues, regulatory T cells are also present in different non-lymphoid tissues in both healthy and diseased conditions (Burzyn et al. 2013). The presence of Tregs are reported in skin (Gratz et al. 2013, Rodriguez et al. 2014), intestinal mucosa (Sefik et al. 2015, Luu et al. 2017), liver (Breous et al. 2009), lung (Arpaia et al. 2015), adipose tissue (Feuerer et al. 2009), skeletal muscle (Burzyn et al. 2013, Villalta et al. 2014), brain (Stubbe et al 2013), tumors (Facciabene et al. 2011) etc. They all possess a unique phenotype and have different functions beyond the modulation of immune responses. Apart from the suppression of effector T cell responses, tissue Tregs also impact other myeloid populations by inhibiting pro-inflammatory macrophages and promoting anti-inflammatory macrophages (Burzyn et al. 2013). They are also involved in the regulation of non-immunological responses including cardiac remodelling after myocardial infarction (Nahrendorf & Swirski 2014), skeletal muscle regeneration (Castiglioni et al. 2015), impact on glucose intolerance and insulin resistance (Eller et al 2011), liver fibrosis (Nunoya et al 2014) and ischemic stroke (Xia et al. 2016).

Each tissue-resident Treg population possesses unique tissue-specific characteristics and differs in their expression markers, chemokine receptors, effector molecules, targets and function. Adipose tissue Tregs express a tissue-antigen specific TCR repertoire and exhibit high levels of PPAR-γ, an adipose tissue-specific transcription factor which is crucial for their development (Cipolletta et al. 2012, Zeng et al. 2018). ST2, the receptor for interleukin-33 (IL-33) is also highly expressed in visceral adipose tissue Tregs. IL-33

signalling is shown to be essential for the development and maintenance of visWAT (visceral white adipose tissue) Tregs and sustaining their transcriptional signature (Vasanthakumar et al. 2015). Recent studies from our lab reported that different adipose depots like visWAT, scWAT and BAT also harbor distinct Treg frequencies (Kalin et al. 2017).

A specific population of Tregs was also identified in the skeletal muscle which displayed a special TCR repertoire and a distinct transcriptome (Burzyn et al. 2013). Muscle specific Tregs possess unique set of genes and chemokine receptors which contribute to their function. Several reports reveal the presence of increased amphiregulin (AREG) levels on Tregs, especially in the regenerating muscles (Burzyn et al. 2013). Amphiregulin is a growth factor and a type 2 cytokine produced by several innate and adaptive immune cell types as well as Tregs (Zaiss et al. 2006, Zaiss et al. 2015). AREG signals through its receptor EGFR (epidermal growth factor receptor). Unlike other EGFR ligands, AREG binds with a low affinity and instead of causing receptor internalization, degradation and negative feedback loops, it induces a sustained signal (Zaiss et al. 2013, Zaiss et al. 2015). AREG can act in an autocrine manner, enhances Treg activity and is critical for the efficient suppressive function of Tregs, tissue repair and regeneration (Zaiss et al. 2015, Dai et al. 2014). Certain muscle-specific antigens may also be involved in recruiting Tregs to the muscle and retaining them (Burzyn et al. 2013). Muscle-specific Tregs exhibit decreased expression of Satb1, which encodes a chromatin organizer and increased levels of ST2, the receptor for interleukin-33 as compared to the lymphoid organs (Burzyn et al. 2013, Kuswanto et al. 2016). These distinct characteristics of Tregs highlight their important biological implications within the specific local tissue environment.

Based on both immunological and non-immunological functions in distinct local environments, tissue-residing Tregs could be used as potential therapeutic targets in different disease conditions. Adipose tissue-resident Tregs are considered as a key regulator in obesity-associated metabolic disorders (Chen et al. 2013). White adipose tissue Tregs were greatly reduced in HFD fed mice and obese animal models (Feuerer et al. 2009). Obese humans also have

decreased circulating regulatory T cells (Wagner et al. 2013). Depletion of adipose tissue Tregs in diet-induced obese mice led to pro-inflammatory profiles and further worsening of metabolic control (Feuerer et al. 2009), whereas the expansion or adoptive transfer of Tregs significantly improved insulin sensitivity in obese mice (Ilan, et al. 2010, Eller et al. 2011). Recent reports from our lab show that cold exposure and adrenergic stimulation can induce regulatory T cells in the adipose tissue and thus could be a potential strategy for the treatment of obesity-related metabolic disorders (Kälin et al. 2017). Similarly, Treg numbers were decreased in the atherosclerotic lesions of both mice and humans (Ait-Oufella et al. 2006, derBoer et al. 2007) and adoptive transfer of Tregs caused a reduction in the lesion development, thus preventing cardiovascular diseases (Ait-Oufella et al. 2006, Foks, et al. 2015).

Muscle-residing Tregs are known to contribute to tissue repair by promoting polarization of anti-inflammatory macrophages or by acting on the satellite cells directly or through the production of IL-10 or AREG (Zhou et al. 2015, Castiglioni et al. 2015, Burzyn et al. 2013). In muscular dystrophy patients and mouse models, an accumulation of regulatory T cells suppresses muscle inflammation (Villalta et al. 2014) and facilitates skeletal muscle regeneration by increasing satellite cells (Burzyn et al. 2013). Another study using co-culture method shows the direct interaction of regulatory T cells and satellite cells leading to their expansion (Castiglioni et al. 2015). In aged injured mice, the decreased muscle regenerative capacity was shown to be associated with a diminished Treg accumulation and defect in their recruitment, proliferation and retention which was IL-33 dependent (Kuswanto et al. 2016). In infectious lung injury, Tregs exhibit a major role in tissue protection which is AREG dependent (Arpaia et al. 2015). Immunosuppressive capacity of the intrahepatic regulatory T cells could also be promoted by AREG through EGF receptor (Dai et al. 2014). Regulatory T cell functions in the intestine were shown to be promoted via ST2 by the release of alarmin IL-33 upon inflammation induced tissue damage (Schiering et al. 2014). A recent study highlights the role of brain regulatory T cells in potentiating neurological recovery during brain injury by suppressing astrogliosis with the help of AREG (Ito et al. 2019). Collectively, all these studies highlight the importance of several factors including IL-33/ST2

and AREG/ EGFR axis in impacting the tissue Treg accumulation, retention and function based on the local microenvironment. As impaired Treg frequencies and function is associated with several disease conditions, restoration of the homeostatic balance has a great therapeutic potential. Different methods to overcome this reduction of immunoregulatory cells could help in the restoration of metabolic homeostasis. Several approaches could thus be employed to target Tregs by promoting Treg cell generation, its expansion, suppressive functions, survival and its stability (Boehmer & Daniel 2013) for the development of novel treatment strategies.

1.5 GLP-1R agonists/co-agonists in metabolic diseases

Glucagon-like peptide-1 (GLP-1) is an incretin hormone released from gut in response to nutrient ingestion. Peripherally, GLP-1 decreases gastric motility (Tolessa et al.1998), stimulates insulin secretion in pancreas and improves insulin sensitivity (McDonald et al. 2002, Holst 2007). Centrally, it induces satiety due to its direct effects on the hypothalamic feeding centers and thus reduces weight gain (Gutzwiller et al. 1999, Turton et al. 1996). Owing to the rapid inactivation of GLP-1 by DPP4 (Dipeptidyl peptidase-4), several DPP4 inhibitors and modified GLP-1R agonists (incretin mimetics) with a prolonged half-life were developed (Pratley & Gilbert 2008, Agerso et al. 2002, Drucker & Nauck 2006). Drugs acting via GLP1R have been shown to be beneficial in improving insulin resistance as well as inflammation associated with metabolic disorders. Moreover, GLP-1R agonists enhance insulin secretion in a glucose-dependent manner (Meloni et al. 2013).

Different strategies are employed in the designing of GLP-1 co-agonists in order to enhance their pharmacological effects. One approach utilized combining multiple peptides into a single peptide in order to target different receptors at once to attain synergistic therapeutic effects as demostrated for a glucagon/GLP-1 co-agonist (Day et al. 2009) and a GLP-1/GIP co-agonist (Finan et al. 2013). Another approach employed GLP-1 as a cargo and was designed in such a way that the GLP-1 is capable of directing and releasing the active moiety specifically to the target tissues, thereby eliminating the unwanted

side effects. GLP-1/ estrogen (Finan et al. 2012) and GLP-1/Dexa (Quarta et al. 2017) were designed in this manner and exhibited enhanced metabolic effects in diet-induced obese mice as compared to their mono agonist controls.

Several studies utilizing GLP-1R agonists improved type 1 diabetes in animal models implicating GLP-1R dependent signaling pathways in immuno-regulatory processes (Zhang et al. 2007, Hadjiyanni et al. 2008). The GLP-1R transcripts were identified in several immune cell populations from lymphoid tissues and these receptors were found to be functionally active (Hadjiyanni et al. 2010). GLP1R activation leads to cAMP production in lymphocytes and regulates lymphocyte proliferation (Hadjiyanni et al. 2010). GLP-1 is shown to inhibit adipose tissue macrophage infiltration and inflammation in obese mouse model (Lee et al. 2012) and inhibited the secretion of inflammatory cytokines in macrophages in-vitro (Guo et al. 2016). However, despite these insights, the role of GLP1R analogs in controlling adaptive immune cells under diet induced obese condition is not much studied. As immune system and metabolism are highly integrated, the impact of GLP-1 agonists and co-agonists on the immune cell level could open up new mechanistic insights into the treatment of metabolic aberrations.

1.6 Exercise and its beneficial metabolic effects

Regular exercise has been known to have significant beneficial effects on various diseases including obesity, diabetes and cardiovascular disease whereas physical inactivity is a major contributing factor for the development of the latter (Pederson 2006, Booth et al. 2012, Must & Tybor 2005, Pederson 2009). High prevalence of obesity arises from the imbalance of energy expenditure and energy consumption (Spiegelman & Flier 2001). Life style interventions including increased physical activity are a primary preventive approach for metabolic diseases. It can also be employed as a treatment strategy. Long-term exercise training is capable of inducing physiological effects in many organs and leads to numerous molecular adaptations in the body (Fiuza-Luces et al. 2013). This includes changes on the transcriptional

and translational level, signaling pathways and in mitochondrial and metabolic adaptations (Egan & Zierath 2013, Howald et al. 1985, Phillips S.M. et al. 1996). Endurance exercise training promotes phenotypic adaptations in skeletal muscle by shifting towards a more oxidative phenotype (Röckl et al. 2007). It also promotes fibre type transformation, mitochondrial biogenesis, angiogenesis and improves insulin sensitivity and metabolic flexibility (Holloszy 1976, Yan et al. 1985). Exercise training also improved metabolic profiles and exerted anti-inflammatory effects in patients with type 2 diabetes (Kadoglou et al. 2007). Regular physical activity increases energy expenditure and reduces fat depots, thereby decreasing the proinflammatory and increasing the antiinflammatory factors (Gleeson et al. 2011). Exercise conditioning also improves regenerative response in the skeletal muscle of aged mice (Joanisse et al. 2016). Metabolic cues like AMP- activated protein kinase (AMPK) activated by contraction and energy deprivation plays an important role in skeletal muscle metabolic adaptations (Long & Zierath 2008). Endurance exercise training has also shown to upregulate fatty acid oxidation and to reduce the triglyceride content in the skeletal muscle of obese subjects (Louche et al. 2013). Thus integrating signals from the physiological stimuli and its co-ordination with the metabolic adaptations involve several players and is highly complex.

1.6.1. Immunomodulatory effects of exercise

Exercise exerts its beneficial effects through diverse mechanisms including modulation of immune cells (Terra et al. 2012). Immunomodulatory changes triggered by exercise depend on its intensity and duration (Schon & Weiskirchen 2016). Exercise is known to increase the number of circulating regulatory T cells (Clifford et al. 2017), anti-inflammatory cytokines, shifts the macrophage polarization to anti-inflammatory and decreases the number of circulating pro-inflammatory monocytes, thus inhibiting the inflammatory reactions within the body (Schon & Weiskirchen 2016, Yeh et al. 2007, Shaw et al. 2018). In the context of metabolic diseases, exercise mediates its anti-inflammatory effects mainly via modulation of immune cells (Lancaster &

Fabbraio 2014). In high-fat diet induced obese mice, exercise training has been shown to reduce macrophage infiltration into adipose tissue and induces its phenotypic switch to an anti-inflammatory profile. It also decreases proinflammatory cytokines, enhances angiogenesis and causes down regulation of toll-like receptors on immune cells leading to the overall reduction of systemic inflammation. (Kawanishi et al. 2010, You et al. 2013). Another study reports the reduction in obesity-induced osteoarthritis by voluntary wheel running in mice (Griffin et al. 2012). A role of immune cells in exercise induced muscle damage and repair is also known. Depending on the intensity of exercise, mechanical stress or impact can cause the release of alarmins which initiates a coordinated system by immune cells with an initial inflammatory phase followed by a resolution phase and finally the tissue repair phase. A range of different innate and adaptive immune cells participate in each phase to facilitate repair and thus restore homeostasis (Jones & Hoyne 2017). A recent study demonstrates that exercise enhances regulatory T cell responses in lungs in an asthma model (Lowder et al. 2010). However, the beneficial effects of exercise mediated via immune cells, especially adaptive immune cells in the context of metabolic diseases are largely unexplored.

1.7 Exercise induced myokines

Skeletal muscle fibres produce several factors including proteins, growth hormones and cytokines during muscle contractions or after exercise training. These muscle-derived factors are termed myokines (Pederson et al. 2003) and they exert autocrine, paracrine and endocrine effects (Pederson & Febbraio 2008, Pederson 2011, Schnyder & Handschin 2015). The endocrine effect of myokines is important in mediating the whole-body effects of exercise. This provides the basis for the crosstalk of skeletal muscle with other organs including adipose tissue, liver, pancreas, bone, brain etc. (Pederson 2006, Schnyder & Handschin 2015).

1.7.1 Role of IL-6 as a myokine

IL-6 is one of the widely studied myokines induced upon exercise. Various reports show a several fold increase in the circulating IL-6 levels upon exercise and it varies with exercise intensity and duration (Pederson & Febbraio 2008). A seminal work by the group of Febbraio revealed that skeletal muscle fibres are the source of exercise-induced IL-6 (Hiscock et al. 2004). In contrast to sepsis, exercise induced IL-6 production is not preceded with an increase in TNFα, a pro-inflammatory cytokine (Pederson & Febbraio 2008, Steensberg et al. 2002). It is mainly regulated by a network of signaling cascades including Ca^{2+}/NFAT (nuclear factor of activated T cells) pathways. Contraction induced IL-6 further triggers the production of IL-10 and IL-1 receptor antagonist (IL-1RA) thus setting up an anti-inflammatory mileu (Pederson & Febbraio 2008). Upon exercise training, the increase in plasma IL-6 levels are downregulated, whereas the skeletal muscle IL-6 receptor expression increases gradually, thereby enhancing the sensitivity to IL-6 (Pederson & Febbraio 2008, Keller et al. 2005).

IL-6 exerts its action locally within the muscles or peripherally in a hormone-like fashion. It can act on several other tissues or cells and can mediate important metabolic, anti-inflammatory and immune modulatory effects directly or indirectly (Karstoft & Pederson 2016, Huh 2018, Pederson 2011). Furthermore, exercise-induced IL-6 can stimulate glucagon-like peptide-1 (GLP-1) secretion, thus improving insulin secretion and glycemia (Ellingagaard et al. 2011). In skeletal muscle, it exerts beneficial effects on glucose homeostasis and lipid metabolism by enhancing peripheral glucose uptake and lipid oxidation via AMPK (Pederson & Febbraio 2008). It also increases hepatic glucose production and lipolysis in adipose tissue (Pederson & Fischer 2007, Pederson et al. 2001).

IL-6 also exhibits a broad effect on the cells of the immune system. IL-6 is essential for maturation, proliferation, differentiation and maintenance of B cells/plasma cells and several T cell subsets (Jourdan et al. 2014, Choi et al. 2013, Tvedt et al. 2007, Hunter & Jones 2015). IL-6 exerts its action via IL-6

receptor which exists in both membrane- bound as well as soluble form (Scheller et al. 2014). The membrane bound form is expressed by a limited number of cell types. The pleiotropic effects of IL-6 may be attributed due to its different signaling modes. The anti-inflammatory properties of IL-6 are associated with classical signaling in which IL-6 exert its action through its membrane receptor associated complex IL6Rα/gp130. In trans signaling, IL-6 binds to the soluble IL-6 receptor and the complex, further binding and activating gp130 and this refers to many of the pro-inflammatory effects of IL-6 (Taga et al. 1989, Rose-John 2012, Scheller et al. 2011, Scheller et al. 2014). Blocking IL-6 trans signaling is shown to prevent HFD-induced adipose tissue macrophage recruitment (Kraakman et al. 2015). The third type of signaling known as trans-presentation (cluster signaling) implies a mechanism in which IL-6 binds to the IL6R on the antigen presenting cells like dendritic cells which presents it to gp130 on T cells (Heink et al. 2017). The effect of IL-6 as a myokine on the immune cells is not much explored. Therefore, the possible cross-talk between myokines and the muscle-resident immune cells upon exercise could unravel new mechanisms underlying the beneficial effects of exercise.

2. Objectives

Chronic Inflammation is regarded as a nexus between obesity and the development of associated comorbidities, promoting dysfunctions in metabolically relevant organs. High calorie diets and physical inactivity are among the major contributing factors in the pathogenesis of obesity. Immune cells play detrimental or beneficial roles in these metabolic aberrations. Inappropriate activation of immune system inducing local inflammation, disturbance of immune tolerance and tissue homeostasis also occur during these metabolic complications. As both immune and metabolic regulations are highly integrated, several metabolic interventions might also involve modulation of immune cells a not much explored issue. Furthermore, understanding the immune metabolic interplay is crucial in gaining more insights and could open up new dimensions in the development of novel therapeutic strategies targeting both arms. Concerning immune-metabolic interventions, here we used a pharmacological and a physiological approach to study the impact on immune cells in metabolic tissues.

(1) The first objective was to study the impact of GLP-1 agonists and co-agonists on adaptive immune cells. Drugs acting via GLP-1 receptor are proven to be beneficial in improving insulin resistance and weight loss, but knowledge about their immunoregulatory role, especially on the adaptive immune cells under diet-induced obese condition is limited. The presence of functionally active GLP-1 receptor on several immune populations also provides a possibility for the involvement of these receptors in immune regulation. Moreover, Dexamethasone being an immunosuppressant, the GLP-1 co-agonist, GLP-1/Dexamethasone may exhibit a profound effect on the immune cell level to improve the inflammatory processes associated with obesity.

(2) The second objective was to study how physical activity may affect immune cell function. Exercise exhibits a multitude of beneficial effects through various mechanisms, including modulation of immune cells. Immune cell function in tissues is a key component in integrating immune metabolic crosstalk and

guiding tissue function and homeostasis. Tissue-residing regulatory T cells, known to mediate immune tolerance and tissue homeostasis, are at least partly disrupted in metabolic complications. In light of this, we hypothesized that some of the beneficial effects of exercise may be exerted via its impact on Treg frequencies and function in a tissue-specific manner that in turn would be beneficial in metabolic diseases. Here we mainly focused on regulatory T cells residing in skeletal muscle as they are metabolically relevant and a directly impacted organ upon exercise. Moreover, these results can also provide new mechanistic insights into the immune metabolic cross talk within the local tissue environment in order to potentially interfere with metabolic diseases.

3. Materials and Methods

Mice

All mice were maintained group-housed with a 12-h/12-h light - dark cycle at 23°C with free access to food and water at the animal facility of Helmholtz Zentrum München, Germany, according to the Institutional Animal Committee Guidelines. All animal experiments were approved and conducted under the guidelines of the Institutional Animal Care and Use Committee of the Helmholtz Center, Munich. Wild type mice used in all experiments were C57BL/6J mice (Charles River). T cell specific IL6RαKO ($IL6R\alpha^{TKO}$) mice (Nish et al.,2014) were kindly provided by Jens Brüning (Max Plank Institute, Cologne). GLP-1 global KO mice ($GLP1r^{-/-}$) were generated in-house (Quarta et al., 2017, Scrocchi et al.,1996). Muscle specific IL6KO mice were generated in-house using MLC^{Cre} mice ($Myl1^{tm1(Cre)Sjb}$/J, Jackson laboratories, stock #024713) expressing Cre recombinase under the control of myosin light chain promoters (Bothe et al., 2000) and $IL6^{f/f}$ mice (European Mouse Mutant Archive- EMMA). All the studies were performed in male mice.

Table 1: List of chemicals, kits and reagents

Reagent	Provider
Bovine Serum Albumin (BSA)	Sigma - Aldrich
Hanks' Balanced Salt Solution (HBSS)	Sigma - Aldrich
Interleukin-2 (IL-2) recombinant human	ReproTech
RPMI medium 1640	Gibco by life technologies
Sodium pyruvate solution 100mM	Sigma - Aldrich
Sodium hydrogen carbonate solution (7.5%w/v)	Biochrom AG
Penicillin- Streptomycin (Pen/Strep)	Sigma - Aldrich
Non essential amino acids (NEAA)	Merck Millipore
Foxp3 staining buffer set	eBioscience
Proteome Profiler Array kit (Mouse cytokine array Panel A)	R and D systems
Collagenase type II	Sigma - Aldrich
Fc block (CD16/CD32)	BD Pharmingen
Fixable Viability Dye eFlour450	eBioscience
Streptavidin PE	Invitrogen
Sytox Blue	Thermo Fischer Scientific
Calcium chloride	Roth
Fetal Calf serum (FCS)	PAA
Ethylene diamine tetra acetic acid (EDTA)	VWR
Collagenase IV	Life Technologies

Dulbecco's Modified Eagle Medium (DMEM/F12)	Invitrogen
Zombie aqua fixable viability kit	Biolegend
Dulbecco's Phosphate Buffered Saline (DPBS)	Life Technologies
RBC lysis buffer	Biolegend
Hydroxy ethyl- piperazine ethane sulfonic acid solution (HEPES) 1M	VWR
Qiazol lysis reagent	Qiagen
QIAshredder	Qiagen
miRNeasy Microkit	Qiagen
SsoFast™ Evagreen Supermix	Bio-Rad
iScript™ Advanced cDNA synthesis kit	Bio-Rad
RNeasy Mini kit	Qiagen
QuantiTect Reverse Transcription kit	Qiagen

Table 2: List of media and buffers used

Media and Buffer	Composition
Hank's Balanced Salt Solution with supplements ($HBSS^+$)	HBSS with 5% FCS, 10mM HEPES
Coating buffer	0.1M Sodium bicarbonate buffer, PH 8.2
Cell Culture medium ($RPMI^+$)	RPMI, 10%FCS, 1x Pen/Strep (100 U/ml Penicillin, 100 µg/ml Streptomycin), 50nM β-mercaptoethanol, 1mM Sodium pyruvate, 1x Non-essential amino acids
MACS-PBS	DPBS supplemented with 0.5% BSA, 2 mM EDTA
PBS+BSA	DPBS, supplemented with 0.5% BSA
Staining buffer	10% FBS in PBS
$DMEM/F12^+$	DMEM/F12 with10%FBS,1%Pen/Strep

Table 3: List of antibodies used

Antibody	Clone	Source
CD3 AF647	17A2	Biolegend
CD45R/B220 AF647	RA3-6B2	Biolegend
Ly6G/Ly6C AF647	RB6-8C5	Biolegend
CD11b PECy7	M1/70	Biolegend
Ly-6C Pacific Blue	HK1.4	Biolegend
F4/80 Pacific Blue	BM8	Biolegend
IgD Pacific Blue	11-26c.2a	Biolegend
IgM PECy7	RMM-1	Biolegend
CD3 Pacific Blue	17A2	Biolegend

CD4 AF488	GK 1.5	Biolegend
CD115(CSF-1R) AF488	AFS98	Biolegend
CD19 AF488	6D5	Biolegend
CD1d Pacific Blue	1B1	Biolegend
Rat IgG2a, Isotype control AF647	RTK2758	Biolegend
CD5 AF647	53-7.3	Biolegend
CD8a PECy7	53-6.7	Biolegend
CD19 FITC	6D5	Miltenyi
CD45R/B220 AF488	RA3-6B2	Biolegend
CD4 Alexa Flour 700	RM4-5	eBioScience
CD25 PerCPCy5.5	PC61	Biolegend
CD62L APC	MEL-14	eBioScience
CD44 PE	IM7	Biolegend
FITC Foxp3	FJK-16s	eBioScience
Ki67 Brilliant violet 605	16A8	Biolegend
CD3e	145-2C11	BD Pharmingen
CD28	37.51	BD Pharmingen
CD8a Pacific Blue	53-6.7	Biolegend
CD11b Pacific Blue	M1/70	BioLegend
B220 Pacific Blue	RA3-6B2	BioLegend
CD14 V450	rmC5-3	BD Biosciences
CD11c BV421	N418	Biolegend
EGFR AF647	D38B1	Cell Signalling
CD126 (IL6Ra)-PE	REA620	Miltenyi Biotech
AREG - Biotinylated	BAF989	R &D systems
ST2 BB700	U29-93	BD OptiBuild
Streptavidin-PE	S21388	ThermoFischer

Table 4: List of primer sequences for qPCR analysis

All the primers for qPCR analysis except 18S ribosomal RNA (Qiagen) were purchased from Sigma-Aldrich.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
Histone	ACTGGCTACAAAAGCCG	ACTTGCCTCCTGCAAAGCAC
Cpt1b	CGTACCAAGTAGCCAAGGCA	CAGGAACGCACAGTCTCAGT
18SribosomalRNA	Cat no# QT02448075	
Pdk4	GATTGACATCCTGCCTGACC	CATGGAACTCCACCAAATCC
SNAT1	TTACCAACCATCGCCTTC	ATGAGAATGTCGCCTGTG

Cpt1b (Carnitine palmitoyl transferase1b), *pdk4* (pyruvate dehydrogenase kinase 4), *SNAT1* (Sodium coupled neutral aminoacid transporter 1)

3.1 Diet and treatment

Mice had ad libitum access to water and standard diet (SD) (Altromin, #1314, Lage, Germany) or high fat high sugar diet (HFHS) composed of 58.0 % kcal from fat, 25.5 % kcal from carbohydrates (including 8 % sucrose) and 16.4 % kcal from protein (Research Diets, #D12331, New Brunswick, NJ). For the HFHS studies, mice were maintained on the diet from the age of 8 weeks for a minimum of 16 weeks before initiation of the pharmacological studies. Mice were randomized into the treatment groups based on the body weights. Compounds used in the study, GLP-1 agonist and GLP-1/Dexa (Finan et al., 2012, Quarta et al., 2017) were injected subcutaneously for 7 days daily at a dose of 100nmol/kg.

3.2 Proteome profiler (cytokine array)

Cytokine and chemokine levels in the plasma were evaluated using a proteome profiler array kit (Mouse cytokine Array Panel A, R&D systems) according to the manufacturer's instruction. Plasma samples (300µl) were incubated for one hour with the biotinylated detection antibody cocktail at room temperature. The membrane was blocked with the blocking buffer supplied by the manufacturer. The blocked membranes were then incubated with the samples overnight at 4°C. After the washings, membranes were incubated with HRP-conjugated Streptavidin at room temperature for 30 min. Signal was acquired using chemiluminescent detection reagents with LI-COR Odyssey and quantified using Image Studio Lite software (LI-COR BioSciences).

3.3 Voluntary wheel running experiments

The mice were randomly divided into sedentary, exercised, pre-exercised and continuous exercised groups at the age of 8 weeks. Mice were double-housed and for the exercised mice, the cages were equipped with voluntary running wheels. Exercised mice remained sedentary for the first 4 weeks and had access to wheels for the last 4 weeks. The pre-exercised mice had access to the wheels only during the first 4 weeks. HFHS diet was introduced to the specific groups during the last 4 weeks. Wheel running activity was monitored

by a computerized activity monitoring system. The number of revolutions was recorded using the wheel manager software (Med Associates Inc., St.Albans, USA). Body weights were monitored regularly. Running wheels of the exercised group were locked 24 hours prior to the study termination.

3.4 Murine cell isolation

For the isolation of PBMCs (Peripheral blood mononuclear cells), blood samples were centrifuged at 3000rpm for 10min at 4°C, supernatant was removed and pellets were further lysed using RBC lysis buffer (BioLegend), centrifuged at 1200rpm for 5min at 4°C and resuspended in staining buffer (10% FBS in PBS) for further staining.

Primary single cell suspensions from spleen and lymph nodes were prepared by gently grinding the tissue through a 70µm cell strainer in $HBSS^{+}$ (Sigma Aldrich-supplemented with 5%FCS and 10mM HEPES).

Skeletal muscles were collected in $HBSS^{+,}$ minced and digested using collagenase II (0.5mg/ml) and calcium chloride (0.18mg/ml) in PBS with 0.5% BSA at 37°C on a rotator for about 20-30 minutes. Cell suspension was passed through 70µM/100µM filter, centrifuged at 400g for 5min at 4°C. Pelleted cells were resuspended in $HBSS^{+}$ and stained for flow cytometric analysis.

3.5 Cell staining and flow cytometry

All single cell suspensions were incubated with Fc blocking reagent for 10 minutes to prevent unspecific binding of antibodies and thereafter incubated with fluorochrome- labeled antibodies for surface staining on ice in the dark for 30 minutes. For intracellular staining, cells were fixed for 30 minutes on ice using fixation buffer followed by permeabilisation with permeabilisation buffer from the Foxp3 staining buffer kit (eBioscience), and staining with intracellular antibodies (Foxp3, Ki67, AREG) for 30 minutes. For the biotinylated antibody (AREG), cells were further stained using a Streptavidin-PE conjugate (ThermoFischer Scientific). Antibodies and clones used are given in table 3. Following staining, cells were passed through a 40µm cell strainer (NeoLab) to remove any debris before acquisition.

3.6 Sample acquisition and analysis

For pharmacological studies, cells were analysed using a MACSQuant VYB (Miltenyi : Biotech, Germany). For exercise studies, cells were sorted and acquired using a BD FACS Aria III with FACS Diva software (Beckton Dickinson) with optimal compensation and gain settings. Dead cells were excluded based on forward and sideward scatter and staining with sytox blue (Thermo Fischer) or zombie aqua (BioLegend) or fixable viability dye eflour450 (eBioscience). All data were analysed using Flowjo software version 7.6.1 (Treestar Inc,OR).

3.7 *In-vitro* Treg induction assay

For Treg induction, murine naïve T cells ($CD4^{+}CD44^{low}$ $CD62L^{hi}$ $CD25^{-}$) from popliteal lymph nodes were FACS sorted on BD FACS Aria III cell sorting system and stimulated in 96 well plates. Plates were pre-coated with 5 µg/ml anti-CD3 and 5 µg/ml anti-CD28 in 0.1M sodium bicarbonate buffer (pH=8.2). Sorted cells were cultured for 18hours in the pre-coated plates with RPMI medium supplemented with 10%FCS, 1 mM sodium pyruvate, 50 mM β-mercaptoethanol (Amimed), 1x non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich) in the presence of 100 U/ml recombinant human IL-2 (Reprotech). To mimic subimmunogenic conditions *in-vitro*, cells were transferred into new uncoated wells after 18hours of stimulation. T cells were then cultured without stimulation for additional 36hours prior to analysis.

3.8 Gene expression analysis from tissues and cells

Total RNA from sorted $CD4^{+}$T cells of popliteal lymphnodes were extracted using Qiazol lysis reagent/miRNeasy Micro Kit according to manufacturer's instructions. mRNA from snap-frozen muscle and adipose tissue was extracted using RNeasy Mini kit (Qiagen). cDNA synthesis was performed with iScript™ Advanced cDNA synthesis Kit (Bio-Rad) for sorted T cells and Quantitect Reverse Transcription Kit (Qiagen) for tissues according to manufacturer's instructions.

Gene expression of T cells was profiled with qPCR technique using SsoFast EvaGreen Supermix (Bio-Rad) on a CFX96 Real-Time system. Expression levels of each gene were normalized to the house keeping genes histone and 18S. Primer sequences used for the qPCR are listed in table 4. For the tissues, qPCR was performed with a ViiA 7 Real-time PCR system (Applied Biosystems) using taqman probe (IL6-Mm00446190_m1, Thermofisher Scientific). Target gene expression was normalized to the reference gene, HPRT (HPRT-Mm01545399_m1, Thermofisher Scientific) and calculated relative to controls.

3.9 Genotyping

Genotyping of KO mice were performed from the ear punches by PCR followed by gel electrophoresis. For IL6Rα^{TKO} mice, following primers were used for IL6R flox (5GK12: CCGCGGGCGATCGCCTAGG, 5IL6E3: CCAGAGGAGCCCAAGCTCTC, 3IL6A: TAGGGCCCAGTTCCTTTAT, Sigma Aldrich) and for CD4 cre (RO289: TGTGGCTGATGATCCGAATA, RO290: GCTTGCATGATCTCCGGTAT). For muscle IL6KO mice, following primer sets were used, IL6flox primers (forward - 5'-CCCACCAAGA ACGATAGTCA -3', reverse - 5'- GGTATCCTCTGTGAAGTCCTC -3', Sigma Aldrich) and Cre (oIMR0042: CTAGGCCACAGAATTGAAAGATCT; oIMR0043: GTAGGTGGAAATTCTAGCATCATCC; oIMR1084: GCGGTCTG GCAGTAAAAACTATC; oIMR1085: GTGAAACAGCATTGCTGTCACTT, Sigma Aldrich).

3.10 Statistics

All statistical analysis was performed using Graphpad prism 8. Results are presented as the mean and standard error of the mean (SEM) or as percentages, wherever appropriate. Comparisons of two groups were done using unpaired student's t test and for more than two groups, one- or two-way ANOVA followed by Tukey's multiple comparisons test was used. $P<0.05$ was considered to be statistically significant. Statistical significance is shown as *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$

4 RESULTS

Targeted modulation of the immune metabolic crosstalk to alleviate metabolic disease and inflammation

Immune cells play a major role in the inflammatory processes associated with obesity (Osborn & Olefsky 2012) in which immune tolerance and tissue homeostasis are also disrupted (Han & Levings 2013). Immune-metabolic interventions could be helpful in modulating immune cell function that are contributing to metabolic disease and inflammation. Here we utilized a pharmacological and an exercise approach to understand whether the immune-metabolic cross-talk can be modulated, an area of research that is still not clearly understood.

4.1 Role of the newly developed endocrine co-agonist GLP-1/Dexamethasone (GLP-1/Dexa) in modulating immune cell function

GLP-1, an incretin hormone reduces the blood glucose levels by enhancing insulin secretion in a glucose-dependent manner. GLP-1R agonists are known to be beneficial in improving insulin resistance as well as obesity-associated inflammation (Lee & Jun 2016). GLP-1R agonists and their impact on immune cells and their influence on the immune metabolic cross talk remains incompletely understood. Previous reports showing that GLP-1 receptor (GLP-1R) is expressed on immune cells (Hadjiyanni et al. 2010) opens up the possibility to target systemic inflammation exclusively in GLP-1 receptor expressing cells. In the first part of this thesis, we looked at the effects of a newly developed GLP-1 co-agonist (GLP-1/Dexa) on peripheral immune cells compared to GLP-1 monoagonist (Quarta, et al. 2017) specifically in peripheral circulation and spleen.

4.1.1 Peripheral immune cell profile changes upon HFHS exposure

HFHS exposure induces several changes in immune cell populations from circulation as well as from tissues (Kosaraju et al. 2017, Zhao et al. 2018). Obese subjects exhibit an increased production of circulating pro- inflammatory cytokines (Dicker et al. 2013). The changes in the different immune cell populations and their frequencies vary depending on the type and duration of high-fat diet. Before addressing pharmacological effects of GLP-1/Dexa on immune cells, we performed immune cell profiling in circulation and spleen of diet-induced obese mice focusing mainly on total T and B lymphocytes, B regulatory cells and on B cell maturation stages and subsets. Total T lymphocytes were increased in PBMC and decreased in spleen upon HFHS diet, while total B cells were not affected (Fig 1,2 a-b). Within the subset of T lymphocytes, $CD4^+$ T cells decreased and $CD8^+$ T cells were significantly increased in circulation under HFHS condition, but not in spleen indicating a more inflammatory milieu in circulation (Fig 1c, 2d).

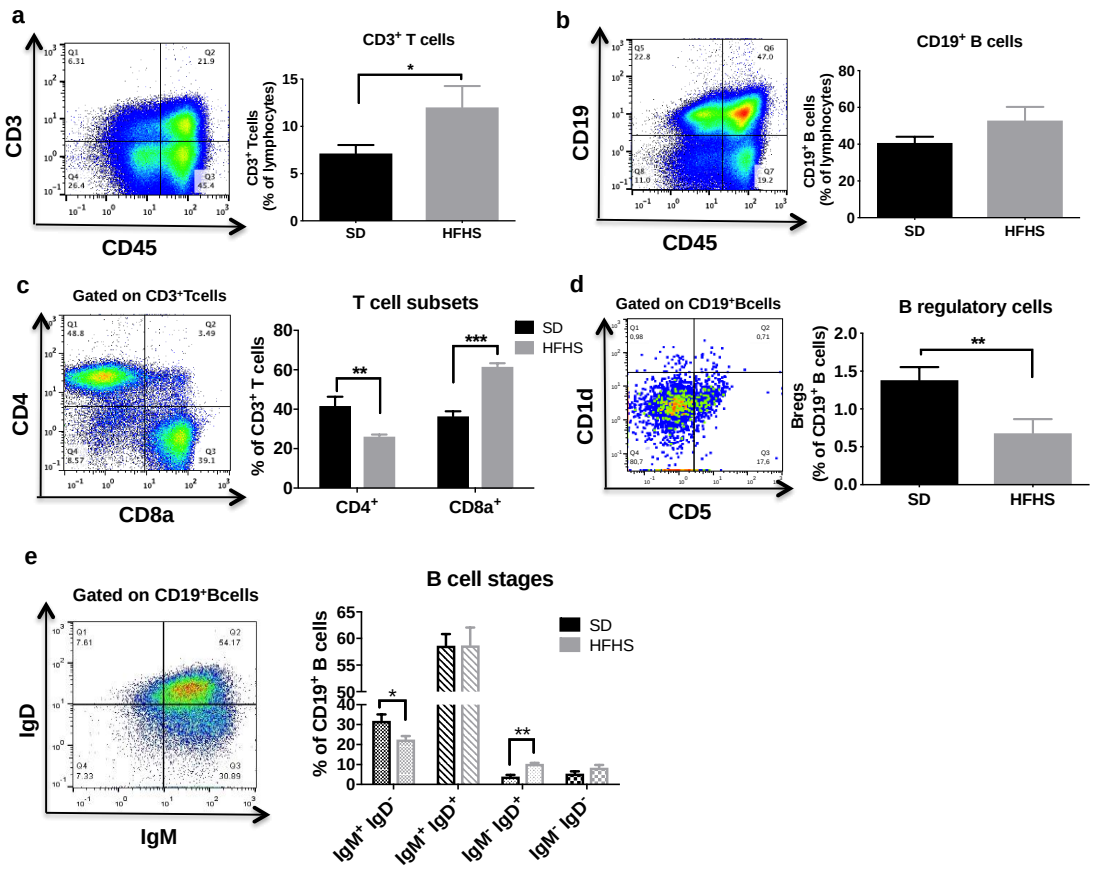


Figure 1: Immune cell profile in PBMC upon HFHS diet
Flow cytometric detection of (a) total CD3$^+$Tcells, (b) total CD19$^+$Bcells, (c) CD4$^+$ and CD8a$^+$ Tcells (d) B regulatory cells (Bregs) identified as CD19$^+$CD5$^+$CD1d$^+$ B cells, (e) the different B cell maturation stages. Data represent mean±SEM (n=3).*P < 0.05, **P < 0.01, ***P< 0.001 based on student's unpaired t test (a,d) or multiple t test (c,e).

B regulatory cells (Bregs) defined as CD19$^+$CD5$^+$CD1d$^+$ and known to be anti-inflammatory (Shaikh et al. 2014), were significantly decreased in both PBMC (Fig 1d) and spleen (Fig 2c) upon HFHS diet.

In circulation we found that immature B cells (IgM$^+$IgD$^-$) decreased and while IgM$^-$IgD$^+$ B cells increased upon HFHS diet feeding (Fig 1e). Similar to our findings in circulation, percentages of IgM$^+$IgD$^-$ B cells in spleen were also reduced. Exclusively in spleens mature B cells (IgM$^+$IgD$^+$) increased significantly (Fig 2e) upon HFHS diet feeding, indicating an imbalance in B cell development.

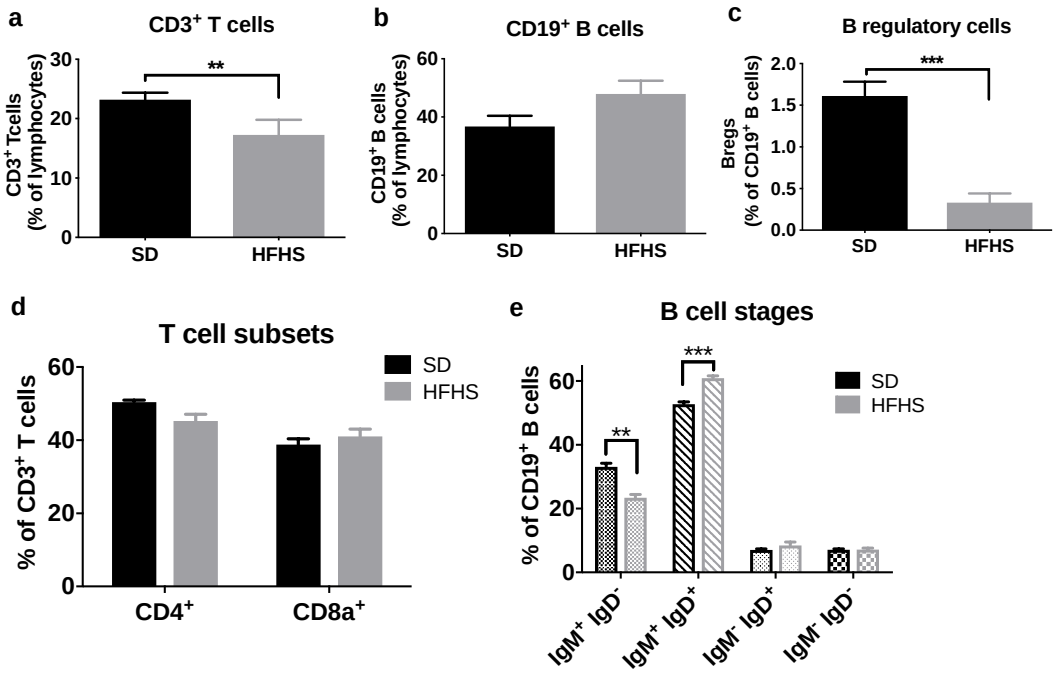


Figure 2: Immune cell profile in Spleen upon HFHS diet
Flow cytometric detection of (a) total CD3$^+$Tcells, (b) total CD19$^+$Bcells, (c) B regulatory cells (Bregs) identified as CD19$^+$CD5$^+$CD1d$^+$ B cells, (d) CD4$^+$ and CD8a$^+$ Tcells (e) the different B cell maturation stages. Data represent mean±SEM (n=3).*P < 0.05, **P < 0.01, ***P< 0.001 based on student's unpaired t test (a,c) and multiple t-test (e).

4.1.2 Loss of GLP-1 receptor causes an increase in mature B cell levels

GLP-1 receptors are expressed in several immune populations and are functionally active (Hadjiyanni et al. 2010). In order to gain a greater understanding of the role of GLP1R in immune regulation, mice with a global deletion of GLP-1 receptor (GLP1r $^{-/-}$) (Quarta et al. 2017) were profiled under standard diet conditions. In KOs, a significant increase in mature B cells (IgM^+IgD^+) was observed in both PBMC and spleen, whereas IgM^+IgD^- B cells were reduced in spleen (Fig 3e,4e) implicating that GLP-1 R may be involved in the B cell maturation process directly or indirectly. There were no differences in total T and B lymphocytes between the genotypes (Fig 3,4 a-b). Bregs, $CD4^+$ and $CD8a^+$ T cells were also unaltered in the absence of GLP-1R (Fig 3,4 c-d).

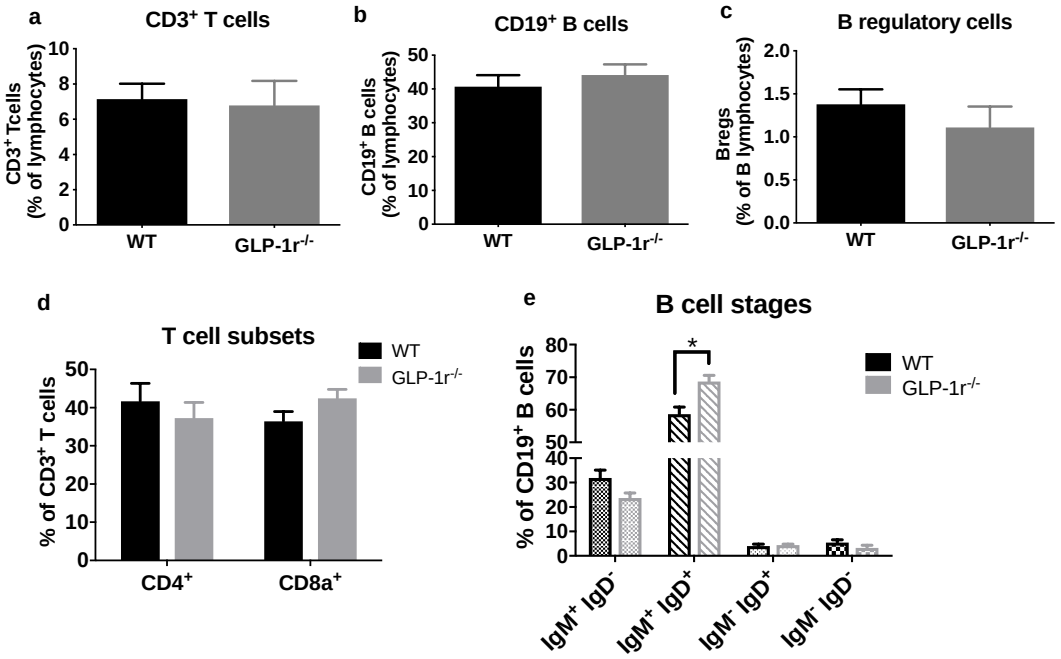


Figure 3: Immune cell profile of PBMC in GLP1r$^{-/-}$ mice
Flow cytometric detection of (a) total $CD3^+$ T cells, (b) total $CD19^+$ B cells, (c) B regulatory cells (Bregs) identified as $CD19^+CD5^+CD1d^+$ B cells, (d) $CD4^+$ and $CD8a^+$ Tcells and (e) the different B cell maturation stages in PBMC. Data represent mean± SEM (n=3).*P < 0.05 based on student's unpaired multiple t test.

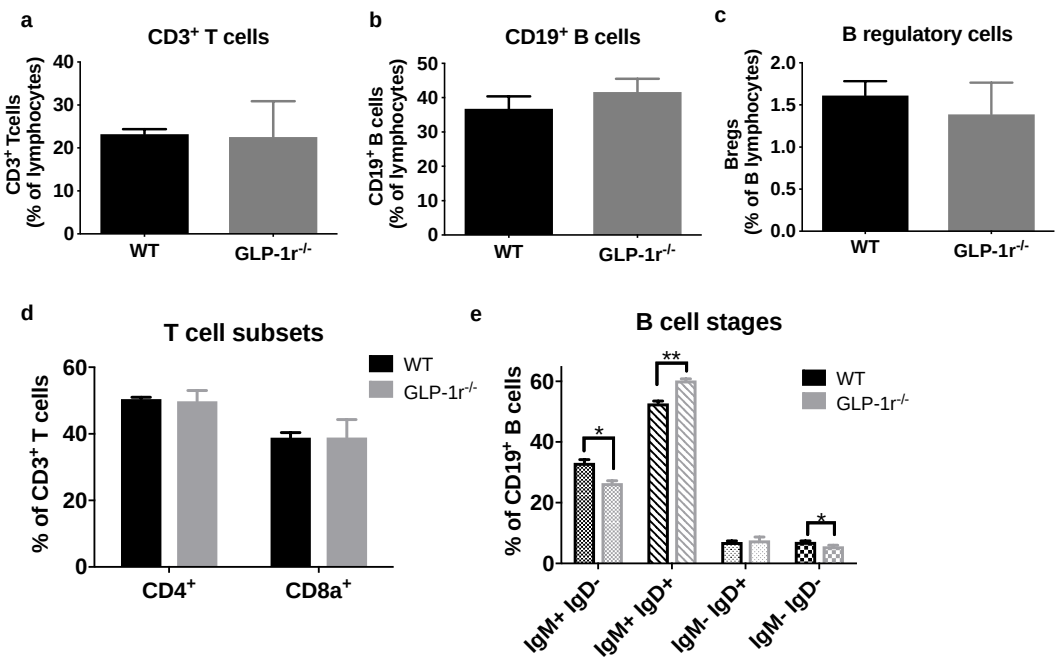


Figure 4: Immune cell profile of Spleen in GLP1r$^{-/-}$ mice
Flow cytometric detection of (a) total CD3$^+$ T cells, (b) total CD19$^+$ B cells, (c) B regulatory cells (Bregs) identified as CD19$^+$CD5$^+$CD1d$^+$ B cells, (d) CD4$^+$ and CD8a$^+$ T cells and (e) the different B cell maturation stages in spleen. Data represent mean± SEM (n=3).*P < 0.05 based on student's unpaired multiple t test.

4.1.3 GLP-1R agonist treatment decreases mature B cells in spleen and increases T cells in circulation

To study the impact of GLP-1R agonists on immune cells, HFHS fed mice were treated *in-vivo* with GLP-1R agonist (Quarta et al. 2017) and the spleen and PBMC were profiled for immune cell populations. Total T cells were increased in circulation (Fig 5a), whereas total B lymphocytes, helper and cytotoxic T cells as well as Bregs were unaltered in both PBMC and spleen (Fig 5b-d,6b-d). Among B lymphocyte subsets in spleen, mature B cells (IgM$^+$IgD$^+$) were significantly decreased upon GLP-1R agonist treatment (Fig 6e).

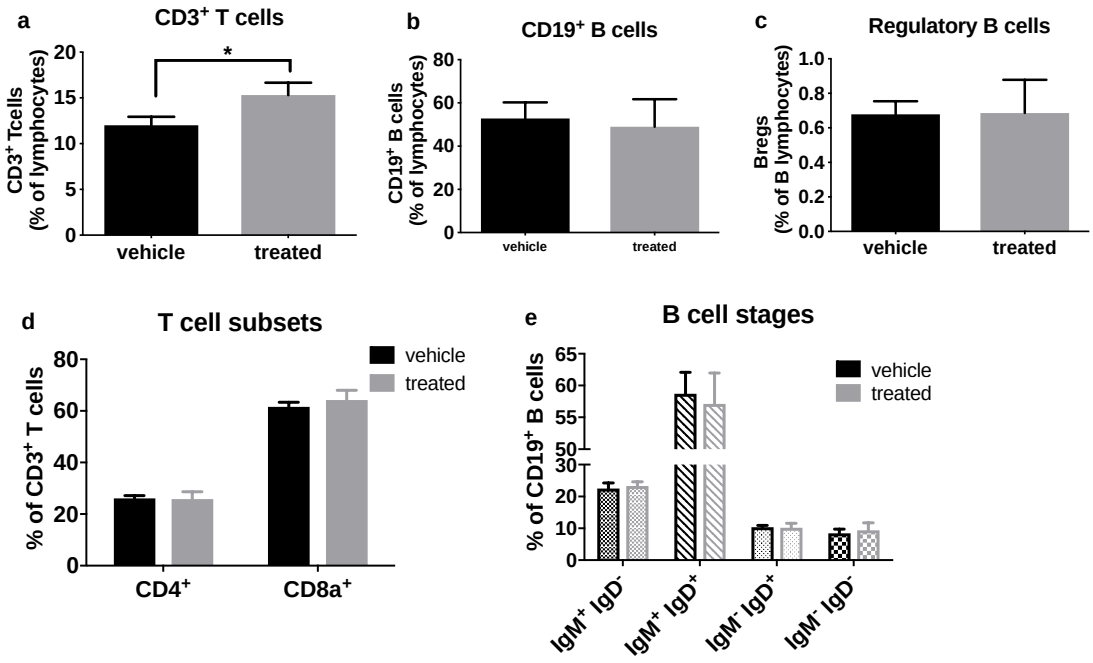


Figure 5: Immune cell profile in PBMC upon GLP-1R agonist treatment
Flow cytometric detection of (a) total $CD3^+$ T cells, (b) total $CD19^+$ Bcells, (c) B regulatory cells (Bregs) identified as $CD19^+CD5^+CD1d^+$ B cells, (d) $CD4^+$ and $CD8a^+$ T cells and (e) the different B cell maturation stages. Data represent mean±SEM (n=6).*P < 0.05, **P < 0.01, ***P< 0.001 based on student's unpaired t test.

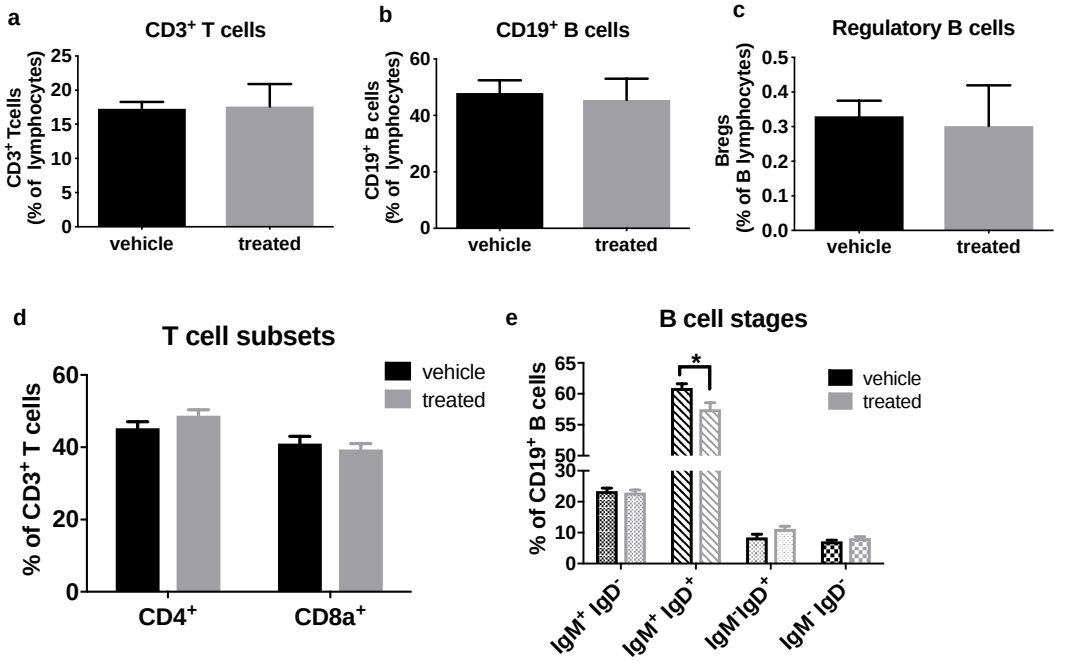


Figure 6: Immune cell profile in Spleen upon GLP-1R agonist treatment
Flow cytometric detection of (a) total $CD3^+$ T cells, (b) total $CD19^+$ B cells, (c) B regulatory cells (Bregs) identified as $CD19^+CD5^+CD1d^+$ B cells, (d) $CD4^+$ and $CD8a^+$ T cells and (e) the different B cell maturation stages. Data represent mean±SEM (n=6).*P < 0.05, **P < 0.01, ***P< 0.001 based on student's unpaired multiple t-test.

4.1.4 GLP-1/Dexa conjugate does not alter total T and B lymphocyte frequencies in PBMC, but increases the total T cells in spleen

GLP-1 co-agonists are designed in such a way that GLP-1 is capable of directing and releasing the active moiety specifically to the GLP-1 target tissues (Finan et al. 2012, Quarta et al. 2017). GLP-1/Dexa is a unimolecular compound comprising of GLP-1 and dexamethasone and shows enhanced metabolic effects as compared to its mono-agonist controls (Quarta et al. 2017). Dexamethasone being an anti-inflammatory agent and due to the presence of GLP-1R on immune cells, the conjugate could be specifically delivered to immune cells, thereby enhancing their action. To address that, we studied the impact of GLP-1/Dexa conjugate on the immune cell populations in HFHS fed mice. Mice were injected with the conjugate subcutaneously for 6 days and PBMCs and spleen were processed and stained. There were no significant changes in total T and B lymphocytes as well as B regulatory cells in circulation, whereas total T lymphocyte frequencies were increased in spleen (Fig 7,8 a-d). We could also see a slight reduction in the splenic cytotoxic $CD8a^+$ T cells ($p=0.08$) upon treatment (Fig 8c).

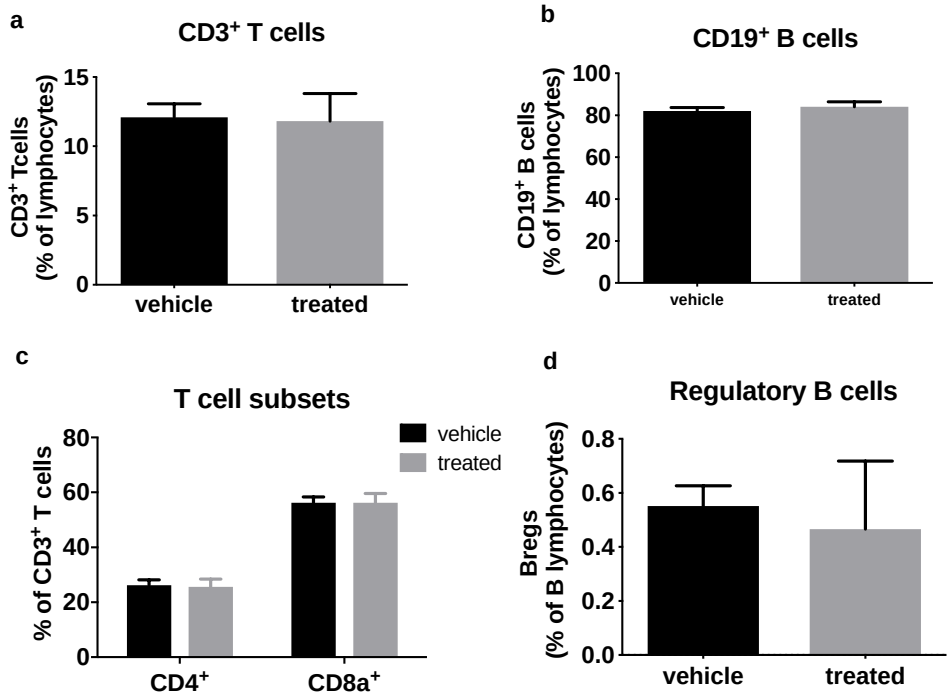


Figure 7: Immune cell profile of PBMC upon GLP-1/Dexa treatment
Summary graph showing the flow cytometric detection of total a) $CD3^+$Tcells b)$CD19^+$Bcells, c) $CD4^+$,$CD8^+$ Tcells and d) B regulatory cells (Bregs) identified as $CD19^+CD5^+CD1d^+$ B cells in PBMC. Data represent mean ± SEM (n=6).

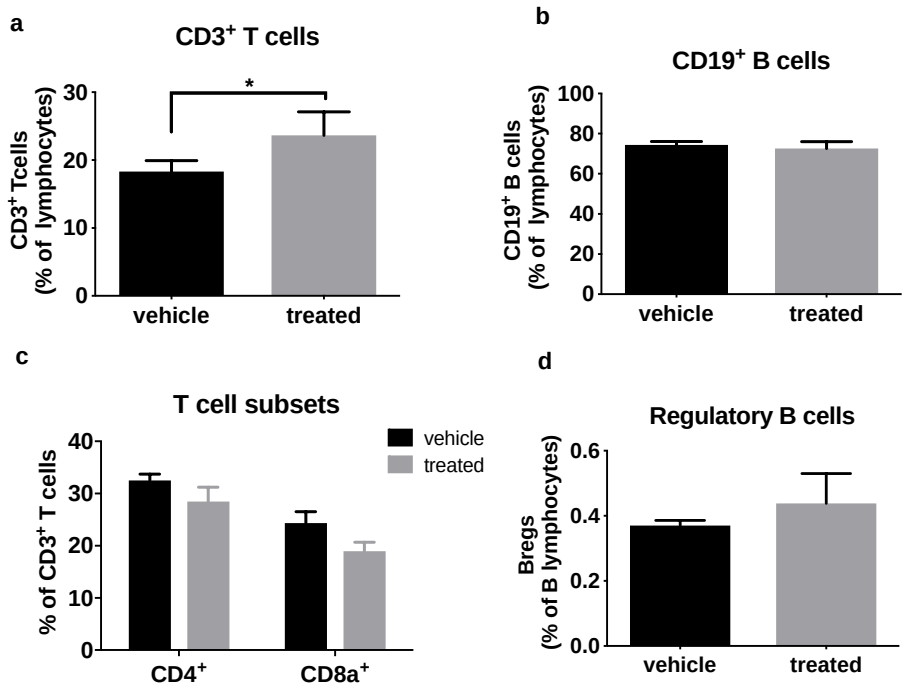


Figure 8: Immune cell profile of Spleen upon GLP-1/Dexa treatment
Summary graph showing flow cytometric detection of total a) $CD3^+$Tcells and b) $CD19^+$Bcells, c) $CD4^+$,$CD8a^+$ Tcells and d) B regulatory cells (Bregs) identified as $CD19^+CD5^+CD1d^+$ B cells in spleen. Data represent mean ± SEM (n=6). *P < 0.05, based on student's unpaired t test.

4.1.5 GLP-1/Dexa conjugate ameliorates diet-induced systemic inflammation

In order to get a broader perspective regarding systemic immunometabolic effects of GLP/Dexa, plasma levels of the inflammatory markers altered during obesity were studied. Plasma from the *in-vivo* GLP-1/Dexa, GLP-1 agonist, Dexamethasone and vehicle treated 16 week HFD fed mice were analyzed using a proteome profiler array. Several pro-inflammatory cytokines/chemokines were reduced upon treatment (Quarta, et al. 2017). GLP-1/Dexa conjugate induced a prominent reduction in the chemotactic peptides C5a and sICAM-1A which are involved in high-fat diet induced vascular inflammation (Osaka et al. 2016, Shai et al. 2006). Chemokines like CXCL1, CXCL10, SDF-1 and interleukin16 are all involved in the development of insulin resistance and diabetes (Dufour et al. 2002, Kim et al. 2014a, Meagher et al. 2010) and were found to be decreased by the treatment. Inflammatory markers known to be upregulated in obesity including TIMP-1, MCSF (Kralisch et al. 2007, Levine et al. 1998) and TNFalpha were also

reduced. The mono-agonistic controls also induced comparable systemic anti-inflammatory actions, but C5a, sICAM1 and TNFalpha were lowered to a greater extent by the conjugate.

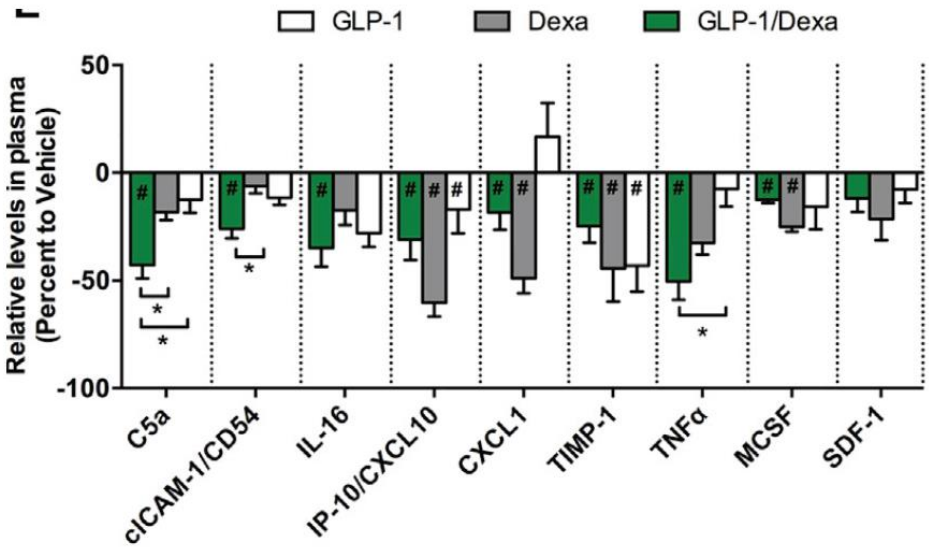


Figure 9: Cytokine profile of plasma upon GLP-1/Dexa treatment
Plasma cytokines analyzed from n=3 independent experiments by cytokine antibody array in mice treated with vehicle, GLP-1/Dexa, GLP-1 and Dexa for 6 days. #p<0.05 comparing compound treatment versus vehicle and *p< 0.05 comparing GLP-1/Dexa versus GLP-1 or Dexa by ANOVA, expressed as mean ± SEM.

These results show that different cytokines and chemokines upregulated in obese conditions are altered by the conjugate, thus lowering the high fat diet induced systemic inflammation. More studies need to be done in order to dissect out the specific immune cell populations involved and the underlying mechanisms.

4.2 Role of physiological interventions (exercise) in modulating local immune cell function

Since physical inactivity is a major contributing factor to metabolic disorders like obesity (Pietiläinen et al. 2008), the importance of exercise in regulating body weight and other metabolic parameters is remarkable. Exercise exerts its beneficial effects through various mechanisms, including modulation of immune

cells. Immune cell function in tissues is a key component in integrating immune-metabolic crosstalk and guiding tissue function and homeostasis (Sharma & Rudra 2018). Focusing on a specific immune cell population at the tissue-specific level is necessary to determine precisely the immune-metabolic interplay in the metabolic target tissues. Regulatory T cells (Tregs), a subset of T lymphocytes are known to mediate immune tolerance and tissue homeostasis (Sakaguchi et al. 2008) which is at least partially disrupted in these metabolic complications (Deng et al. 2017). So, here we addressed whether exercise can impact Treg frequencies and functions in a tissue-specific manner which in turn would be beneficial in metabolic disorders like obesity, specifically focusing on skeletal muscle-residing Tregs.

4.2.1 Identification of $CD4^+$T cells and Tregs of skeletal muscles in the steady state

Initially, the presence of T cells and Tregs in the skeletal muscles was evaluated in the steady state. For this, different fast and slow-twitch muscles like soleus, EDL (Extensor Digitorium Longus), TA (Tibialis anterior) and gastrocnemius were isolated from mice and digested in order to isolate lymphocyte populations. $CD4^+$T cells and regulatory T cells ($CD4^+Foxp3^+$ T cells) present in all muscles could be identified in the steady state (Fig 10a,b). Treg frequencies differ in all muscle types, the highest being present in soleus, the predominantly oxidative muscle which was significantly different from the other predominantly glycolytic muscles (Fig 10c).

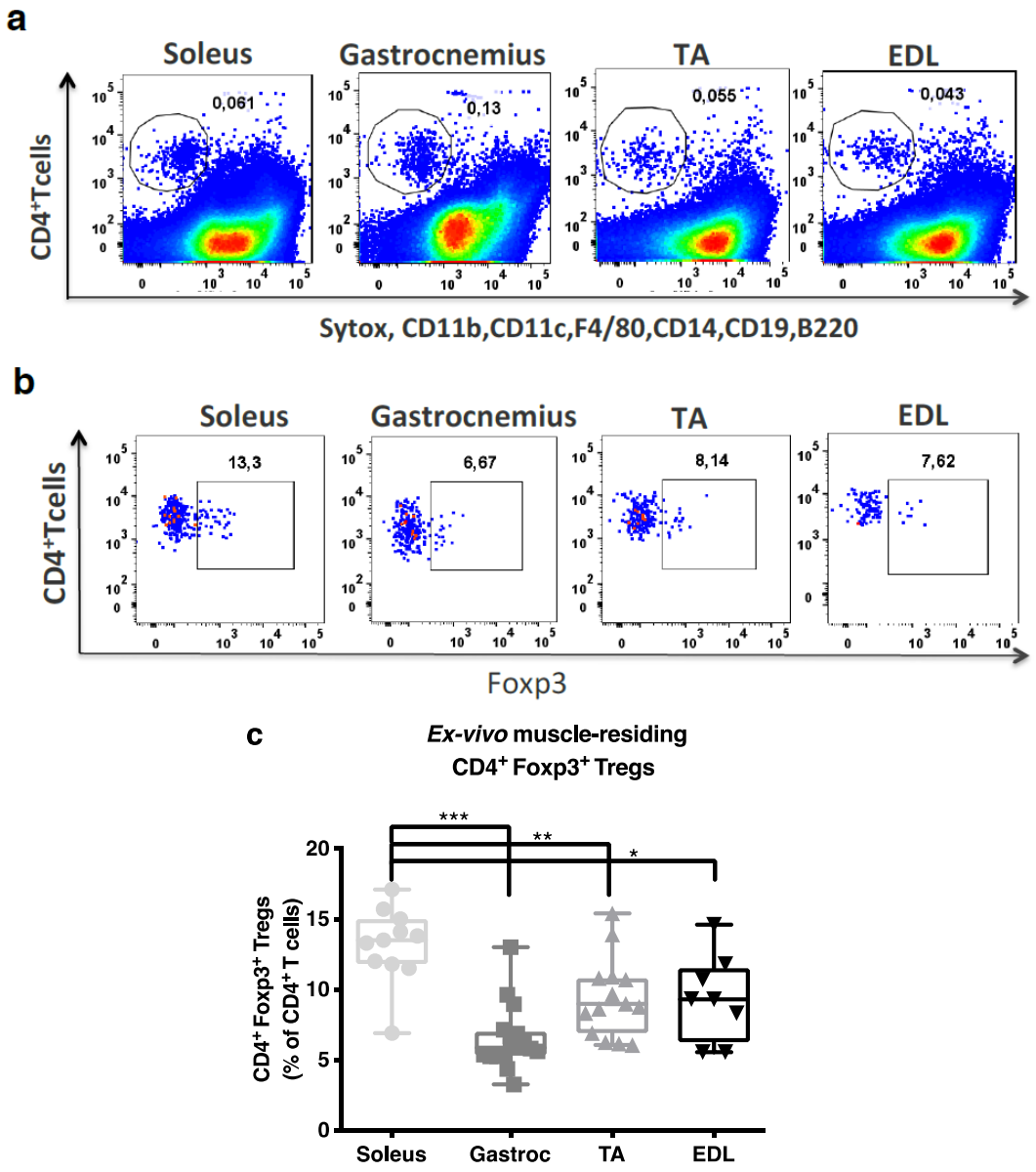


Figure 10: Skeletal muscle-residing CD4⁺ T cells and Tregs a) Representative FACS plot showing muscle-residing CD4⁺ T cells and b) Tregs (Foxp3⁺CD4⁺ T cells) in Soleus, Gastrocnemius, TA (Tibialis anterior) and EDL (Extensor Digitorum Longus). c) Summary graph of *ex-vivo* Tregs (% of CD4⁺ T cells) in different muscles in the steady state in male 16 week od C57Bl/6J mice : soleus (n = 11), gastrocnemius (n= 17), TA (n= 15), EDL (n= 8). In soleus and EDL, each point corresponds to 2 mice pooled together. Data represent mean± SEM. *P < 0.05, **P < 0.01, ***P< 0.001 analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

4.2.2 Impact of exercise on muscle-residing Tregs

In order to define whether muscle-residing Tregs have any role in directing some of the beneficial effects of exercise, mice were randomized into groups of sedentary, exercised and pre-exercised mice on standard diet as well as on

HFHS diet for a period of 8 weeks. To understand whether voluntary running has also a memory effect on T cell function that is detectable also weeks after an exercise period mice were given access to voluntary wheels for the first 4 weeks and then the wheels were removed for the last 4 weeks of the study (pre-exercised mice). To determine exercise related effects in T cell function another group of mice had access to running wheels for the last 4 weeks only (exercised mice). For the HFHS diet groups, mice were exposed to HFHS during the last 4 weeks (Fig 11a). Body weights were monitored regularly for the entire study period (Fig 11b). Visceral fat pads were weighed upon experiment termination and fat pads were reduced in exercised mice as compared to the controls (Fig 11c). There were no significant changes in the muscle mass upon exercise (Fig 11d,e). Fat pads and muscle weights were normalized to the body weights. Running profiles of the mice were monitored using a computerized system and the number of revolutions were recorded on the wheel manager software and analyzed. Average running distance in km per day per cage were calculated (Fig 11f) and running frequency actogram shows that running occurred almost exclusively in the dark cycle (Fig 11g).

4.2.3 Exercised and pre-exercised mice show a significant increase in muscle-residing Tregs

In order to address the effects of exercise on Treg frequencies in muscles, voluntary wheel running was performed utilizing sedentary, exercised and pre-exercised mice. Mice were sacrificed, skeletal muscles (soleus and gastrocnemius) were isolated and digested in order to isolate lymphocyte populations. Cells were stained and analyzed for Treg frequencies. Upon standard diet, exercised and pre-exercised mice showed a significant increase in muscle-residing Tregs as compared to the sedentary mice. A more pronounced increase was evident in the pre-exercised group in both the muscles (Fig 12).

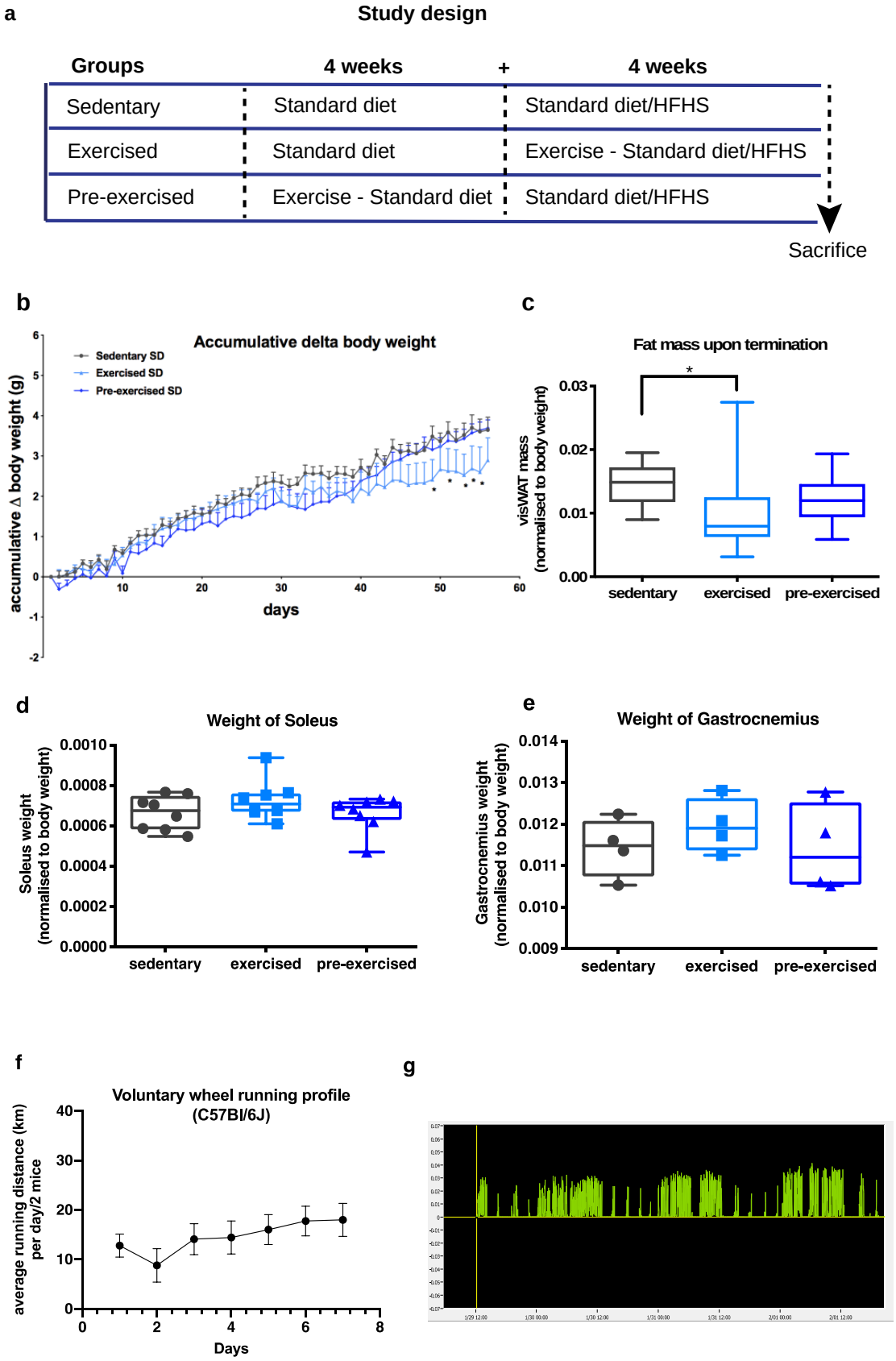


Figure 11: Impact of exercise on body weight, tissue weights and running profile of C57BL/6J mice a) Experimental study design b) Accumulative delta body weight of sedentary, exercised and pre-exercised mice for 8 weeks upon standard diet (n=14) c) visWAT mass normalized to body weight upon termination (n = 20 per group) d) Weight of soleus (n=

8) and e) gastrocnemius (n= 4) normalized to body weight in sedentary, exercised and pre-exercised mice upon standard diet. Data are expressed as mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by one-way ANOVA followed by Tukey's multiple comparison. f) Voluntary wheel running profile of C57BL/6J mice represented as average running distance per day in km for 2 mice (from three independent experiments). g) Running frequency actogram showing the wheel activity mostly in the dark cycle.

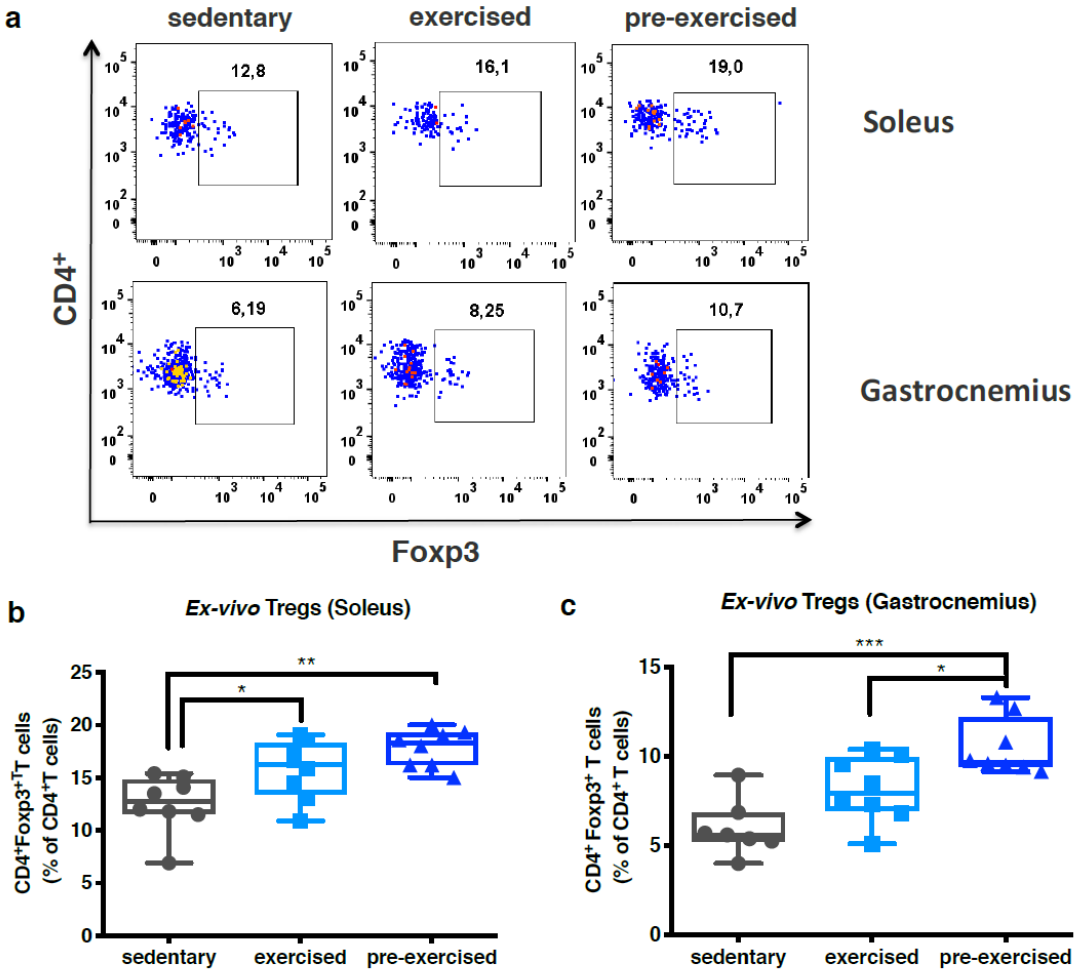


Figure 12: Impact of exercise on muscle-residing Tregs a) Representative FACS plots showing *ex-vivo* $CD4^+Foxp3^+$Tregs from soleus and gastrocnemius in sedentary, exercised and pre-exercised mice. b) Summary graph of *ex-vivo* $CD4^+Foxp3^+$Tregs from soleus (n= 8) and c) gastrocnemius (n= 8) in sedentary, exercised and pre-exercised mice. In Soleus, 2 mice were pooled together. All data are represented as mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by one-way ANOVA followed by Tukey's multiple comparison.

4.2.4 Phenotypic characterization of muscle-residing Tregs upon exercise

Tissue-residing Tregs differ from lymphoid Tregs in their phenotype and function. Tissue-resident Tregs possess unique characteristics in accordance with their respective target tissue. Among these are the expression of certain transcription factors, receptors or functional markers and they behave closely to the immediate microenvironment integrating local cues (Burzyn et al. 2013). Muscle-residing Tregs are reported to possess certain specific molecules like AREG, ST2, EGFR etc. which distinguish them from their lymphoid counterparts (Zhou et al. 2015) and in addition enhances their suppressive capacity (Zaiss et al. 2013). Initially, we identified these markers on muscle-residing Tregs (Fig 13). Since exercise releases a lot of myokines, these could also signal through their receptors present on T cells resulting in a cross talk between myokines and T cells. Hence we determined expression of AREG, EGFR and ST2 on the muscle-residing T cells to see whether these phenotypic characteristics change with exercise.

4.2.4.1 Treg proliferation is increased in skeletal muscles of pre-exercised mice

In order to address whether increased Treg frequencies are due to the increased proliferation of muscle- residing Tregs, we used Ki67 as a proliferative marker. Treg proliferation was significantly increased in pre-exercised mice in both soleus and gastrocnemius (Fig 14a,b) indicating that the local muscle environment supports a specific expansion of muscle Tregs upon exercise.

4.2.4.2 Amphiregulin expressed by the muscle Tregs increases upon exercise

Amphiregulin (AREG), a growth factor expressed by several immune and epithelial cells is a key factor involved in tissue repair and homeostasis, suppressing local inflammation and restoring tissue integrity (Zaiss et al. 2015). Muscle-specific Tregs produce more amphiregulin as compared to lymphoid

tissues. Amphiregulin positive Tregs are reported to possess more suppressive and regenerative function and also enhance regulatory T cell function. (Burzyn et al. 2013, Zaiss et al. 2015). Here, we first could confirm that muscle Tregs expresses amphiregulin (Fig 13). In order to see whether exercise had any influence on the AREG expression in Tregs, sedentary, exercised and pre-exercised mice skeletal muscles were stained intracellularly for amphiregulin. In both soleus and gastrocnemius muscles, AREG^{+}Foxp3^{+} Tregs were found to be significantly increased only in pre-exercised mice (Fig 14c,d). In contrast, AREG did not increase in exercised mice compared to sedentary mice.

4.2.4.3 EGFR^{+}Foxp3^{+} Tregs increases in muscle upon exercise

Amphiregulin enhances regulatory T cell function through its receptor, EGFR (epidermal growth factor receptor) (Zaiss et al. 2013). Reports show a more immunosuppressive capacity for EGFR^{+} Tregs in an intrahepatic environment (Dai et al. 2014). Muscle-residing Tregs also possess EGFR (Fig 13). We found that the EGFR^{+} Tregs decreased in the exercised mice and increased in the pre-exercised mice in both the muscles compared to sedentary and exercised groups (Fig 14e,f).

4.2.4.4 Skeletal muscle ST2^{+} Treg frequencies were enhanced in pre-exercised mice

ST2, the receptor for cytokine IL33, is a transmembrane receptor and is strongly upregulated in tissue Tregs as compared to the lymphoid-tissue Tregs (Kuswanto et al. 2016, Han et al. 2015). Previous reports show the presence of IL33^{+} or ST2^{+}Foxp3^{+} Tregs in muscle-residing Tregs and their function in impacting muscle Treg accumulation and regeneration. (Kuswanto et al. 2016). We also show that ST2 is present on muscle-residing Tregs (Fig 13). Since IL33 is also reported to be released upon exercise (Little et al. 2018), we determined whether there is any difference in the ST2 expression levels on muscle T cells. Pre-exercised mice, but not exercised mice showed an increase in ST2^{+}Treg frequency in gastrocnemius muscle (Fig 14g,h). ST2 expression

in soleus muscle remained unchanged upon exercise in all groups compared to sedentary mice (Fig 14g).

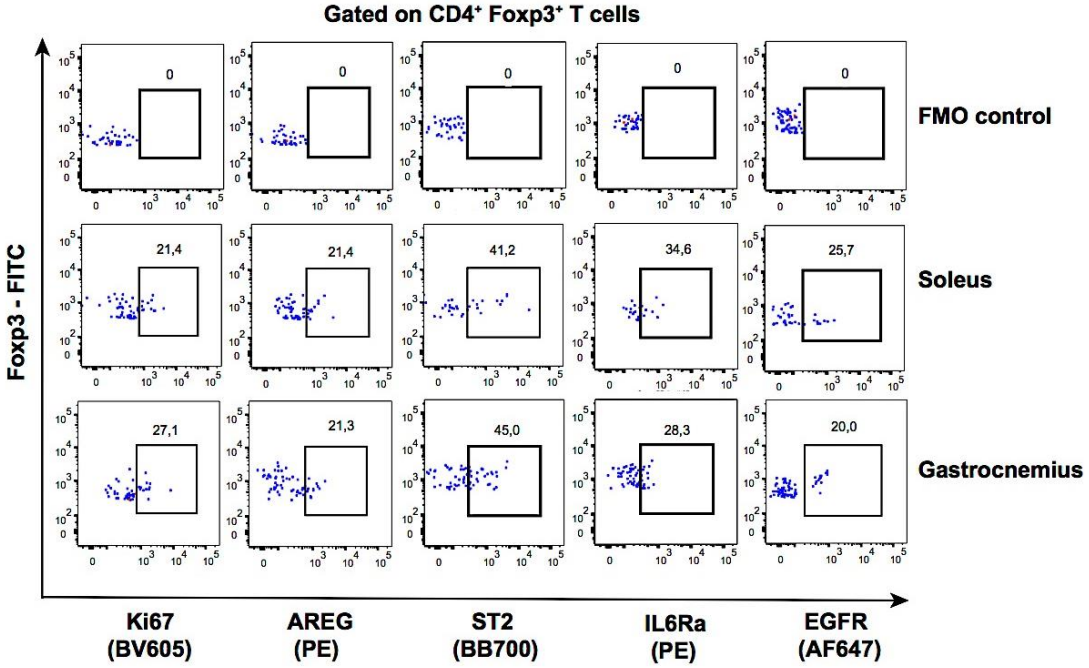
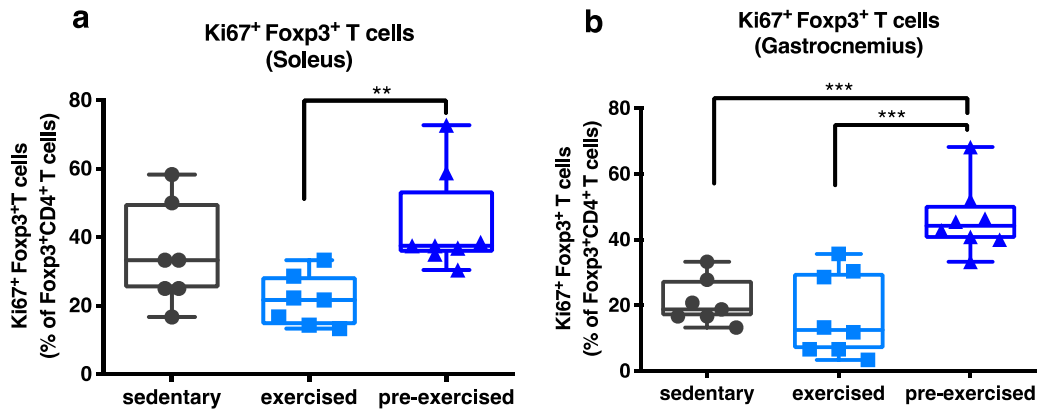


Figure 13: Representative FACS plots showing the presence of Ki67, AREG, ST2, IL6Ra and EGFR on muscle–residing CD4⁺Foxp3⁺Tregs (in soleus and gastrocnemius) and the corresponding FMO controls.



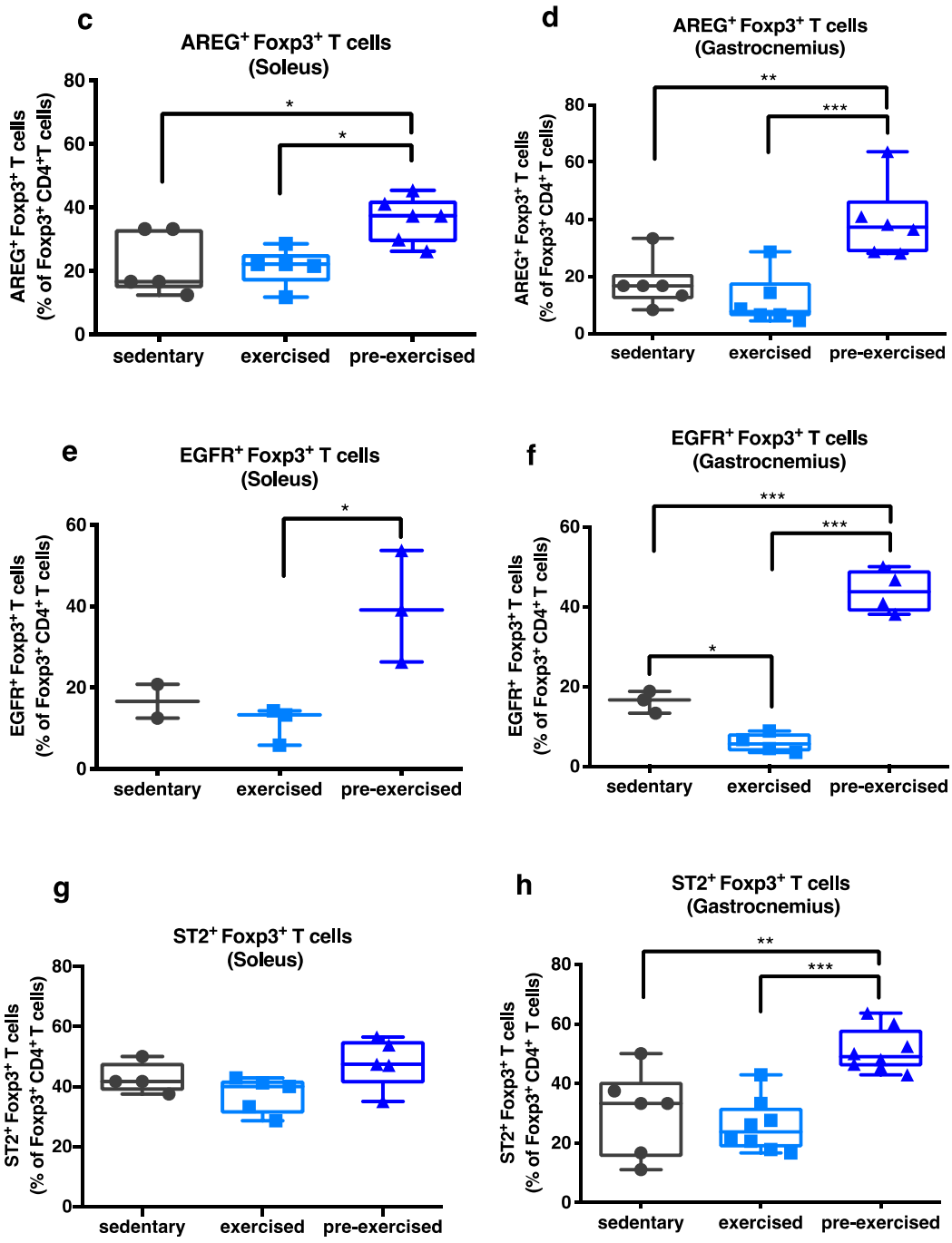


Figure 14: Phenotypic characterization of muscle-residing Tregs upon exercise. a) Summary graph for the *ex-vivo* proliferative status of Tregs (Ki67$^+$Foxp3$^+$Tregs) in soleus (n = 7) and b) in gastrocnemius (n= 8). c) Summary graph of *ex-vivo* AREG$^+$Foxp3$^+$Tregs from soleus (n = 5-6) and d) gastrocnemius (n = 6) in sedentary, exercised and pre-exercised mice e) Summary graph of EGFR$^+$Foxp3$^+$Tregs in soleus (n= 3) and f) gastrocnemius (n = 3-4). g) Summary graph of *ex-vivo* ST2$^+$Foxp3$^+$Tregs in soleus (n = 4 - 5) and h) gastrocnemius (n = 6 - 8). In soleus, each point corresponds to 2 mice. Data are expressed as mean ± SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by one-way ANOVA followed by Tukey's multiple comparison.

4.2.5 Impact of exercise on muscle-residing Tregs upon HFHS diet

In order to understand the effect of exercise on Tregs upon exposure to HFHS diet, sedentary, exercised and pre-exercised mice were fed standard diet for 4 weeks followed by 4 weeks of HFHS diet as shown (Fig 11a). Exercised and pre-exercised mice showed a significant reduction in body weight as compared to the sedentary group. Pre-exercised mice showed a reduction in body weight during the exercise phase; once we removed the wheels pre-exercised mice gained weight at the same rate as sedentary mice (Fig 15a). Visceral fat mass was reduced in the exercised mice (Fig 15b). The increase in *ex-vivo* Tregs we observed in pre-exercised mice fed standard diet (Fig 12) was maintained upon exposure to HFHS diet in soleus muscle (Fig 15c). Pre-exercised mice also showed an increased tendency in the proliferative status and AREG$^+$ Tregs (Fig 15d,e).

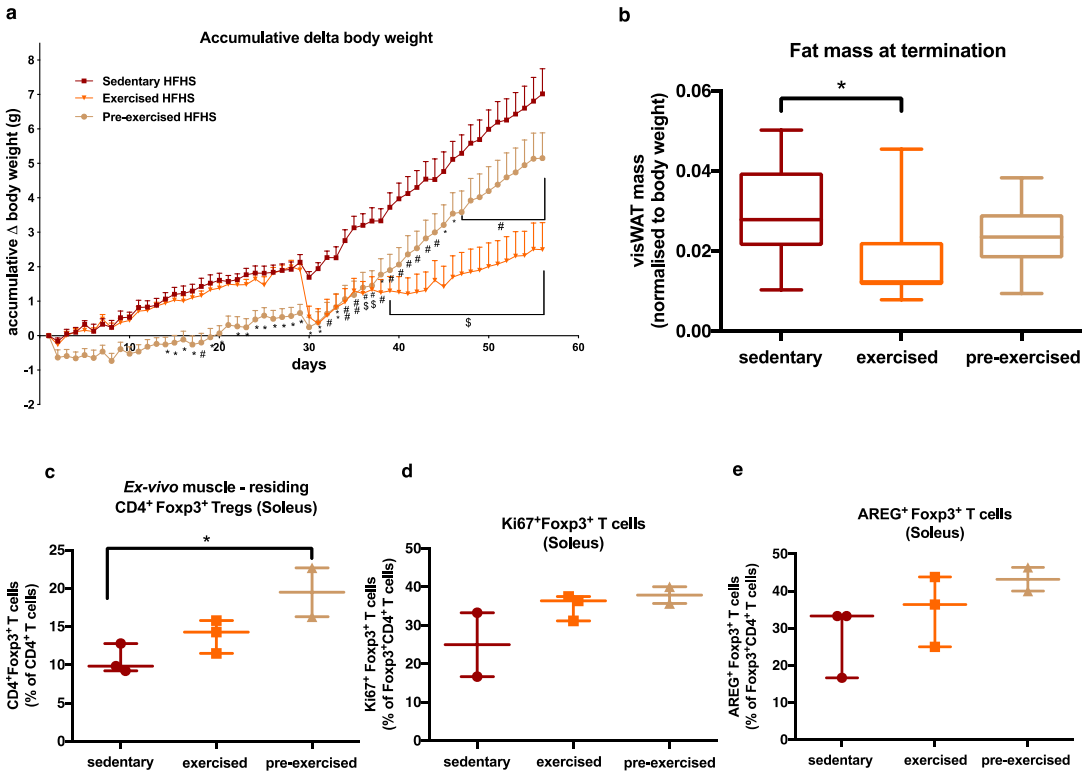


Figure 15: *Ex-vivo* muscle-residing Tregs upon HFHS diet

a) Accumulative delta body weight of sedentary, exercised and pre-exercised mice for 4 weeks on standard diet followed by 4 weeks of HFHS diet (n=14) *(p< 0.05), # (p< 0.01),$ (p< 0.001) compared to sedentary group. b) VisWAT mass normalized to body weight upon termination (n =14) c) Summary graph showing the *ex-vivo* muscle-residing CD4$^+$ Foxp3$^+$ Tregs in soleus (n = 2-3). d) Summary graph of Treg proliferation

(Ki67^{+}Foxp3^{+}Tregs) and e) AREG^{+}Foxp3^{+}Tregs in soleus upon HFHS diet (n = 2 - 3). Each data point corresponds to 2 mice. Data are expressed as mean±SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by one -way ANOVA followed by Tukey's multiple comparison.

4.2.6 Pre-exercised mice exhibit a significant increase in the *de novo* induction of Tregs

Apart from the thymic derived ones, Foxp3^{+} Tregs can also develop in the periphery from conventional CD4^{+} T cells under different conditions known as induced Tregs (Sakaguchi et al. 2008, Bilate & Lafaille 2012). Several experiments show that naive T cells can acquire Foxp3 expression *in-vitro* more efficiently with a strong agonistic ligand under subimmunogenic conditions (Gottschalk et al. 2010, Daniel et al. 2010). In order to assess Treg induction capacity upon exercise, naive CD4^{+} Tcells (CD4^{+}CD44^{low} CD62L^{hi} CD25^{-}) from popliteal lymph nodes, the draining lymph nodes of muscles were used since it was really difficult to obtain adequate amounts of naïve T cells from the skeletal muscles per se for the *in-vitro* culture. Naïve T cells from sedentary, exercised and pre- exercised groups were sorted and seeded on 96 well plates pre-coated with anti-CD3 and anti-CD28 followed by stimulation with IL-2 for 18 hours. Cells were then cultured for a further 36 hours without stimulation and then stained and analyzed for Treg frequencies. Pre-exercised mice showed a significant increase in their *de-novo* induction capacity as compared to the other groups under standard diet. Under HFHS conditions, mice exhibits a trend towards increased Treg induction upon exercise. (Fig 16). Since about 50 % of HFHS fed mice within the exercised groups did exhibit an increase in de-novo induction of Tregs upon exercise, it is plausible to assume that there are individual differences in the response to exercise.

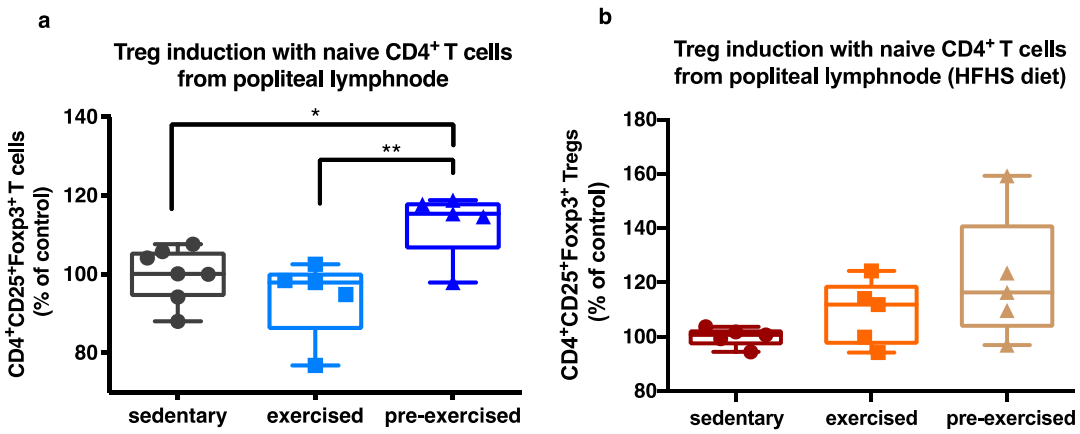


Figure 16 : *In-vitro* Treg induction assays upon SD and HFHS diet
Graphs showing $CD4^+CD25^+Foxp3^+$Tregs (represented as % of control) from the *in-vitro* Treg induction assays using limited TCR stimulation of naive T cells from popliteal lymphnodes upon a) standard diet and b) HFHS diet in sedentary, exercised and pre- exercised mice (n= 5 per group). Data are expressed as mean±SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by one-way ANOVA followed by Tukey's multiple comparison.

4.2.7 Gene expression analysis of the sorted $CD4^+$ T cells from popliteal lymph nodes upon exercise

Multiple changes occur in muscles due to exercise and since we saw that Treg induction potential of naive T cells from popliteal lymphnodes was also increased in pre-exercised mice fed standard diet, some changes might have occurred in the naive T cell stage itself upon exercise. Metabolic switching happens during T cell differentiation and development (Windt and Pierce 2012). Hence we looked at gene expression profiles of total $CD4^+$ T cells, isolated and sorted from popliteal lymph nodes of sedentary, exercised and pre-exercised mice. Certain genes associated with T cell metabolic pathways involved in T cell differentiation like Cpt1b, involved in fatty acid oxidation, SNAT1, an amino acid transporter and PDK1, a major checkpoint in the glycolytic pathway (Patsoukis et al. 2015) were analyzed. There were no significant changes upon exercise under both dietary conditions (Fig 17).

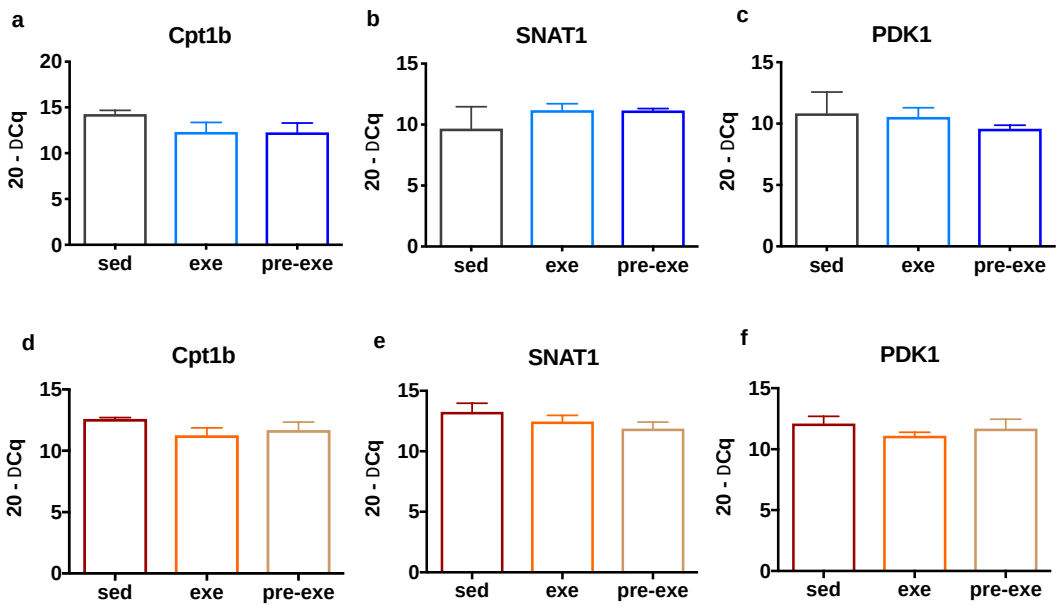


Figure 17: Gene expression data of CD4⁺ T cells from popliteal lymphnodes
a) qPCR data showing the expression of Cpt1b, SNAT1, PDK1 mRNA in the sorted CD4⁺ T cells from the popliteal lymphnodes of sedentary, exercised and pre-exercised mice on standard diet (a-c) and HFHS diet (d-f), n=3.

4.3 Role of IL-6 as a myokine and its functions in T cell differentiation and expansion

4.3.1. Role of IL-6 receptor signaling in muscle-residing T cells

Previous reports show that lymphocytes possess IL6 receptor (Jones 2005) and here we confirmed the expression of IL6R on muscle-residing Tregs (Fig 13). IL6R expression on skeletal muscles has been shown to be increased upon exercise training. (Keller et al. 2005, Pederson & Febbraio 2008). Hence, to dissect out the relevance of IL-6R signaling in guiding exercise-mediated changes of muscle-residing Tregs, we determined IL6R levels on T cells residing within the muscles. In soleus IL-6R levels did not change significantly between sedentary and both exercised groups (Fig 18a). In gastrocnemius muscle we observed a marked increase in IL-6R expression only in pre-exercised mice compared to sedentary and exercised mice (Fig 18b). In order to address the effects of IL-6R and its signaling on T cells, and to dissect

whether IL6R has an impact on Tregs, a T cell specific IL6RαKO mice was used (Xu et al. 2016).

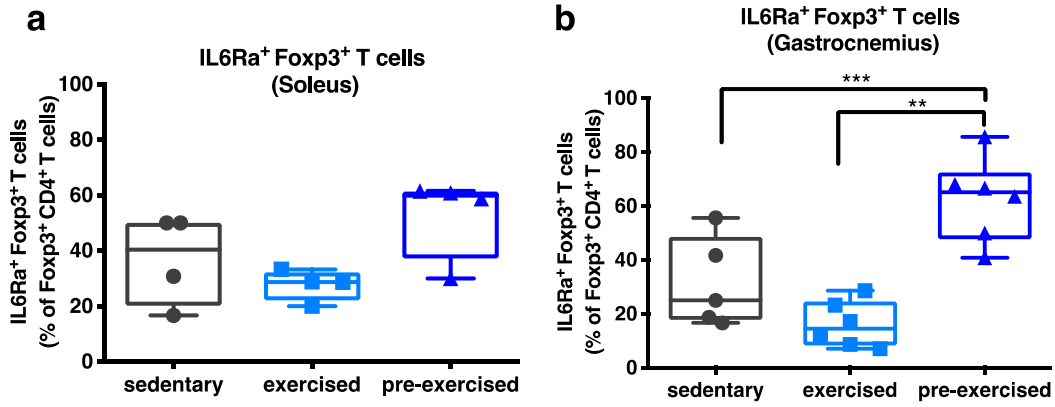


Figure 18: Impact of exercise on IL6Rα⁺ Foxp3⁺ muscle-residing Tregs
a) Summary graph of IL6Ra⁺Foxp3⁺Tregs from soleus (n= 4) and f) gastrocnemius (n= 5 - 6) in sedentary, exercised and pre-exercised mice. In soleus, each point corresponds to 2 mice. Data are expressed as mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by one-way ANOVA followed by Tukey's multiple comparison.

4.3.1.1 Characterization of T cell specific IL6RαKO mice

T cell specific deletion of IL6R was confirmed by genotyping (Fig 19b) using PCR of CD4cre and IL6R floxed allelles and reconfirmed by flow cytometric staining of IL6Rα on T cells (Fig19a).

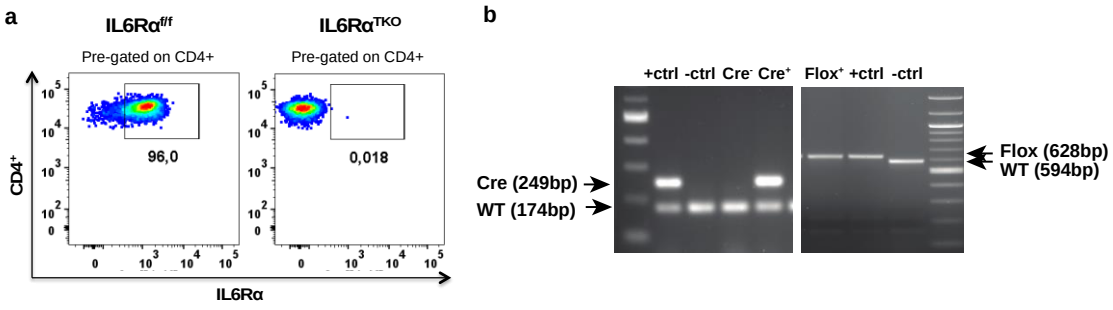


Figure 19 : Characterization of Tcell specific IL6RαKO mice a) FACS staining plot of IL6Rα on CD4⁺ T cells from inguinal lymphnodes in IL6RαTKO mice and floxed control and b) genotypic characterisation of IL6RαTKO mice

4.3.1.2 *Ex-vivo* muscle-residing Tregs in T cell IL6RαKO in the steady state

Treg frequencies of T cell specific IL6RαKOs were compared with floxed controls in the steady state. Since we saw exercise induced differences in IL6Rα levels on Tregs more prominent in Gastrocnemius than Soleus (Fig 18), we further focused on Gastrocnemius muscle to determine the role of IL6 signalling using the KO mice models. We could not detect any changes in total Treg frequencies between the genotypes (Fig 20a), whereas $ST2^{+}Foxp3^{+}$ Tregs were significantly reduced in KOs as compared to floxed controls (Fig 20b).

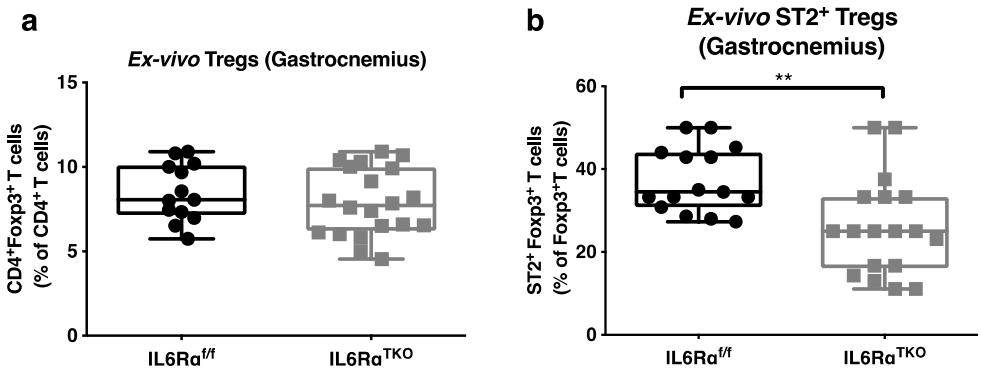


Figure 20: Steady state *ex-vivo* muscle-residing Tregs in IL6Rα^{TKO} mice
Ex-vivo $CD4^{+}Foxp3^{+}$Tregs in gastrocnemius from IL6Rα^{TKO} (n=20) and floxed controls (n=13) in the steady state. b) *Ex-vivo* $ST2^{+}$ $Foxp3^{+}$ Tregs in gastrocnemius from IL6Rα^{TKO} (n=15) and floxed controls (n= 18) in the steady state. Data are expressed as mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by unpaired t test.

4.3.1.3 Muscle-residing Treg frequencies were unaltered in T cell-specific IL6Rα KO mice upon exercise

Considering the effect of IL6 as a myokine together with our observation that IL6Ra expression on Tregs was increased exclusively in pre-exercised mice, we hypothesized that exercise-released IL-6 may partially be responsible for the increased Treg frequencies. In order to address that, T cell IL6RαKOs and its floxed controls were randomized into sedentary and pre-exercised groups for a period of 8 weeks. As reported previously, pre-exercised mice had access to the running wheels for the first 4 weeks and remained sedentary for the last 4 weeks. Body weights and running profiles were recorded. We did not observe any significant changes in body weight and fat mass between sedentary and pre-exercised IL6RαTKOs and also between sedentary and pre-exercised floxed controls. Analysis of running profiles revealed no significant differences

in the running distance between KOs and floxed controls. (Fig 21a-c). Gastrocnemius muscles were digested, lymphocytes were isolated and stained for Tregs and other phenotypic markers. As expected, pre-exercised controls showed a significant increase in Tregs. In contrast this increase was abolished in exercised KO mice (Fig 22) indicating that exercise induced IL-6 or the signaling via IL6Rα may play a role in augmenting Treg frequencies.

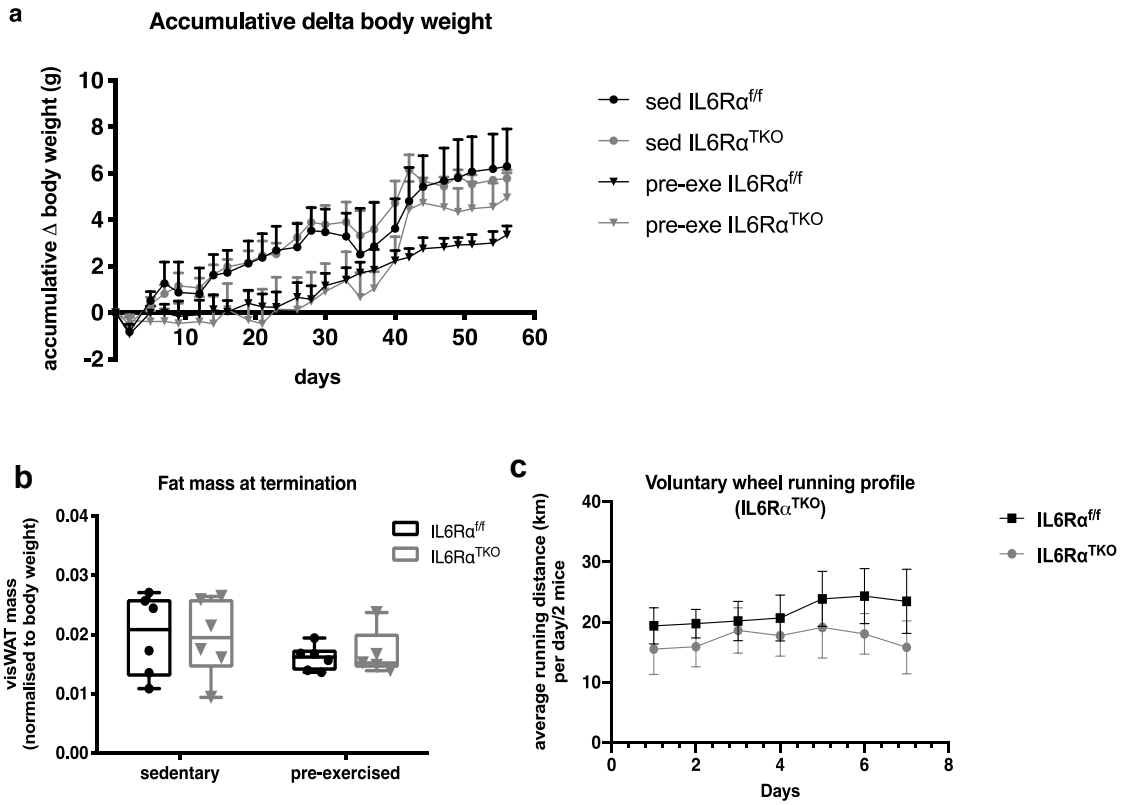


Figure 21: Impact of exercise on body weight, fat weight and running profile of IL6Ra^{TKO} mice a) Accumulative delta body weight of sedentary and pre-exercised IL6Ra^{TKO} and floxed controls for 8 weeks upon standard diet (n=4). b) visWAT mass normalized to body weight of sedentary and pre-exercised IL6Ra^{TKO} and flox controls (n=6). Data are expressed as mean± SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by two-way ANOVA followed by Tukey's multiple comparison. c) Voluntary wheel running profile mice of IL6Ra^{TKO} and IL6Rα$^{fl/fl}$ mice represented as average running distance per day in km for 2 mice (from three independent experiments).

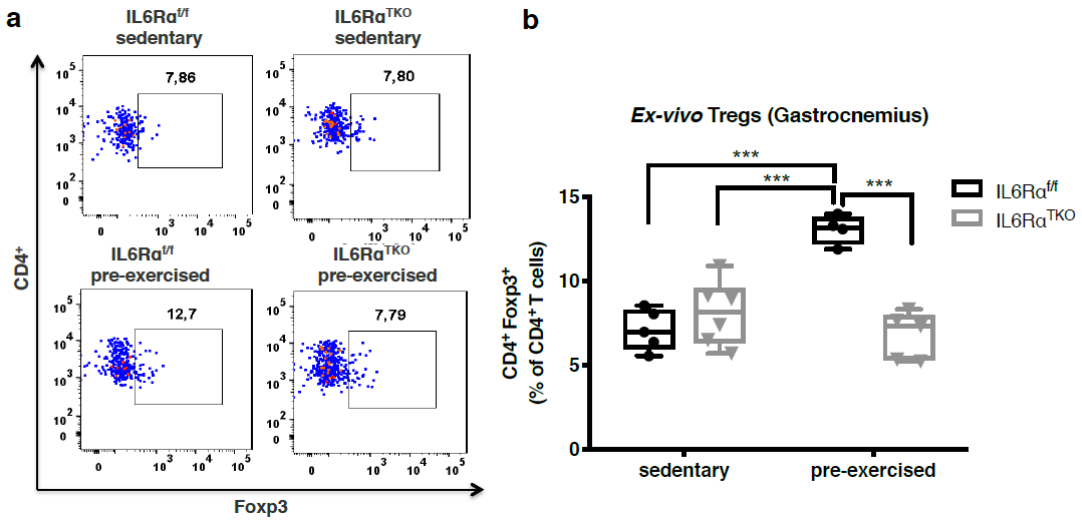


Figure 22: Impact of exercise on muscle-residing Tregs in IL6Ra^{TKO} mice
a) Representative FACS plots of CD4$^+$Foxp3$^+$Tregs in IL6Ra^{TKO} vs floxed controls upon exercise in gastrocnemius b) Summary graph showing the *ex-vivo* CD4$^+$Foxp3$^+$Treg frequencies in IL6Ra^{TKO} and floxed controls of sedentary and pre-exercised mice in gastrocnemius (n= 4 - 6). Data are expressed as mean ± SEM *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by by two-way ANOVA followed by Tukey's multiple comparison.

4.3.1.4 Phenotypic characterisation of Tregs in T cell specific IL6RαKOs upon exercise

Treg proliferation was increased significantly in pre-exercised floxed controls, but not in KOs (Fig 23a). We observed a significant decrease in ST2$^+$Tregs in gastrocnemius muscle of KO mice as compared to floxed controls in the sedentary state. (Fig 23b). In floxed control mice we replicated the earlier finding (see Fig 13 c – f) that AREG and its receptor EGFR were significantly higher in pre-exercised floxed controls compared to sedentary floxed controls. In contrast we did not observe a pre-exercise effect on AREG and EGFR expression of Tregs in KO mice (Fig 23c,d). These data indicate that IL6 receptor signaling is contributing to the increase of AREG and EGFR on Tregs upon exercise.

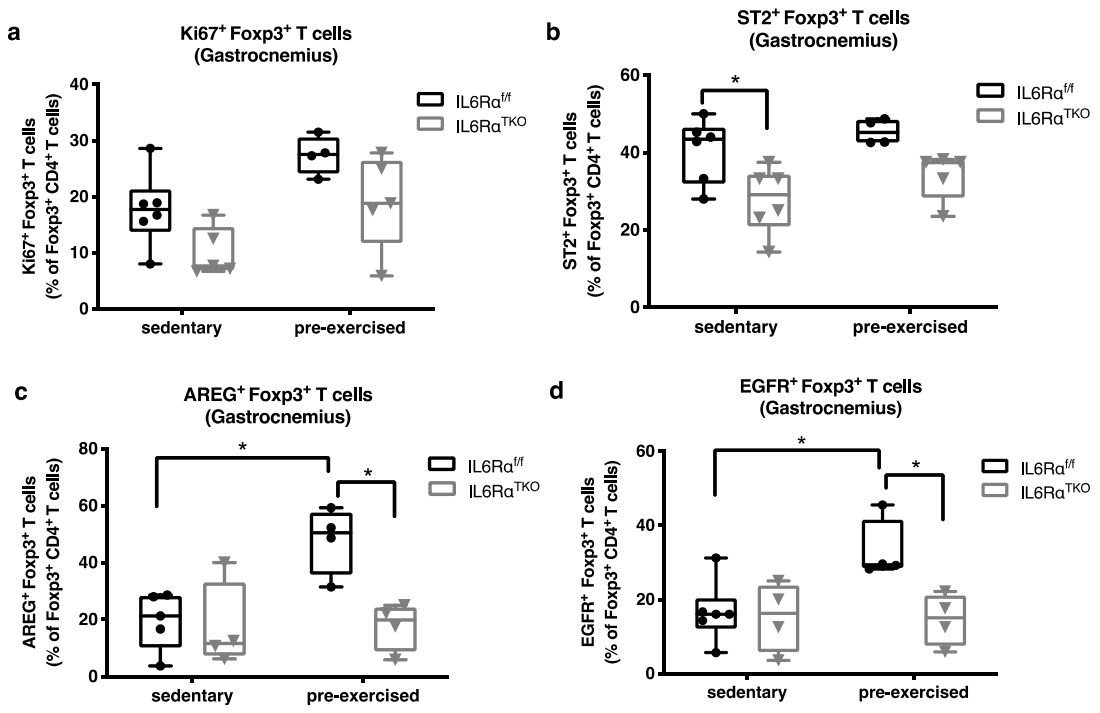


Figure 23: Phenotypic characterization of muscle-residing Tregs upon exercise in IL6Ra^{TKO} mice a) Summary graph representing Treg proliferation (Ki67$^+$Foxp3$^+$Tregs), b) ST2$^+$Foxp3$^+$Tregs c) AREG$^+$Foxp3$^+$Tregs, and d) EGFR$^+$Foxp3$^+$Tregs in IL6Ra^{TKO} and floxed controls of sedentary and pre-exercised mice in gastrocnemius (n= 4 - 6). Data are expressed as mean±SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by two-way ANOVA followed by Tukey's multiple comparison.

4.3.2. Impact of IL6 as a myokine on the muscle-residing Tregs

IL-6 is a well-known myokine released from muscle fibres upon exercise with autocrine and paracrine effects (Febbraio & Pederson 2002). IL-6 affects T cells expressing IL6R directly. Due to our observation that IL6R expression on Tregs is increased upon exercise that is abolished in T cell specific IL6RαKOs, it is plausible to assume that signaling via IL6R may modulate Treg function. In order to address this hypothesis we performed our exercise experiments in mice with a muscle-specific IL6 deletion.

4.3.2.1 Characterization of muscle specific IL6KO mice

Muscle IL6KO mice were generated by crossbreading MLC^{Cre} mice (Gothe et al. 2000) expressing a Cre recombinase with the skeletal muscle-specific myosin light chain promoter with IL6$^{f/f}$ mice (Quintana et al. 2013, Molinero et al. 2017). Muscle specific deletion of IL-6 was confirmed by genotyping using

PCR for MLCcre and IL-6 floxed allelles (Fig 24b) and mRNA IL-6 expression levels were significantly reduced in the IL-6 KO muscles compared to other tissues (adipose tissue was used as a positive control) (Fig 24a).

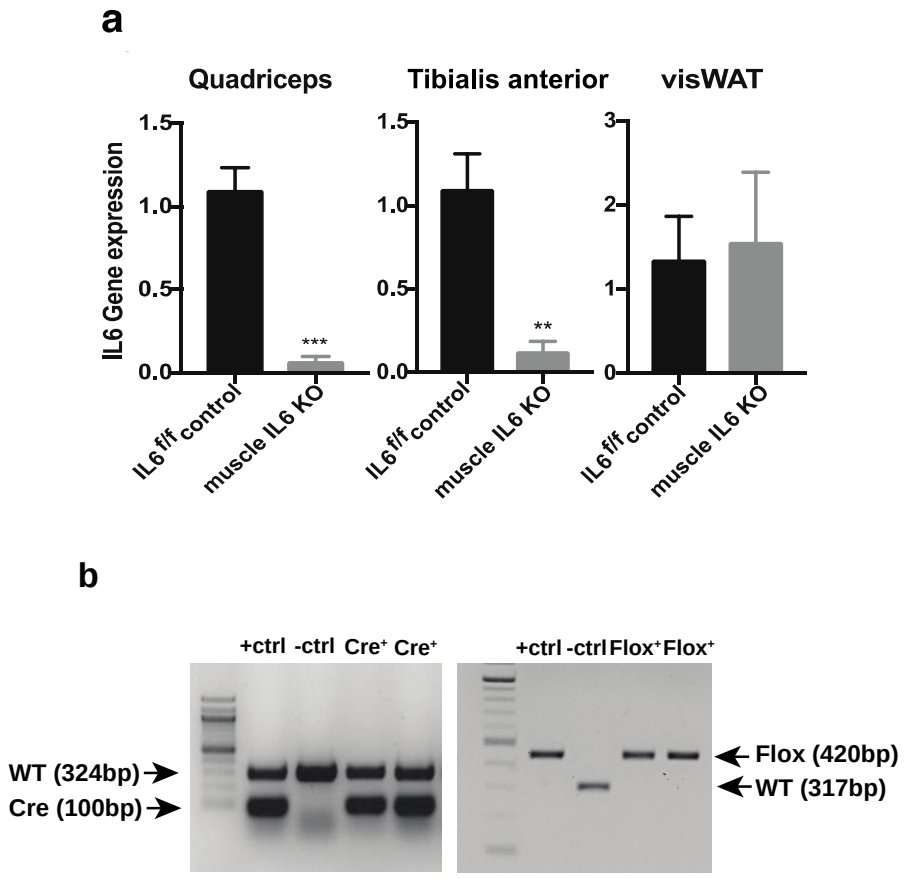


Figure 24: Characterization of muscle specific IL6KO mice a) Graph showing IL6 mRNA levels in muscles (Quadriceps, Tibialis anterior) and adipose tissue of IL6KOs and flox controls by qPCR (n= 8). Data represents the fold change, expressed as mean ± SEM, analyzed by unpaired t test with Welch's correction. b) Genotypic characterization of muscle specific IL6 KO mice.

4.3.2.2 Muscle-specific IL6 KO mice exhibited an increased muscle Treg frequency upon exercise

In order to determine the role of IL6 in the exercise-mediated increase of muscle-residing Treg frequencies, muscle specific IL6KO mice and their IL6 floxed controls were randomised into sedentary and pre-exercised mice on a standard diet for 8 weeks. Pre-exercised mice had access to the wheels for the first 4 weeks. Body weights and the running profile were monitored. Fat mass

was also measured upon study termination. We observed a significant difference in body weight between sedentary IL6KO mice and sedentary fl/fl control mice. Since the visceral fat pads were only slightly increased in KOs as compared to their floxed controls, we assume that lean mass may be a contributor to this body weight difference. Fat mass was slightly reduced in the pre-exercised groups compared to the sedentary groups regardless of genotype. Running profiles showed no significant differences in running distance between muscle IL6KO and floxed controls (Fig 25 a-c). Floxed controls and muscle specific IL6 KOs exhibited a similar increase in Treg frequency in gastrocnemius muscle in pre-exercised mice of both genotypes (Fig 26) indicating that skeletal muscle derived cytokine IL6 as such does not have a direct influence on increased Treg frequencies attained upon exercise.

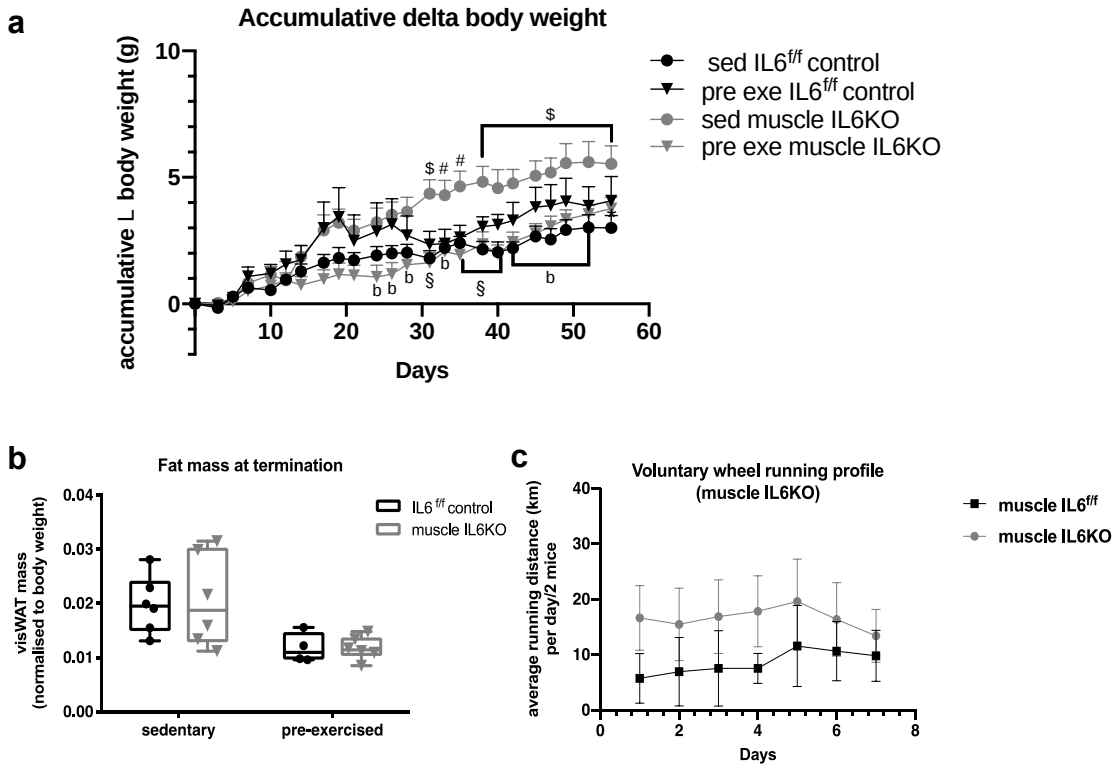


Figure 25: Impact of exercise on body weight, fat weight and running profile of muscle IL6KO mice a) Accumulative delta body weight of sedentary and pre-exercised muscle IL6KOs and flox controls upon standard diet (n=6). # ($p< 0.01$), $ ($p< 0.001$) comparing sedentary IL6KO to sedentary control; β ($p< 0.01$), § ($p< 0.001$) comparing sedentary IL6KO to pre-exercised IL6KO. b) visWAT mass normalized to body weight of sedentary and pre-exercised muscle IL6KO and flox controls (n=6). Data are expressed as mean±SEM *$p<0.05$, **$p< 0.01$, ***$p< 0.001$, analyzed by two-way ANOVA followed by Tukey's multiple comparison. c) Voluntary wheel running profile mice of muscle IL6KO mice and $IL6^{fl/fl}$ mice represented as average running distance per day in km for 2 mice (from three independent experiments).

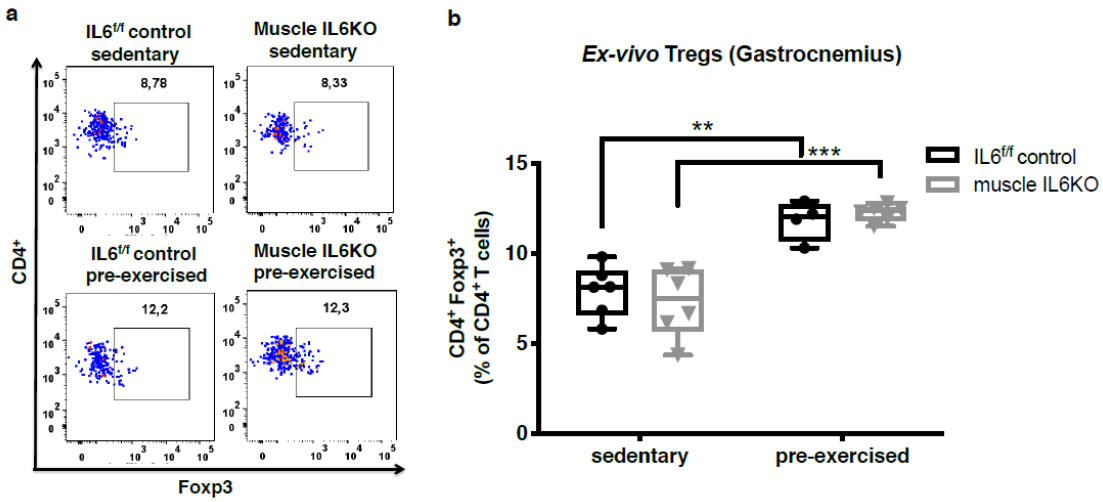


Figure 26: Impact of exercise on muscle-residing Tregs in muscle IL6KO mice a) Representative FACS plots identifying muscle-residing CD4$^+$Foxp3$^+$Tregs from muscle IL6KO and floxed controls upon exercise in gastrocnemius b) Summary graph showing the *ex-vivo* CD4$^+$Foxp3$^+$Tregs in muscle IL6KO and floxed controls of sedentary and pre-exercised mice in gastrocnemius (n= 4 - 6). Data are expressed as mean±SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by two-way ANOVA followed by Tukey's multiple comparison.

4.3.2.3 Phenotypic characterization of muscle- residing Tregs in muscle specific IL6KOs upon exercise

Although we did not detect differences in exercise-mediated increases of T cell frequencies between IL6KO and their floxed control mice, IL6 may still modulate specific phenotypic characteristics of muscle-residing Tregs upon exercise. Treg proliferation was not significantly changed in gastrocnemius of muscle IL6KOs upon exercise (Fig 27a) compared to the increase detected in floxed control mice, suggesting that local proliferation might not be the sole mechanism promoting the muscle-residing Tregs. Similarly, exercise-induced increase in ST2$^+$ Tregs was not detected in muscle IL6KO mice in contrast to floxed control mice (Fig 27b). AREG$^+$ Tregs and EGFR$^+$ Tregs were also not significantly increased upon exercise in muscle IL6KOs compared to floxed control mice (Fig 27c,d). These data point out to the fact that IL6 as such may not be required for the increased Tregs, but it influences the phenotypic characteristic of the muscle-residing Tregs upon exercise.

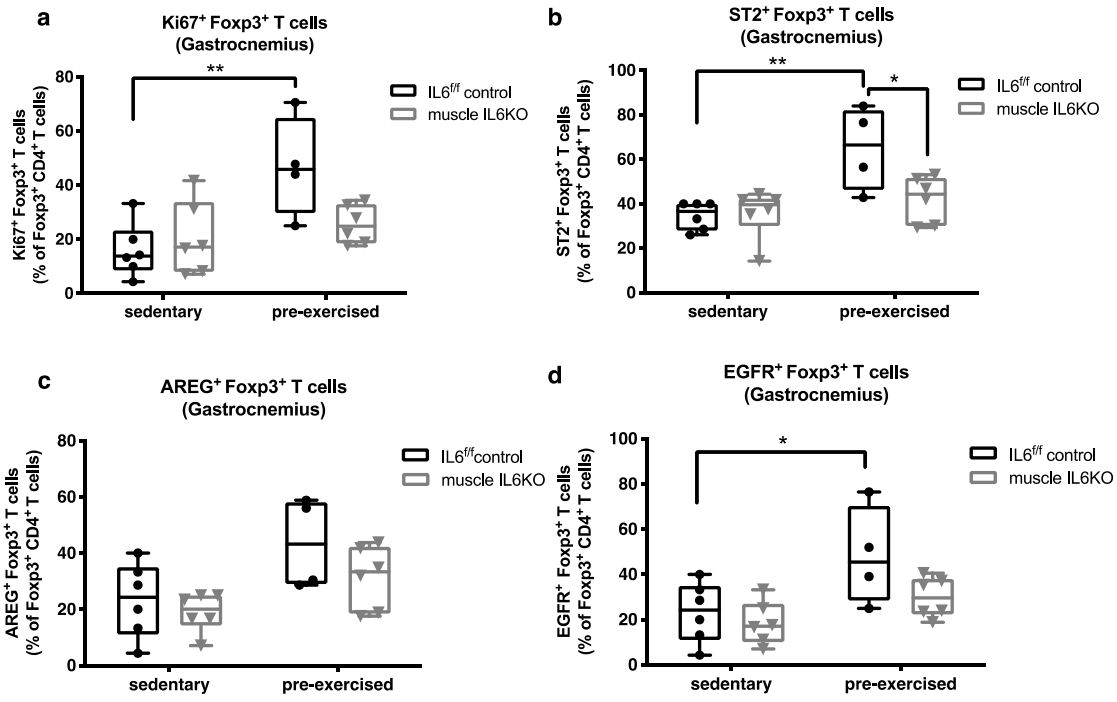


Figure 27: Phenotypic characterization of muscle-residing Tregs upon exercise in muscle IL6KO mice a) Summary graph showing Treg proliferation ($Ki67^+Foxp3^+$Tregs), b) $ST2^+Foxp3^+$ Tregs and c) $AREG^+Foxp3^+$ Tregs, d) $EGFR^+Foxp3^+$ Tregs in muscle IL6KO and floxed controls of sedentary and pre-exercised mice in gastrocnemius (n= 4 - 6). Data are expressed as mean±SEM *p<0.05, **p< 0.01, ***p< 0.001, analyzed by two - way ANOVA followed by Tukey's multiple comparison.

5. Discussion

The present thesis reports the involvement of adaptive immune cells in metabolic homeostasis and demonstrates that immune cells could be modulated using different pharmacological as well as physiological approaches. The present findings highlight the relevance of immune-metabolic crosstalk regulated by GLP-1 co-agonists on inflammatory markers and T and B cells in diet-induced obese mice and by exercise in skeletal muscle and its locally residing Tregs. Moreover, it provides novel mechanistical insights into exercise induced tissue-residing regulatory T cells which are crucial in maintaining tissue homeostasis. These are of particular importance since immune cell perturbations and disturbances of immune tolerance (Anderson et al. 2016) due to infiltration of effector immune cells (Lumeng & Saltiel 2011) and a reduction of immunoregulatory cells underlies many chronic diseases like obesity and diabetes (Feurer et al. 2009, Khan et al. 2005).

Upon exposure to high fat high sugar diet, we saw changes in lymphocytes pointing to an inflammatory milieu evidenced by an increase in the cytotoxic $CD8a^+$ T cells in circulation and significantly reduced regulatory B cells in both circulation and spleen. Mature B cells (IgM^+IgD^+) were increased in spleen upon high calorie diet and is in agreement with previous studies in obese subjects (Zhai et al. 2016, Winer et al. 2014). The decrease in immature B cells and the increase in IgM^+IgD^+ cells also depicts a state of imbalance in the homeostatic control of B cell development that is regulated by a feedback exerted by mature B cells (Shahaf et al. 2016). Apart from the bone marrow derived B cells, the increase in the peripheral B cell compartment could also be due to antigen-driven proliferation (Shahaf et al. 2016). Elevated surface IgD levels and reduced surface IgM levels could also be indicative of anergic B cells (Gutzeit et al. 2018)

Here we used mice with a global deletion of GLP-1R ($GLP1r^{-/-}$) to decipher the role of GLP-1R on immune regulation. A significant increase in mature B cells was observed upon standard diet in both PBMC and spleen in GLP-1RKOs

depicting a direct /indirect involvement of GLP-1R in the B cell maturation process. In line with that, HFHS diet fed mice treated with GLP-1R agonist exhibited a reduction in the $IgM^{+}IgD^{+}$ mature B cells in spleen. The changes we see within the B cell subsets could be either due to the direct involvement of GLP1 receptor or an indirect effect owing to the overall reduction in inflammation. However, we didn't see any changes in the regulatory B cells upon GLP-1R agonist treatment. Upon treatment with GLP-1R agonists, the total T cells in circulation were increased. As per recent reports, GLP-1 treatment increases Tregs (Hadjiyanni et al. 2010) as well as iNKT cells (Lynch et al. 2016) and the total increase in T cells we saw may be due to any of these subtypes which has to be confirmed with more specific markers.

Here we also report the anti-inflammatory effect of GLP-1/Dexa conjugate, a unimolecular compound integrating incretin and glucocorticoid, in diet-induced obese mice (Quarta et al. 2017). Analysis of plasma from co-agonist and mono agonist treated HFHS fed mice compared with vehicle treated mice highlights their effect on high-fat diet induced inflammation. The treatment caused a prominent reduction in several chemokines and chemotactic peptides involved in high fat diet induced inflammation. Several inflammatory markers were lowered by both GLP-1 agonist and GLP-1/Dexa, but most prominently by the conjugate. GLP-1/Dexa treatment also increased the total T cell frequencies in spleen. We couldn't see any changes in the numbers of regulatory B cells, but its functionality has to be checked since Bregs are known to release IL-10, an anti-inflammatory cytokine (Mizoguchi & Bhan 2006, Iwata et al. 2011). Taken together, these results suggest that both GLP-1 agonist and GLP-1/Dexa conjugate helps in lowering obesity-associated systemic inflammation and also impact both T and B lymphocyte populations that contributes to the production of these cytokines. But as there are several subsets within each lymphocyte populations having either inflammatory or anti-inflammatory effects, more markers are needed to define each population to get a more conclusive idea.

The environment within the individual tissues differ from that of the circulation, therefore a closer look on a metabolically relevant tissue level may give more insights into the local regulation of immune cells and could help in

understanding and dissecting out the individual players, thereby contributing to the impact on adiposity, insulin sensitivity and glucose metabolism.

So with the second approach of physiological intervention like exercise, we focused on a particular subset of T lymphocytes in a tissue-specific level so as to more directly modulate the immune-metabolic interplay in the metabolic target tissues. T cells, more specifically Tregs residing within the skeletal muscle was studied since it is a metabolically relevant organ contributing to the whole body metabolism and the most directly impacted organ upon exercise. Here we could demonstrate that exercise causes a durable induction of skeletal muscle residing Tregs.

Here we show that in steady state, distinct skeletal muscles harbor different Treg frequencies, highest being observed in predominantly oxidative soleus muscle. These are in line with the concept that the microenvironment within the tissues can shape the local Treg characteristics and is in agreement with recent studies showing the highest Treg frequencies in the more oxidative brown fat as compared to other white adipose tissues (Källin et al. 2017).

Here we used voluntary wheel running as the mode of exercise to study the training effects on immune cells. Exercised and pre-exercised groups were evaluated with regard to the immediate effects and the maintenance effects of prior exercise, respectively. Prior exercise training has shown to exert different benefits like attenuating the short term HFD induced weight gain in mice (Snook et al. 2016) and enhancing beta-adrenergic signaling in adipose tissue of HFD fed mice (Snook et al. 2017). Furthermore, voluntary wheel running prior to tumor inoculation reduced the tumor growth to a greater extent than post exercise through regulation of immune cells in a tumor model (Pederson et al. 2016). Here we demonstrate that muscle-residing Treg frequencies were significantly enhanced upon exercise with maximum frequencies observed in pre-exercised mice. These findings emphasize the maintenance of Tregs even after termination of exercise and suggest a memory effect of exercise in driving local Tregs. Exercise-mediated rise in Treg frequencies was also maintained

under hypercaloric diet conditions. We could not notice any alteration in the *ex-vivo* Treg frequencies due to HFHS diet as such, which might be due to its shorter duration.

These increased Treg frequencies could be due to several possibilities including the expression of certain chemotactic molecules by tissues or certain receptors on Tregs required for the recruitment of circulating Tregs and retaining them therein. Tissue-specific antigens can also cause either the expansion of Tregs or the conversion of conventional T cells into induced Tregs able to adapt to the new tissue environment (Burzyn et al. 2013). Based on the significant increase in the proliferative marker Ki67 on Tregs in pre-exercised mice, local expansion of muscle-residing Tregs may be one mechanism contributing to the enhanced Treg frequencies. In line with the observed enhanced muscle Treg frequencies, pre-exercised mice also exhibited a significant increase in the *de-novo* Treg induction capacity in the popliteal lymph nodes, indicating possible alterations in the naïve T cell itself following exercise. To get a clearer picture, Treg induction capacity of the T cells from skeletal muscle should be analyzed.

For a mechanistical dissection of the increased Treg frequencies in pre-exercised mice, phenotypic characteristics of Tregs needed for an adaption to the local environment in the residing tissues were studied. Each tissue-residing Tregs possess unique characteristics and functions and express specific molecules that render them different from the lymphoid Tregs (Burzyn et al. 2003). Here we show that AREG, EGFR and ST2 are present on the muscle-residing Tregs and these molecules are known to enhance the suppressive capacity of Tregs (Zhou et al 2015, Zaiss et al. 2013, Siede et al. 2016). Exercise elevated the AREG, EGFR and $ST2^{+}$Tregs, implying that those Tregs possess high suppressive capacity.

Amphiregulin can act in an autocrine manner and can further enhance Treg activity (Zaiss et al. 2015). In the context of muscle residing Tregs, AREG has shown to facililtate muscle repair processes and differentiation of muscle satellite cells and Treg levels are also elevated and retained within the muscles

for a longer period of time (Burzyn et al. 2013). Role of immune cells in exercise induced muscle damage and repair is also reported. Depending on the intensity of exercise, mechanical impact can cause the release of alarmins which trigger a well co-ordinated cascade by different immune cells participating in the initial inflammatory phase followed by resolution and tissue repair phase (Jones & Hoyne 2017, Deyhle & Hyldahl 2018). Tregs are reported to participate in the resolution and repair phase following injury and amphiregulin is a major factor facilitating these processes (Villalta et al. 2014, Arpaia et al. 2015). A recent study by Minutti et al. demonstrates that macrophage derived amphiregulin induces TGF-β activation on pericytes, following tissue injury and leads to re-vascularization and wound healing (Minutti et al. 2019). Treg derived amphiregulin contributes to the expansion and survival of tissue-resident Tregs upon injury (Zaiss et al. 2019), but whether it acts via the same mechanism despite its source is not known.

Exercise-induced skeletal muscle damage is thought to be necessary for long-term muscle strengthening adaptations. It has been reported in different mice strains that with voluntary wheel running exercise, hind limb muscle fibre damage can occur at the onset of running followed by muscle regeneration (Irintchev & Wernig 1987). Furthermore, another study shows that voluntary running can induce the proliferation of satellite cells without causing muscle hypertrophy (Kurosaka et al. 2009). Since Tregs and AREG are shown to be elevated following muscle injury (Burzyn et al. 2013) and Tregs directly activate satellite cells facilitating muscle regeneration (Castiglioni et al. 2015, Schiaffino et al. 2017), the increase in AREG^{+}Tregs we observed following exercise might have been possibly triggered from the slight damage induced by exercise. But, further studies need to be done in order to investigate this. Dissecting out the mechanisms underlying the exercise-induced changes is difficult since exercise is a highly complex process which simultaneously involves several responses in multiple tissues and organs at the cellular and systemic level. Among several factors released upon exercise, some of them may trigger the induction of Tregs directly or indirectly and certain muscle-specific antigens might help in retaining the Tregs or expanding them, which might be the case happening in the pre-exercised mice.

In order to identify exercise triggered factors that might have contributed to the enhanced Tregs, we focused primarily on the well known pleiotropic myokine, IL-6 (Febbraio &Pederson 2002, Pal et al. 2014) and its signaling on T cells. We were able to identify the presence of IL6Rα on muscle-residing Tregs and the IL6Rα levels were found to be significantly increased in pre-exercised mice. This is in line with the observation that upon exercise training, the levels of IL6 receptor alpha (IL6Rα) on skeletal muscle is elevated (Keller et al. 2005, Pederson & Febbraio 2008. From the T cell-specific IL6RαKO mice employed to study the role of IL6Rα signaling on T cells, the significant increase in the Treg frequencies in pre-exercised floxed controls were not evident in pre-exercised KOs. The Treg frequencies in pre-exercised KOs were rather similar as that of the sedentary KO mice indicating that signaling via IL6Rα in T cells contributes to the increased Treg frequencies upon exercise. Eventhough the absence of IL6Rα on T cells blocks the classical IL-6 signaling, IL-6 trans-signaling can still happen which is initiated by binding of IL-6 to sIL6R. But from the result of T cell IL6RaKOs, since we do not see any increase in Tregs, we could speculate that trans-signaling does not play any role in the upregulation of Tregs. The phenotypic changes including the upregulation of ST2, AREG and EGFR were also absent in these mice deficient in IL6Rα signalling in T cells indicating the contribution of IL6 receptor signaling in the upregulation of these markers on T cells.

In order to further understand whether exercise induced IL-6 has any direct impact on IL6R signalling in T cells causing the increased Treg frequencies in pre-exercised mice, muscle-specific IL-6 KO mice were utilized. Pre-exercised muscle-specific IL-6 KOs exhibited a similar increase in the Treg frequencies as that of pre-exercised flox controls, indicating that IL-6 as such does not have any direct impact on the increased Treg frequencies in pre-exercised mice. But the phenotypic characterization of muscle specific IL-6 KOs upon exercise implicates that IL-6 influences the expression of ST2, AREG and EGFR on Tregs and its proliferation as there was no increase in these markers in pre-exercised IL-6 KOs. Since IL-6 directly does not have any impact on IL6R signaling in Tregs for enhancing the Treg frequencies and specifically Foxp3

expression, one underlying possibility is that other factors released upon exercise may signal via their respective receptors and those receptors or their downstream signaling molecules might activate the IL6Rα signaling in T cells. Since studies using both KO models demonstrate the contribution of IL-6 as well as IL6Rα signaling in increasing AREG, ST2 and EGFR, we could speculate that these signals are somehow integrated. Several studies report cross talk of signals between EGFR and IL6R (Colomiere et al. 2009, Stolarczyk et al. 2016). Another report shows that IL6R induced STAT3 activation is prolonged by the binding of IL6R with EGFR (Wang et al. 2013) which point out to the fact that IL-6 itself may not be a sole requirement for the activation of IL-6R signaling. Furthermore, several factors can induce the production of AREG which further activates the EGF receptor. IL-33, ligand for ST2 receptor is also reported to be produced in skeletal muscle upon different exercise paradigms in a recent study (Little et al. 2018). Upon exercise, we also saw an elevation in AREG, EGFR, ST2 and IL6Rα expressing Tregs. In Th2 cells, the signaling complex between EGFR and ST2, and IL-33 induced EGFR-mediated signaling is also reported (Minutti et al. 2017). Different studies report the possible involvement of IL-33/ST2 axis in muscle Treg cell accumulation and function (Kuswanto et al. 2016, Biton et al. 2016). Monticelli et al. reports that IL-33 can induce the release of AREG from gut associated innate lymphoid cells, highlighting the involvement of IL-33-AREG-EGFR pathway in tissue protection (Monticelli et al. 2015). AREG is known to act through EGFR signaling pathway, thereby enhancing the Treg suppressive function (Zaiss et al. 2013, Zaiss et al. 2015). All these aspects point to the fact that there may be multiple factors contributing in a co-ordinated manner to enhance the IL6Rα signaling in Tregs even in the absence of the IL-6 cytokine itself, thereby leading to the durable induction of skeletal muscle-residing regulatory T cells.

As exercise-induced upregulation of Foxp3 is abolished upon IL6Rα deletion in T cells, signaling cascades downstream of IL6Rα also might play a role in the enhancement of Foxp3. Previous reports show the direct role of STAT3 in the maintenance of Treg phenotype and function (Pallandre et al. 2007). Foxp3 can act as a co-transcription factor with STAT3, thereby enhancing IL10 gene transcription in tumour-induced Tregs (Hossain et al. 2013). Regulatory T cells

are also reported to control pathogenic Th17 responses in a STAT3 dependent manner (Chaudhry et al. 2009). Being a downstream factor in IL6Rα signaling, and owing to its association with Foxp3, STAT3 might be involved in exercise induced upregulation of Tregs and is worth analyzing.

Together, we demonstrate that exercise supports a stable induction of skeletal muscle-residing Foxp3^{+}Tregs with high functional activity as evidenced by the increased expression of AREG, EGFR and ST2. The significant increase we saw in pre-exercised mice and their ability to sustain and adapt to the local environment under standard diet and 4 weeks of HFHS diet specifically points out to the memory effect of exercise. Exposure to a long term HFHS diet should also be addressed in order to see whether this affects the maintenance of Tregs. By maintaining a homeostatic environment, Tregs might also help in withstanding the metabolic challenges thereafter and could therefore delay the onset of metabolic diseases. Using T cell specific loss of function models, we show that exercise induced Tregs and its phenotypic alterations require IL6R on T cells. Our results also unravel a view into the crosstalk between the complex signaling network integrating IL6/IL6R-AREG/EGFR/ST2 in enhancing the muscle Tregs upon exercise. Overall, this thesis contributes to the understanding of immune cell regulation by different interventions to interfere with the inflammation associated with metabolic diseases. Furthermore, it provides mechanistic insights into the induction of immunomodulatory cells as a therapeutic intervention so as to maintain or restore the metabolic tissue homeostasis.

6. Conclusion & perspectives

The relevance of targeting immune modulation to improve metabolic diseases arises due to the highly integrated and inter-dependent immune metabolic interactions. Understanding the potential players involved and the underlying mechanisms will help in developing therapeutic strategies to enhance or suppress them in order to attain a homeostatic environment, thereby reducing the inflammation. In this thesis, two distinct strategies were employed to address the impact on adaptive immune cells.

Exposure to a high calorie diet altered the systemic immune cell profiles to an inflammatory phenotype. Pharmacological strategies targeting GLP-1 receptor demonstrated that GLP-1R is involved in the regulation of immune cells and in the lowering of systemic inflammation. GLP-1R agonists impacted total T lymphocytes as well as the B cell maturation process, by lowering the mature B cells which were upregulated upon high calorie diet. Furthermore, studies using mice with a deletion of GLP-1R also revealed its possible involvement in the B cell maturation process. As several transitional stages are involved in the B cell developmental processes, more markers are needed to be included to clearly distinguish between the different B cell stages. GLP-1/Dexa conjugate lowered the high calorie-diet induced systemic inflammation to a greater extent than its monoagonist control demonstrating the combined effect of the co-agonist. In order to identify the key players, more studies need to be done focusing at the subset level rather than looking at the total lymphocyte populations. Furthermore, studies on a tissue basis apart from circulation could give more insights into the local regulation.

Data obtained from the physiological strategy like exercise demonstrates that endurance exercise training supports a durable induction of skeletal muscle-residing regulatory T cells which is dependent on T cell specific IL6Rα signaling and not on muscle-derived IL-6. Predominantly oxidative muscle exhibited a more prominent increase in Treg frequencies when compared to the glycolytic ones. Even upon exposure to 4 weeks of HFHS diet, the Treg frequencies were

elevated following exercise training. The higher Treg frequencies in pre-exercised mice demonstrates that Tregs are maintained even after termination of exercise under both dietary conditions. However, studies under a long term HFHS diet should be addressed to see how the induction of Tregs by prior exercise training protects against the deleterious effects of high calorie diet. The enhanced Treg induction capacity of the naïve T cells in popliteal lymph nodes from pre-exercised mice indicate that certain metabolic changes might also be occurring at a naïve T cell level, but has to be confirmed using the T cells from muscle. T cells could also sense the immediate surrounding environment thus altering the metabolic activity within the T cells shifting it to an effector or a regulatory phenotype which is also worth addressing.

The significant increase of AREG, ST2, EGFR and Ki67 expressing Tregs in pre-exercised mice implicates its high suppressive and proliferative capacity and the data from the KO mice models demonstrates that the expression of these markers are dependent on both IL-6 and Tcell IL6Rα signalling. Since IL-6 does not have any direct impact on the increased Treg frequencies, these results together with several other studies highlighting the signal cross-talk and complex formation of EGFR with IL6R and ST2 (Colomiere et al. 2009, Wang et al. 2013, Minutti et al. 2017) indicates that certain factors apart from IL-6 might also support the IL6Rα signaling in Tregs upon exercise. Further studies in this respect, based on the deletion of either EGFR or ST2 as well as their ligands AREG or IL33 will help in delineating the complex networks involved with T cell IL6Rα signaling causing the increased Treg frequencies. Likewise, the downstream signaling factors of IL6Rα signaling (specifically STAT3) enhancing Foxp3 is also required to unravel the complete picture. Moreover, the functionality and therapeutic potential of exercise induced Tregs as a protective as well as a treatment strategy under diet-induced obese conditions should be analyzed. Due to the paracrine and endocrine properties of the myokines, the impact of exercise on Tregs residing within other metabolically relevant tissues is also worth addressing. These mechanistic findings could contribute to the understanding of tissue Treg maintenance and could help in identifying the right players involved and in the development of novel

therapeutic strategies aiming at immunomodulation with the goal to reduce the inflammation and lower/prevent the incidence of metabolic insults.

7. References

Agerso, H., Jensen, L.B., Elbrond, B. Rolan, P. & Zdravkovic, M. The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy man. *Diabetologia.* 2002. 45, 195-202.

Ait-Oufella, H., Salomon, B.L., Potteaux, S., Robertson, A.K., Gourdy, P., Zoll, J., Merval, R., Esposito, B., Cohen, J.L., Fisson, S., Flavell, R.A., Hansson, G.K., Klatzmann, D., Tedgui, A. & Mallat, Z. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat. Med.* 2006. 12,178-180.

Andersen, C.J., Murphy, K.E. & Fernandez, M.L. Impact of obesity and metabolic syndrome on obesity. *Adv Nutr.* 2016. 7, 66-75.

Apostolou, I. & von Boehmer, H. In vivo instruction of suppressor commitment in naïve T cells. *J Exp. Med.* 2004. 199, 1401-1408.

Arpaia, N., Green, J.A., Moltedo, B., Arvey, A., Hemmers, S., Yuan, S., Treuting, P.M., Rudensky, A.Y. A distinct function of Regulatory T cells in tissue protection. *Cell.* 2015.162**,**1078- 1089*.*

Asseman, C., Mauze, S., Leach, M.W., Coffman, R.L. & Powrie, F. An essential role for interleukin10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med.* 1999. 190, 995-1004.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Feguson, P.J., Whitesell, L., Kelly, T.E., Saulsbry, F.T., Chance, P.F. & Ochs, H.D. The immune dysregulation, polyendocrinopathy, enteropathy, x- linked syndrome (IPEX) is caused by mutations of Foxp3. *Nat. Genet.* 2001. 27, 20-21.

Berger, N.A. Obesity and cancer pathogenesis. *Ann N Y Acad Sci.* 2014. 1311, 57-76.

Bilate, A.M & Lafaille, J.J. Induced CD4+Foxp3+ Regulatory T cells in immune tolerance. *Annu. Rev. Immunol.* 2012. 30, 733-758.

Biton, J., Athari, S.K., Thiolat, A., Santinon, F., Lemeiter, D., Herve, R., Delavallee, L., Levascot,A., Rogs, S., Decker, P., Girard, J.P., Herbelin, A., Boissier, M.C. & Bessis, N. In vivo expansion of activated Foxp3+ regulatory T cells and establishment of a type 2 immune response upon Il-33 treatment protect against experimental arthritis. *J Immunol.* 2016. 197, 1708-1719.

Booth, F.W., Roberts, C.K. & Laye, M.J. Lack of exercise is a major cause of chronic diseases. *Compr Physiol.* 2012. 2, 1143-1211.

Bothe, G.W., Haspel, J.A., Smith, C.L., Wiener, H.H. & Burden, S.J. Selective expression of Cre Recombinase in skeletal muscle fibres. *Genesis* 2000. 26, 165-166.

Breous, E., Somanathan, S., Vendenberghe, L.H. & Wilson, J.M. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. *Hepatology.* 2009. 50, 612-621.

Brestoff, J.R. & Artis, D. Immune regulation of metabolic homeostasis in health and disease. *Cell.* 2015. 161, 146-160.

Brunkow, M.E., Jeffery, E.W., Hjerrlid, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F. & Ramsdell, F. Disruption of a new forkhead/winged–helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet*. 2001. 27, 68-73.

Burzyn, D., Kuswanto, W., Kolodin, D., Shadrach, J.L., Cerletti, M., Jang, Y., Sefik, E., Tan, T.G., Wagers, A,J., Benoist, C. & Mathis, D. A special population of Regulatory T cells potentiates muscle repair. *Cell.* 2013. 155, 1282-1295.

Burzyn, D., Benoist, C & Mathis, D. Regulatory T cells in nonlymphoid tissues. *Nat. Immunol.* 2013. 14, 1007-1013.

Cai, D., Yuan, M., Frantz, D.F., Melendez, P.A., Hansen, L. Lee, J. & Shoelson, S.E. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat. Med.* 2005. 11,183-190.

Campbell, D.J. & Koch, M.A. Phenotypic and functional specialization of $Foxp3^{+}$ regulatory T cells. *Nat. Rev. Immunol.* 2011.11,119-130.

Cancello, R., Tordjman, J., Poitou, C., Guilhem, G., Bouillot, J.L., Hugol, D., Coussieu,C., Basdevant, A., Bar Hen, A., Bedossa, P., Guerre- Millo, M. & Clement, K. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes.* 2006. 55,1554-1561.

Cao, X., Cai, S.F., Fehniger, T.A., Song, J., Collins, L.I., Piwnica-Worms, D.R. & Ley, T.J. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity.* 2007. 27, 635-646.

Caridade, M., Graca, L. & Ribeiro, R.M. Mechanisms underlying CD4+Treg immune regulation in the adult : from experiments to models. *Front. Immunol.* 2013. 4: 378.

Castiglioni, A., Corna, G., Rigamonti, E., Basso, V., Vezzoli, M., Monno, A., Almada, A.E., Mondino, A., Wagers, A.J., Manfredi, A.A. & Rovere- Querini, P. $Foxp3^{+}$ T cells recruited to sites of sterile skeletal muscle injury regulate the fate of satellite cells and guide effective tissue regeneration. *PLoS ONE.* 2015. 10, e0128094.

Chaudhry, A., Rudra, D., Treuting, P., Samstein, R.M., Liang, Y., Kas, A. & Rudensky, A.Y. CD4+ regulatory T cells control Th17 responses in a STAT-3 dependent manner. *Science*. 2009. 326, 986-991.

Chen, J., Guan, Z., Wang, L., Song, G., Ma, B. & Wang, Y. Meta-analysis: overweight , obesity, and Parkinson's disease. *Int J Endocrinolo.* 2014. 203930.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G. & Wahl, S.M. Conversion of peripheral CD4+CD25- naïve T cells to CD4+CD25+ regulatory T cells by TGF- beta induction of transcription factor Foxp3. *J Exp. Med.* 2003. 198, 1875- 1886.

Chen, X., Wu, Y. & Wang, L. Fat-resident Tregs: an emerging guard protecting from obesity-associated metabolic disorders. *Obes Rev.* 2013. 14, 568-578.

Choi, Y.S., Eto, D., Yang, J.A., Lao, C. & Crotty, S. Cutting edge: STAT1 is required for IL-6 mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol.* 2013. 190, 3049- 3053.

Cipolletta, D., Feuerer, M., Li, A., Kamei, N., Lee, J., Shoelson, S.E., Benoist, C. & Mathis, D. PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature.* 2012. 486, 549-553.

Clifford, T., Wood, M.J., Stocks, P., Howatson, G., Stevenson, E.J. & Hilkens, C.M.U. T- regulatory cells exhibit a biphasic response to prolonged endurance exercise in humans. *Eur J Appl Physiol.* 2017. 117, 1727-1737.

Colomiere, M., Ward, A.C., Riley, c., Trenerry, M.K., Cameron- Smith, D., Findlay, J., Ackland, L. & Ahmed, N. Cross talk of signals between EGFR and IL-6R through JAK2/STAT3 mediate epithelial- mesenchymal transition in ovarian carcinomas. *Br J Cancer.* 2009. 100, 134-144.

Dai, K., Huang, L., Chen, J., Yang, L. & Gong, Z. Amphiregulin promotes the immunosuppressive activity of intrahepatic $CD4^+$ regulatory T cells to impair $CD8^+$ T cell immunity against hepatitis B virus infection. *Immunology*, 2014. 144, 506-517.

Daniel, C. & Boehmer, H. Extrathymic generation of regulatory T cells- change and challenges for prevention of autoimmune diseases. *Adv. Immunol*. 2012. 112, 117-213.

Daniel, C., Wennhold, K., Kim, H.J. & von Boehmer, H. Enhancement of antigen-specific Treg vaccination in-vivo. *PNAS*. 2010. 107, 16246 – 251.

Day, J.W., Ottaway, N., Patterson, J.T., Gelfanov, V., Smiley, D., Gidda, J., Findeisen, H., Bruemmer, D., Drucker, D.J., Chaudhary, N., Holland, J., Hembree, J., Abplanalp, W., Grant, E., Ruehl, J., Wilson, H., Kirchner, H., Lockie, S.H., Hofmann, S., Woods, S.C., Nougueiras, R., Pfluger, P.T., Perez-Tilve, D., DiMarchi, R. & Tschöp, M.H. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat. Chem. Biol.* 2009. 5, 749-757.

De Souza, C.T., Araujo, E.P., Bordin, S., Ashimine, R., Zollner, R.L., Boschero, A.C., Saad, M.J. & Velloso, L.A. Consumption of a fat-rich diet activates a

proinflammatory response and induces insulin resistance in hypothalamus. *Endocrinology.* 2005. 146, 4192-4199.

Deng, T., Liu, J., Deng, Y., Minze, L., Xiao, X., Wright, V., Yu, R., Li, X.C., Blaszczak, A., Bergin, S., DiSilvestro, D., Judd, R., Bradley, D., Caligiuri,M., Lyon, C.J. & Hsueh, W.A. Adipocyte adaptive immunity mediates diet – induced adipose inflammation and insulin resistance by decreasing adipose Treg cells. *Nat. Commun.* 2017. 8, 15725.

de Boer, O.J., van der Meer, J.J., Teeling, P., van der Loos, C.M. & van der Wal, A.C. Low numbers of Foxp3 positive regulatory T cells are present in all developmental stages of human atherosclerotic lesions. *PLoS One.* 2007. 2, e779.

Deyhle, M.R. & Hyldahl, R.D. The role of T lymphocytes in skeletal muscle repair from traumatic and contraction- induced injury. *Front. Physiol.* 2018. 9, 768.

Dicker, D., Salook, M.A., Marcoviciu, D., Djaldetti, M. & Bessler, H. Role of peripheral blood mononuclear cells in the predisposition of obese individuals to inflammation and infection. *Obes. Facts.* 2013. 6(2),146-151.

Donath, M.Y. & Shoelson, S.E. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol.* 2011. 11, 98-107.

Drucker, D.J & Nauck, M.A. The incretin system: glucagon-like peptide -1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet.* 2006. 368, 1696 -1705.

Dufour, J. H., Dziejman, M., Liu, M.T., Leung, J.H., Lane, T.E. & Luster, A.D. IFN–gamma inducible protein 10 (IP10, CXCL10) - deficient mice reveal a role for IP10 in effector T cell generation and trafficking. *J. Immunol.* 2002.168, 3195- 3204.

Egan, B. & Zierath, J.R. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab.* 2013. 17, 162-184.

Ehses, J.A., Perren, A., Eppler, E., Ribaux, P., Pospisilik, J.A., Maor-Cahn, R., Gueripel, X., Ellingsgaard, H., Schneider, M.K., Biollaz, G., Fontana, A., Reinecke, M., Homo- Delarche, F. & Donath, M.Y. Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes.* 2007. 56, 2356-2370.

Eller, K., Kirsch, A., Wolf, A.M., Sieghart, S., Andrea, T., Stanzl, U., Wolf, D., Patsch, W., Rosenkranz, A.R. & Eller, P. Potential role of regulatory T cells in reversing obesity-linked insulin resistance and diabetic nephropathy. *Diabetes.* 2011. 60, 2954-2962.

Ellingsgaard, H., Hauselmann, I., Schuler, B., Habib, A.M., Baggio, L.L., Meier, D.T., Eppler, E., Bouzakri, K., Wueest, S., Muller, Y.D., Hansen, A.M., Reinecke, M., Konrad, D., Gassmann, M., Reimann, F., Halban, P.A.,

Gromada, J., Drucker, D.J., Gribble, F.M., Ehses, J.A. & Donath, M.Y. Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. 2011. 17, 1481-1489.

Facciabene, A., Peng, X., Hagemann, I.S., Balint, K., Barchetti, A., Wang, L.P., Gimotty, P.A., Gilks, C.B., Lal, P., Zhang, L. & Coukos, G. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature.* 2011. 475, 226-230.

Fahlen, L., Read, S., Gorelik, L., Hurst, S.D., Coffman, R.L. Flavell, R.A. & Powrie, F. T cells that cannot respond to TGF-beta escape control by CD(+)CD25(+) regulatory T cells. *J Exp Med.* 2005. 201, 737-746.

Febbraio, M.A. & Pederson, B.K: Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB J.* 2002. 16, 1335-1347.

Festa, A., D'Agostino, R.Jr., Williams, K., Karter, A.J., Mayer-Davis, E.J., Tracy, R.P., Haffner, S.M. The relation of body fat mass and distribution to markers of chronic inflammation. *Int.J. Obes Relat Metab Disord.* 2001. 25,1407-1415.

Feuerer, M., Herrero, L., Cipolleta, D., Naaz, A., Wong, J., Nayer, A., Lee, J., Goldfine, A.B., Benoist, C., Shoelson, S. & Mathis, D. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* 2009*.* 15, 930-939.

Finan, B., Yang, B., Ottaway, N., Stemmer, K., Müller, T.D., Yi, C.X., Habegger, K., Schriever, S.C., Garcia-Caceres, C., Kabra, D.G., Hembree, J., Holland, J., Raver, C., Seelev, R.J., Hans, W., Irmler, M., Beckers, J., de Angelis, M.H., Tiano, J.P., Mauvais-Jarvis, F., Perez-Tilve, D., Pfluger, P., Zhang, L., Gelfanov, V., DiMarchi, R.D. &Tschöp, M.H. Targeted estrogen delivery reverses the metabolic syndrome. *Nat. Med.* 2012. 18,1847-1856.

Finan, B., Ma, T., Ottaway, N., Müller, T.D., Habegger, K.M., Heppner, K.M., Kirchner, H., Holland, J., Hembree, J., Raver, C., Lockie, S.H., Smiley, D.L., Gelfanov, V., Yang, B., Hofmann, S., Bruemmer, D., Drucker, D.J., Pfluger, P.T., Perez-Tilve, D., Gidda, J., Vignati, L., Zhang, L., Hauptman, J.B., Lau, M., Brecheisen, M., Uhles,S., Riboulet, W., Hainaut, E., Sebokova, E., Conde-Knape, K., Konkar, A., DiMarchi, R.D. & Tschöp, M.H. Unimolecular dual incretins maximize metabolic benefits in rodents, monkeys and humans. *Sci.Trans. Med.* 2013. 5, 209ra151.

Fiuza- Luces, C., Garatachea, N., Berger, N.A. & Lucia, A. Exercise is the real polypill. *Physiology.* 2013. 28, 330-358.

Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K., Chang, H-D., Bopp, T., Schmitt, E., Klein-Hessling, S., Serfling, E., Hamann, A. & Huehn, J. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol.* 2007. 5, e38.

Foks, A.C., Lichtman, A.H. & Kuiper, J. Treating atherosclerosis with regulatory T cells. *Arterioscler Thromb Vasc Biol.* 2015. 35, 280-287.

Fontenot, J.D., Gavin, M.A. & Rudensky, A.Y. Foxp3 programms the development and function of cD4+CD25+ regulatory T cells. *Nat. Immunol.* 2003. 4, 330-336.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima,M., Matsuda, M. & Shimomura, I. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest.* 2004. 114, 1752-1761.

Garcia- Hernandez, M.H., Rodriguez-Varela, E., Garcia- Jacobo, R.E., Hernandez-De Ia Torre, M., Uresti- Rivera, E.E., Gonzalez-Amaro, R. & Portales-Perez, D.P. Frequency of regulatory B cells in adipose tissue and peripheral blood from individuals with overweight, obesity and normal-weight. *Obes Res Clin Pract.* 2018.12, 513-519.

Gleeson, M., Bishop, N.C., Stensel, D.J., Lindley, M.R., Mastana, S.S. & Nimmo, M.A. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat. Rev. Immunol.* 2011. 11, 607-615.

Gottschalk, R.A. & Corse E Allison, J.P. TCR ligand density and affinity determine peripheral induction of Foxp3 in-vivo. *J. Exp. Med.* 2010. 207, 1701-1711.

Gratz, I.K., Truong, H.A., Yang, S.H., Maurano, M.M., Lee, K., Abbas, A.K. & Rosenblum, M.D. Cutting edge: memory regulatory T cells require IL-7 and not IL-2 for their maintenance in peripheral tissues. *J. Immunol.* 2013. 190, 4483-4487.

Griffin, T.M., Huebner, J.L., Kraus, V.B., Yan, Z. & Guilak, F. Induction of osteoarthritis and metabolic inflammation by a very high fat diet in mice: effects of short-term exercise. *Arthritis Rheum.* 2012. 64, 443-453.

Guo, F. & Garvey, W.T. Trends in Cardiovascular health metrics in obese adults: National health and nutrition examination survey (NHANES), 1988-2014. *J Am Heart Assoc.* 2016. 5.

Guo, C., Huang, T., Chen, A., Chen, X., Wang, L., Shen, F. & Gu, X. Glucagon-like peptide 1 improves insulin resistance in vitro through antiinflammation of macrophages. *Braz J Med Biol Res.* 2016. 49, e5826.

Gutzeit, C., Chen, K. & Cerutti, A. The enigmatic function of IgD: some answers at last. *Eur J Immunol.* 2018. 48, 1101-1113.

Gutzwiller, J.P., Drewe, J., Göke, B., Schmidt, H., Rohrer, B., Lareida, J. & Beglinger, C. Glucagon- like peptide-1 promotes satiety and reduces food

intake in patients with diabetes mellitus type 2. *Am. J. Physiol.*1999. 276, R1541- 1544.

Hadjiyanni, I., Baggio, L.L., Poussier, P. & Drucker, D.J. Exendin-4 modulates diabetes onset in nonobese diabetic mice. *Endocrinology.* 2008. 149, 1338-1349.

Hadjiyanni, I., Siminovitch, K.A., Danska, J.S. & Drucker, D.J. Glucagon-like peptide-1 receptor signaling selectively regulates murne lymphocyte proliferation and maintenance of peripheral regulatory T cells. *Diabetologia.* 2010. 53, 730-740.

Han, J.M. & Levings, M.K. Immune regulation in obesity-associated adipose inflammation. *J. Immunol.* 2013. 191,527-532.

Han, J.M., Wu, D., Denroche, H.C., Yao, Y., Verchere, C.B. & Levings, M.K. IL-33 reverses and obesity- induced deficit in visceral adipose tissue ST2+ T regulatory cells and ameliorates adipose tissue inflammation and insulin resistance. *J. Immunol.* 2015. 194, 4777- 4783.

Heink, S., Yogev, N., Garbers, C., Herwerth, M., Aly, L., Gasperi, C., Husterer, V., Croxford, A.L., Möller-Hackbarth, K., Bartsch, H.S., Sotlar, K., Krebs, S., Rgen, T., Blum, H., Hemmer, B., Misgeld, T., Wunderlich, T.F., Hidalgo, J., Oukka, M., Rose-John, S., Schmidt.Supprian, M., Waisman, A. & Kor, T. Trans-presentation of interleukin-6 by dendritic cells is required for priming pathogenic T_H17 cells. *Nat Immunol.* 2017. 18, 74-85.

Hill, J.O., Wyatt, H.R. & Peters, J.C. Energy balance and obesity. *Circulation.* 2012. 126, 126-132.

Hiscock, N., Chan, M.H.S., Bisucci, T., Darby, I.A. & Febbraio, M.A. Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. *FASEB J.* 2004.18, 992-994.

Holloszy, J.O. Biochemical adaptations in muscle, effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem.*1967. 242, 2278-2282.

Holst, J.J. The physiology of Glucagon-like peptide-1. *Phys Rev.* 2007. 87, 1409-1439.

Hong, E.G., Ko, H.J., Cho, Y.R., Kim, H.J., Ma, Z., Yu, T.Y., Friedline, R.H., Kurt-Jones, E., Finberg, R., Fischer, M.A., Granger, E.L., Norbury, C.C., Hauschka, S.D., Philbrick, W.M., Lee, C.G., Elias, J.A. & Kim, J.K. Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle. *Diabetes.* 2009. 58, 2525-2535.

Hossain, D.M., Panda, A.K., Manna, A., Mohanty, S., Bhattacharjee, P., Bhattacharyya, S., Saha, T., Chakraborty, S., Kar, R.K., Das, T., Chatterjee, S.

& Sa, G. Foxp3 acts as a cotranscription factor with STAT3 in tumour- induced regulatory T cells. *Immunity*. 2013. 39, 1057-1069.

Hotamisligil, G.S. Inflammation and metabolic disorders. *Nature.* 2006. 444, 860-867.

Hotamisligil, G.S., Murray, D.L., Choy, L.N. & Spiegelman, B.M. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *PNAS.* 1994. 91,4854-4858.

Hotamisligil, G.S., Shargill, N.S. & Spiegelman, B.M. Adipose expression of tumor necrosis factor- alpha: direct role in obesity- linked insulin resistance. *Science.* 1993. 259, 87-91.

Howald, H., Hoppeler, H., Claassen, H., Mathieu, O. & Straub, R. Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans. *Pflugers Arch.* 1985. 403, 369-376.

Huh, J.Y. The role of exercise-induced myokines in regulating metabolism. *Arch.Pharm.Res.* 2018. 41, 14-29.

Hunter, C.A. & Jones, S.A, IL-6 as a keystone cytokine in health and disease. *Nat Immunol.* 2015. 16, 448-457.

Ilan, Y., Maron, R., Tukpah, A.M., Maioli, T.U., Murugaiyan, G., Yang, K., Wu, H.Y. & Weiner, H.L. Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice. *PNAS.* 2010. 107, 9765-9770.

Irintchev, A. & Wernig, A. Muscle damage and repair in voluntarily running mice: strain and muscle differences. *Cell Tissue Res.* 1987. 249, 509-521.

Ito, M., Komai, K., Mise-Omata, S., Iizuka-Koga, M., Noguchi, Y., Kondo, T., Sakai, R., Matsuo, K., Nakayama, T., Yoshie, O., Nakatsukasa, H., Chikuma, S., Shichita, T. & Yoshimura, A. Brain regulatory T cells suppress astrogliosis and potentiate neurological recovery. *Nature.* 2019. 565, 246-250.

Iwata, Y., Matsushita, T., Horikawa, M., Dilillo, D.J., Yanaba, K., Venturi, G.M., Szabolcs, P.M., Bernstein, S.H., Magro, C.M., Williams, A.D., Hall, R.P., St Clair, E.W. & Tedder, T.F. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood.* 2011. 117, 530-541.

Janeway, C.A. Jr., Travers, P. Walport, M. & Shlomchik, M.J. Generation of lymphocytes in bonemarrow and thymus. *Immunobiology:* The immune system in health and disease. 2001. 5th Edition.

Joanisse, S., Nederveen, J.P., Baker, J.M., Snijders, T., Iacono, C. & Parise, G. Exercise conditioning in old mice improves skeletal muscle regeneration. *FASEB J.* 2016. 30, 3256-3268.

Jones, B. & Hoyne, G.F. The role of the innate and adaptive immunity in exercise induced muscle damage and repair. *J Clin Cell Immunol.* 2017.8: 482.

Jones, S.A. Directing transition from innate to acquired immunity: Defining a role for IL-6. *J. Immunol.* 2005. 175, 3463-3468.

Jourdan, M., Cren, M., Robert, N., Bollore, K., Fest, T., Duperray, C.Guilloton, F., Hose, D., Tarte, K. & Klein, B. IL-6 supports the generation of human long-lived plasma cells in combination with either APRIL or stromal cell-soluble factors. *Leukemia.* 2014. 28, 1647-1656.

Kadoglou, N.P., Iliadis,F., Angelopoulou, N., Perrea, D.,Ampatzidis, G., Liapis, C.D. & Alevizos, M. The anti-inflammatory effects of exercise training in patients with type 2 diabetes mellitus. *Eur J Cardiovasc Prev Rehabil.* 2007. 14, 837-843.

Kälin, S., Becker, M., Ott, V.B., serr, I., Hosp, F., Mollah, M.M.H., Keipert, S., Lamp, D., Rohner- Jeanrenaud, F., Flynn, V.K., Scherm, M.G., Nascimento, L.F.R., Gerlach, K., Popp, V., Dietzen, S., Bopp, T., Krishnamurthy, P., Kaplan, M.H., Serrano, M., Woods, S.C., Tripal, P., Palmisano, R., Jastroch, M., Blüher, M., Wolfrum, C., Weigmann, B., Ziegler,A.G., Mann, M., Tschöp, M.H., & Daniel, C. A Stat6/Pten axis links regulatory T cells with adipose tissue function. *Cell Metab.* 2017. 26, 475-492.

Karstoft, K. & Pederson, B.K. Exercise and type 2 diabetes : focus on metabolism and inflammation. *Immunology and Cell Biology.* 2016. 94, 146-150.

Kawanishi, N., Yano, H., Yokogawa, Y. & Suzuki, K. Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. *Exerc immunol Rev.* 2010. 16, 105-118.

Keller, C., Steensberg, A., Hansen, A.K., Fischer, C.P., Plomgaard, P. & Pederson, B.K. Effect of exercise, training, and glycogen availability on IL-6 receptor expression in human skeletal muscle. *J Appl Physiol.* 2005. 99, 2075-2079.

Khan, I.M., Perrard, X.y., Brunner, G., Lui, H., Sparks, L.M., Smith, S.R., Wang, X., Shi, Z.Z., Lewis, D.E., Wu, H., Ballantyne, C.M. Intermuscular and perimuscular fat expansion in obesity correlates with skeletal muscle T cell and macrophage infiltration and insulin resistance. *Int. J. Obes.* 2015. 39, 1607-1618.

Kim, D., Kim, J., Yoon, J.H., Ghim, J., Yea, K., Song, P., Park, S., Lee, A., Hong, C.P., Jang, M.S., Kwon, Y., Park, S., Jang, M.H., Berggren, P.O., Suh, P.G. & Ryu, S.H. CXCL12 secreted from adipose tissue recruits macrophages and induces insulin resistance in mice. *Diabetologia* 2014. 57, 1456 – 1465.

Kolb, R., Kluz, P., Tan, Z.W., Borcherding, N., Bormann, N., Vishwakarma, A., Balcziak, L., Zhu, P., Davies, B.S., Gourronc, F., Liu, L.Z., Ge, X., Jiang, B.H., Gibson-Corley, K., Klingelhutz, A., Tan, N.S., Zhu, Y., Sutterwala, F.S., Shen,X. & Zhang, W. Obesity- associated inflammation promotes angiogenesis and breast cancer via angiopoietin-like 4. *Oncogene.* 2019. 38, 2351-2363.

Koppe, S.W. Obesity and the liver: nonalcoholic fatty liver disease. *Transl Res.* 2014. 164, 312-322.

Kosaraju, R., Guesdon, W., Crouch, M.J., Teague, H.A., Sullivan, E.M., Karlsson, E.A., Schultz-Cherry, S., Gowdy, K., Bridges, L.C., Reese, L.R., Neufer, P.D., Armstrong, M., Reisdorph, N., Milner, J.J., Beck, M. &Shaikh, S.R. B cell activity is impaired in human and mouse obesity and is responsive to an essential fatty acid upon murine influenza infection. *J Immunol.* 2017. 198 (12), 4738- 4752.

Kraakman, M.J., Kammoun, H.L., Allen, T.L., Deswaerte, V., Henstridge, D.C., Estevez, E., Matthews, V.B., Neill, B., White, D.A., Murphy, A.J., Peijs, L., Yang, C., Risis, S., Bruce, C.R., Du, X.J., Bobik, A., Lee-Young, R.S., Kingwell, B.A., Vasanthakumar, A., Shi, W., Kallies, A., Lancaster, G.I., Rose- John, S. & Febbraio, M.A. Blocking IL-6 trans-signaling prevents high-fat diet-induced adipose tissue macrophage recruitment but does not improve insulin resistance. *Cell Metab.* 2015. 21, 403-416.

Kralisch, S., Bluher, M., Tonjes, A., Lossner, U., Paschke, R., Stumvoll, M., Fasshauer, M. Tissue inhibitor of metalloproteinase-1 predicts adiposity in humans. *Euro. J. Endocrinol.* 2007. 156, 257 – 261.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M.C. & von Boehmer, H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 2005. 6, 1219-1227.

Kurosaka, M., Naito, H., Ogura, Y., Kojima, A., Goto, K. & Katamoto, S. Effects of voluntary wheel running on satellite cells in the rat plantaris muscle. *J Sports Sci Med.* 2009. 8, 51-57.

Kuswanto, W., Burzyn, D., Panduro, M., Wang, K.K., Jang, Y.C., Wagers, A.J., Benoist, C. & Mathis, D. Poor repair of skeletal muscle in aging mice reflects a defect in local, interleukin-33-dependent accumulation of regulatory T cells. *Immunity.* 2016. 44, 355-367.

Lancaster, G.I. & Febbraio, M.A. The immunomodulating role of exercise in metabolic disease. *Trends in Immunology.* 2014. 35, 262- 269.

LeBien, T.W. & Tedder, T.F. B lymphocytes: how they develop and function. *Blood.* 2008. 112,1570-1580.

Lee, Y.S., Park, M.S., Choung, J.S., Kim, S.S., Oh, H.H., Choi, C.S., Ha, S.Y., Kang, Y., Kim, Y. & Jun, H.S. Glucagon-like peptide-1 inhibits adipose tissue

macrophage infiltration and inflammation in an obese mouse model of diabetes. *Diabetologia.* 2012. 55, 2456-2468.

Lee, YS. & Jun, HS, Anti –inflammatory effects of GLP-1 based therapies beyond glucose control. Mediators of Inflammation. 2016. 3094642

Levine, J.A., Jensen, M. D., Eberhardt, N.L. & O'Brien, T. Adipocyte macrophage colony- stimulating factor is a mediator of adipose tissue growth. *J. Clin. Invest.* 1998. 101(8), 1557 – 1564.

Little, H.C., Tan, S.Y., Cali, F.M., Rodriguez, S., Lei, X., Wolfe, A., Hug, C. & Wong, G.W. Multiplex quantification identifies novel exercise-regulated myokines/cytokines in plasma and in glycolytic and oxidative skeletal muscle. *Mol Cell Proteomics.* 2018. 17, 1546-1563.

Loder, F., Mutschler, B., Ray, R.J., Paige, C.J., Sideras, P., Torres, R., Lamers, M.C. & Carsetti, R. B cell development in the spleen take place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med.*1999. 190, 75-89.

Long, Y.C. & Zierath, J.R. Influence of AMP-activated protein kinase and calcineurin on metabolic networks in skeletal muscle. *Am J Physiol Endocrinol Metab.* 2008. 295, E545-552.

Louche, K., Badin, P.M., Montastier, E., Laurens, C., Bourlier, V., de Glisezinski, I., Thalamas, C., Viguerie, N., Langin, D., Moro, C. Endurance exercise training up-regulates lipolytic proteins and reduces triglyceride content in skeletal muscle of obese subjects. *J Clin Endocrinol Metab.* 2013. 98, 4863-4871.

Lowder, T., Dugger, K., Deshane, J., Estell, K. & Schwiebert, L.M. Repeated bouts of aerobic exercise enhance regulatory T cell responses in a murine asthma model. *Bain Behav Immun.* 2010. 24, 153-159.

Lumeng, C.A. & Saltiel, A.R. Inflammatory links between obesity and metabolic disease. *J Clin Invest.* 2011. 121, 2111-2117.

Lumeng, C.N., Bodzin, J.L. & Saltiel, A.R. Obesity induces phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* 2007. 117,175-184.

Lustig, R.H. Childhood obesity: behavioral aberration or biochemical drive? Reinterpreting the first law of thermodynamics. *Nat Clin Pract Endocrinol Metab.* 2006. 2, 447-458.

Luu, M., Steinhoff, U. & Visekruna, A. Functional heterogeneity of gut-resident regulatory T cells. *Clinical & Translational Immunology.* 2017. 6, e156.

Lynch, L., Hogan, A.E., Duquette, D., Lester, C., Banks, A., LeClair, K., Cohen, D.E., Ghosh, A., Lu, B., Corrigan, M., Stevanovic, D., Maratos- Flier, E., Drucker, D.J., O'Shea, D. & Brenner, M. iNKT cells induce FGF21 for

thermogenesis and are required for maximal weight loss in GLP-1 therapy. *Cell Metab.* 2016. 24, 510-519.

Matthias, N. & Swirski, F.K. Regulating repair: Tregs in myocardial infarction. *Circ Res.* 2014. 115, 7-9.

McArdle, M.A. Mechanisms of obesity-induced inflammation and insulin resistance : insights into the emerging role of nutritional strategies. *Front. Endocrinol*. 2013. 4, 52.

McDonald, P.E., El-Kholy, W. Riedel, M.J., Salapatek, A.M., Light, P.E., Wheeler, M.B.The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. *Diabetes.* 2002. Suppl 3: S434-442.

Meagher, C., Beilke, J., Arreaza, G., Mi, Q.S., Chen, W., Salojin, K., Horst, N., Cruikshank, W.W. & Delovitch, T.L. Neutralization of interleukin-16 protects non-obese diabetic mice from autoimmune type-1 diabetes by a CCL4-dependent mechanism. *Diabetes* 2010. 59, 2862-2871.

Meloni, A.R., DeYoung, M.B., Lowe, c. & Parkes, D.G. GLP-1 receptor activated insulin secretion from pancreatic β-cells: mechanism and glucose dependence. *Diabetes Obes Metab.* 2013. 15, 15-27.

Minutti, C.M., Drube, S., Blair, N., Schwartz, C., McCrae, J.C., McKenzie, A.N., Kamradt, T., Mokry, M., Coffer, P.J., Sibilia, M., Sijts, A.J., Fallon, P.G., Maizels, R.M. & Zaiss, D.M. Epidermal growth factor receptor expression licenses type-2 helper T cells to function in a T cell receptor- independent fashion. *Immunity.* 2017. 47, 710-722.

Minutti, C.M., Modak, R.V., Macdonald, F., Li, F., Smyth, D.J., Dorward, D.A., Blair, N., Husovsky, C., Muir, A., Giampazolias,E., Dobie, R., Maizels, R.M., Kendall, T.J., Griggs, D.W., Kopf, M., Henderson, N.C., Zaiss, D.M. A macrophage-pericyte axis directs tissue restoration via amphiregulin-induced transforming growth factor beta activation. *Immunity*. 2019. 50, 645-654.

Mishra, A.K., Dubey, V. & Ghosh, A.R. Obesity: An overview of possible roles of gut hormones, lipid sensing and gut microbiota. *Metabolism.* 2016. 65, 48-65.

Mizoguchi, A. & Bhan, A.K. J. A case of regulatory B cells. *J. Immunol.* 2006. 176, 705-710.

Molinero, A., Fernandez- Perez, A., Mogas, A., Giralt, M., Comes, G., Fernandez-Gayol, O., Vallejo, M. & Hidalgo, J. Role of muscle IL-6 in gender-speciifc metabolism in mice. PLoS ONE 12(3): e0173675. https://doi.org/10.1371/journal.pone.0173675

Must, A. & Tybor, D.J. Physical activity and sedentary behavior: a review of longitudinal studies of weight and adiposity in youth. *Int J Obes.* 2005. 29, S84-S96.

Naderli, E.K., Ratcliffe, S.H. & Dale, M.C. Obesity and Alzheimer's disease: a link between body weight and cognitive function in old age. *Am J Alzheimers Dis Other Demen.* 2009. 24, 445-449.

Nishimura, S., Manabe, I., Takaki, S., Nagasaki, M., Otsu, M., Yamashita, H., Sugita, J., Yoshimura, K., Eto, K., Komuro, I., Kadowaki, T. & Nagai, R. Adipose natural regulatory B cells negatively control adipose tissue inflammation. *Cell Metabolism.* 2013.18, 759-766.

Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., Otsu, M., Hara, K., Ueki, K., Sugiura, S., Yoshimura, K., Kadowaki, T. & Nagai, R. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* 2009. 15, 914-920.

Nunoya, J., washburn, M.L., Kovalev, G.I. & Su, L. Regulatory T cells prevent liver fibrosis during HIV type 1 infection in a humanized mouse model. *J Infect Dis.* 2014. 209, 1039-1044.

Nutt, S.L., Hodgkin, P.D., Tarlinton, D.M. & Corcoran, L.M. The generation of antibody-secreting plasma cells. *Nat. Rev. Immunol*. 2015. 15,160-171.

Ortega, F.B., Lavie, C.J. & Blair, S.N. Obesity and cardiovascular disease. *Circulation Research.* 2016. 118, 1752-1770.

Osaka, M., Shunsuke, I., Honda, M., Inomata, Y., Kensuke, E. & Yoshida, M. Critical role of C5a activated neutrophils in high-fat diet-induced vascular inflammation. *Sci. Rep.* 2016. 6, 21391.

Osborn, O. & Olefsky, J.M. The cellular and signaling networks linking the immune system and metabolism in disease. *Nat. Med.* 2012. 18, 363-374.

Pal, M., Febbraio, M.A. & Whitham, M. From cytokine to myokine: the emerging role of interleukin-6 in metabolic regulation. *Immunol Cell Biol.* 2014. 92, 331-339.

Pallandre, J.R., Brillard, E., Crehange, G., Radlovic, A., Remy-Martin, JP., Saas, P., Rohrlich, PS., Pivot, X., Ling, X., Tiberghien, P., Borg, C. Role of STAT3 in CD4+CD25+Foxp3+ regulatory lymphocyte generation: implications in graft-versus-host disease and antitumor immunity. *J Immunol.* 2007. 179, 7593-7604.

Patsoukis, N., Bardhan, K., Chatterjee, P., Sari, D., Liu, B., Karoly, E.D., Freeman, G.J., Petkova, V., Seth, P., Li, L., Boussiotis, V.A. PD-I alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. *Nat. Commun.* 2015. 6: 6692.

Pederson, B.K. & Febbraio, M.A. Muscle as an endocrine organ: focus on muscle- derived interleukin-6. *Physiol. Rev.* 2008. 88, 1379-1406.

Pederson, B.K. & Fischer, C.P. Beneficial health effects of exercise- the role of IL-6 as a myokine. *Trends Pharmacol Sci.* 2007. 28, 152-156.

Pederson, B.K., Steensberg, A., Fischer, C., Keller, C., Keller, P., Plomgaard, P., Febbraio, M. & Saltin, B. Searching for the exercise factor : is IL-6 a candidate? *J Muscle Res Cell Motil.* 2003. 24, 113-119.

Pederson, B.K. Exercise-induced myokines and their role in chronic diseases. *Brain Behav Immun*. 2011. 25, 811-816.

Pederson, B.K. Muscles and their myokines*. J Exp Biol.* 2011. 214, 337-346.

Pederson, B.K. The anti-inflammatory effect of exercise: its role in diabetes and cardiovascular disease control. *Essays Biochem.* 2006. 42, 105-117.

Pederson, B.K. The diseasome of physical inactivity – and the role of myokines in muscle-fat cross talk. *J Physiol.* 2009. 587, 5559-5568.

Pederson, B.K., Steensberg, A. & Schjerling, P. Muscle-derived interleukin-6: possible biological effects. *J Physiol.* 2001. 536.2, 329-337.

Pederson, L., Idorn, M., Olofsson, G.H., Lauenborg, B., Nookaew, I., Hansen, R.H., Johannesen, H.H., Becker, J.C., Pederson, K.S., Dethlefsen, C., Nielsen, J., Gehl, J., Pedersen, B.K., Thor Straten, P. & Hojman, P. Voluntary running suppresses tumor growth through epinephrine and IL-6 dependent NK cell mobilization and redistribution. *Cell Metabolism.* 2016. 23, 554-562.

Perry, R.J., Camporez, J.G., Kursawe, R., Titchenell, P.M., Zhang, D., Perry, C.J., Jurczak, M.J., Abudukadier, A., Han, M.S., Zhang, X.M., Ruan, H.B., Yang, X., Caprio, S., Kaech, S.M., Sul, H.S., Birnbaum, M.J., Davis, R.J., Cline, G.W., Petersen, K.F. & Shulman, G.I. Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes. *Cell.* 2015. 160, 745-758.

Phillips, S.M., Green, H.J., Tarnopolsky, M.A., Heigenhauser, G.J. & Grant, S.M. Progressive effect of endurance training on metabolic adaptations in working skeletal muscles. *Am J Physiol.* 1996. 270, E265-72.

Pietiläinen, K.H., Kaprio, J., Borg, P., Plasqui, G., Yki-Järvinen, H., Kujala, U.M., Rose, R., J., Westerterp, K.R. & Rissanen, A. Physical inactivity and obesity : A vicious circle. 2008. *Obesity* 16 (2), 409-414.

Pratley, R.E. & Gilbert, M. Targeting incretins in type 2 diabetes: Role of GLP-1 receptor agonists and DPP-4 inhibitors. *Rev. Diabet. Stud.* 2008. 5, 73-94.

Quarta, C. et al. Molecular integration of incretin and glucocorticoid action reverses immunometabolic dysfunction and obesity. *Cell. Metab*. 2017. 26, 1-13.

Quintana, A., Erta, M., Ferrer, B., Comes, G., Giralt, M. & Hidalgo, J. Astrocyte-specific deficiency of interleukin-6 and its receptor reveal specific roles in survival, body weight and behavior. *Brain Behav Immun.* 2013. 27, 162-173.

Roche, S.L. & Silversides, C.K. Hypertension, obesity, and coronary artery disease in the survivors of congenital heart disease. *Can J Cardiol.* 2013. 29, 841-848.

Röckl, K.S.C., Hirshman, M.F., Brandauer, J., Fujii, N., Witters, L.A. & Goodyear, L.J. Skeletal muscle adaptation to exercise training. AMP-activated protein kinase mediates muscle fiber type shift. *Diabetes.* 2007. 56, 2062-2069.

Rodriguez, R.S., Pauli, M.L., Neuhaus, I.M., Yu, S.S., Arron, S.T., Harris, H.W., Yang, S.H., Anthony, B.A., Sverdrup, F.M., Krow-Lucal, E., ManKenzie, T.C., Johnson, D.S., Meyer, E.H., Löhr, A., Hsu, A., Koo, J., Liao, W., Gupta, R., Debbaneh, M.G., Butler, D., Huynh, M., Levin, E.C., Leon, A., Hoffman, W.Y., McGrath, M.H., Alvarado, M,D., Ludwig, C.H., Truong, H.A., Murano, M.M., Gratz, I.K., Abbas, A.K., & Rosenblum, M.D. Memory regulatory T cells reside in human skin. *J. Clin. Invest.* 2014. 124, 1027-1036.

Rose- John, S. IL-6 trans- signaling via the soluble IL-6 receptor: Importance for the pro-inflammatory activities of IL-6. *Int J Biol Sci.* 2012. 8, 1237-1247.

Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T cells and immune tolerance. *Cell.* 2008. 133, 775-787.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing Il-2 receptor alpha-chains (Cd25). Breakdown of a single mechanism of self- tolerance causes various autoimmune diseases. *J Immunol.* 1995. 155, 1151-1164.

Sakaguchi, S., Wing, K. & Miyara. M. Regulatory T cells – a brief history and perspective. *Eur. J. Immunol.* 2007. Suppl 1: S116-23.

Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta* 2011. 1813, 878-888.

Scheller, J., Garbers, C. & Rose-John, S. Interleukin-6: from basic biology to selective blockade of pro-inflammatory activites. *Semin Immunol.* 2014. 26, 2-12.

Schiaffino, S., Pereira, M.G., Ciciliot, S. & Rovere-Querini, P. Regulatory T cells and skeletal muscle regeneration. *FEBS J.* 2017. 284, 517-524.

Schiering, C., Krausgruber, T., Chomka, A., Fröhlich, A., Adelmann, K., Wohlfert, E.A., Pott, J., Griseri, T., Bollrath, J., Hegazy, A.N., Harrison, O.J., Owens, B.M.J., Löhning, M., Belkaid, Y., Fallon, P.G. & Powrie, F. The alarmin IL-33 promotes regulatory T cell function in the intestine. *Nature.* 2014. 513, 564-568.

Schmidt, A., Oberle, N. & Krammer, P.H. Molecular mechanisms of Treg-mediated T cell suppression. *Front Immunol.* 2012. 3: 51.

Schnyder, S & Handschin, C. Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise. *Bone.* 2015. 80, 115-125.

Schon, H-T & Weiskirchen, R. Exercise-induced release of pharmacologically active substances and their relevance for therapy of hepatic injury. *Front. Pharmacol.* 2016. 7, 283.

Sefik, E., Geva-Zatorsky, N., Sungwhan, O., Konnikova, L., Zemmour, D., McGuire, A.M., Burzyn, D., Ortiz-Lopez, A., Lobera, M., Yang, J., Ghosh, S., earl, A., Snapper, S.B., Jupp, R., Kasper, D., Mathis, D. & Benoist, C. Individual intestinal symbionts induce a distinct population of RORγ+ regulatory T cells. *Science*. 2015. 349, 993-997.

Shahaf, G., Zisman-Rozen, S., Benhamou, D., Melamed, D. & Ramit, M. B cell development in the bone marrow is regulated by homeostatic feedback exerted by mature B cells. *Front. Immunol*. 2016. 7:77.

Shai, I., Pischon, T., Hu, F.B., Ascherio, A., Rifai, N. & Rimm, E.B. Soluble intercellular adhesion molecules, soluble vascular adhesion molecules and risk of coronary artery disease. *Obesity*. 2006. 14, 2099 – 2106.

Shaikh, S.R., Haas, K.M., Beck, M.A. & Teague, H. The effects of diet-induced obesity on B cell function. *Clin Exp Immunol.* 2014. 179, 90-99.

Sharma, A. & Rudra, D. Emerging functions of Regulatory T cells in tissue homeostasis. *Front. Immunol.* 2018. 9: 883.

Shaw, D.M., Merien, F., Braakhuis, A. & Dulson, D. T cells and their cytokine production: The anti-inflammatory and immunosuppressive effects of strenuous exercise. *Cytokine*. 2018. 104,136-142.

Siede, J., Fröhlich, A., Datsi, A., Hegazy, A.N., Varga, D.V., Holecska, V., Saito, H., Nakae, S & Löhning, M. IL-33 receptor-expressing regulatory T cells are highly activated, Th2 biased and suppress CD4+ T cell proliferation through IL10 and TGFb release. *PLoS ONE.* 2016. 11(8): e0161507.

Snook, L.A., MacPherson, R.E., Monaco, C.M., Frendo-Cumbo, S., Castellani, L., Peppler, W.T., Anderson, Z.G., Buzelle, S.L., LeBlanc, P.J., Holloway, G.P. & Wright, D.C. Prior exercise training blunts short-term high-fat diet-induced weight gain. *Am J Physiol Regul Integr Comp Physiol.* 2016. 311, R315-324.

Snook, L.A., Trottier, S.K., Worndl, E.A., Bombardier, E., Tupling, A.R. & MacPherson, R.E.K. Prior endurance training enhances beta- adrenergic signaling in epididymal adipose from mice fed a high-fat diet. *Obesity*. 2017. 25, 1699-1706.

Spiegelman, B.M. & Flier, J.S. Obesity and the regulation of energy balance. *Cell.* 2001. 104, 531-543.

Steensberg, A. et al. IL-6 and TNF-alpha expression in, and release from, contracting human skeletal muscle. *Am J Physiol Endocrinol Metab.* 2002. 283, E1272-1278.

Stolarczyk, M., Amatngalim, G.D., Yu, Xiao, Veltman, M., Hiemstra, P.S. & Scholte, B.J. ADAM17 and EGFR regulate IL-6 receptor and amphiregulin mRNA expression and release in cigarette smoke-exposed primary bronchial epithelial cells from patients with chronic obstructive pulmonary disease (COPD). *Physiol. Rep.* 2016. 4(16), e 12878.

Stubbe, T., Ebner, F., Richter, D., Engel, O., Klehmet, J., Royl, G., Meisel, A., Nitsch, R., Meisel, C. & Brandt, C. Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *J Cere Blood Flow Metab.* 2013. 33, 37-47.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. & Kishimoto, T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell.* 1989. 58, 573-581.

Tedder, T.F. & Isaacs, C.M. Isolation of cDNAs encoding the CD19 antigen of human and mouse B lymphocytes. A new member of the immunoglobulin superfamily. *J. Immunol.* 1989. 143, 712-717.

Terra, R., Silva, S.A. G. da.,Pinto, V.S. & Dutra, P.M.L. Effect of exercise on the immune system: Response, adaptation and cell signaling. *Rev Bras Med Esporte* 2012.18, 208-214.

Thornton, A.M. & Shevach, E.M. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol.* 2000. 164, 183-190.

Tolessa,T., Gutniak, M., Holst, J.J., Efendic, S. & Hellström, P.M. Inhibitory effect of glucagon-like peptide-1 on small bowel motility. Fasting but not fed motility inhibited via nitric oxide independently of insulin and somatostatin. *J. Clin. Invest.* 1998. 102, 764-774.

Turton, M.D., O'Shea, D., Gunn, I., Beak, S.A., Edwards, C.M., Meeran, K., Choi, S.J., Taylor, G.M., Heath, M.M., Lambert, P.D., Wilding, J.P., Smith, D.M., Ghatei, M.A., Herbert, J., Bloom, S.R. A role for glucagon- like peptide-1 in the central regulation of feeding. *Nature.* 1996. 379, 69-72.

Tvedt, H.A., Ersvaer, E., Tveita, A.A. & Bruserund, O. Interleukin-6 in allogenic stem cell transplantation: its possible importance for immunoregulation and as a therapeutic target. *Front. Immunol.* 2007. 8, 667.

Van der Windt, G.J.W. & Pearce, E. L. Metabolic switching and fuel choice during T-cell differentiation and metabolic development. *Immunol. Rev.* 2012. 249, 27-42.

Vasanthakumar, A., Moro, K., Xin, A., Liao, Y., Gloury, R., Kawamoto, S., Fagarasan, S., Mielke, L.A., Afshar-Sterle,S., Masters, S.L., Nakae, S., Saito, H., Wentworth, J.M., Li, P., Liao, W., Leonard, W.J., Smyth, G.K., Shi, W., Nutt, S.L., Koyasu, S. & Kallies, A. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nat. Immunol.* 2015. 16, 276-285.

Villalta, S.A., Rosenthal, W., Martinez, L., Kaur, A., Sparwasser, T., Tidball, J.G., Margeta, M., Spencer, M.J. & Bluestone, J.A. Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med.* 2014. 6 (258) : 258ra142.

von Boehmer, H. & Daniel, C. Therapeutic opportunities for manipulating Treg cells in autoimmunity and cancer. *Nat Rev Drug Disc.* 2013. 12, 51-63.

Wagner, N.M., Brandhorst, G., Czepluch, F., Lankeit, M., Eberle, C., Herzberg, S., Faustin, V., Riggert, J., Oellerich, M., Hasenfuss, G., Konstantinides, S. & Schäfer, K. Circulating regulatory T cells are reduced in obesity and may identify subjects at increased metabolic and cardiovascular risk. *Obesity.* 2013. 21, 461-468.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L. & Ferrante, A.W.Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin.Invest.* 2003. 112, 796-1808.

Wilding, J.P. The importance of weight management in type 2 diabetes mellitus. *Int J Clin Pract.* 2014. 68, 682-691.

Winer, D.A., Winer, S., Chng, M.H., Shen, L. & Engleman, E.G. B lymphocytes in obesity related adipose tissue inflammation and insulin resistance. *Cell Mol Life Sci.* 2014. 71,1033-1043.

Winer, D.A., Winer, S., Shen, L., Wadia, P.P., Yantha, J., Paltser, G., Tsui, H., Wu, P-. Davidson, M.G., Alonso, M.N., Leong, H.X., Glassford, A., Caimol, m., Kenkel, J.A., Tedder, T.F., McLaughlin, T., Miklos, D.B., Dosch, H.M., Engleman, E.G. B lymphocytes promote insulin resistance through modulation of T lymphocytes and production of pathogenic IgG antibody. *Nat. Med.* 2011. 17, 610-617.

Winer, S., Chan, Y., Paltser, G., Truong, D., Tsui, H., Bahrami, J., Dorfman, R., Wang, Y., Zielenski, J., Mastronardi, F., Maezawa, Y., Drucker, D.J., Engleman, E., Winer, D., & Dosch, H.M. Normalization of obesity- associated insulin resistance through Immunotherapy: CD4+T cells control glucose homeostasis. *Nat. Med.* 2009. 15, 921-929.

Xia, Y., Cai, W.,Thomson, A.W. & Hu, X. Regulatory T cell therapy for ischemic stroke: how far from clinical translation? *Transl Stroke Res.* 2016. 7, 415-419.

Xu, E., Pereira, M.M.A., Karakasilioti, I., Theurich, S., Al-Maarri, M., Rappl, G., Waisman, A.,Wunderlich, F.T. & Brüning, J.C. Temporal and tissue- specific requirements for T-lymphocyte IL-6 signalling in obesity- associated inflammation and insulin resistance. *Nat. Commun*. 2017. 8, 14803.

Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A. & Chen, H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest.* 2003.112, 1821-1830.

Yan, Z., Okutsu, M., Akhtar, Y.N. & Lira, V.A. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis and angiogenesis in skeletal muscle. *J Appl Physiol.* 1985. 110, 264-274.

Yanaba, K., Bouaziz, J.D., Haas, K.M., Poe, J.C., Fujimoto, M. & Tedder, T.F. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity.* 2008. 28, 639-650.

Yeh, S.H., Chuang, H., Lin, L.W., Hsia, C.Y., Wang, P.W. & Yang, K.D. Tai chi chuan exercise decreases A1C levels along with increase of regulatory T cells and decrease of cytotoxic T-cell population in type 2 diabetic patients. *Diabetes Care.* 2007. 30, 716-718.

You, T., Arsenis, N.C., Disanzo, B.L.& LaMonte, M.J. Effects of exercise training on chronic inflammation in obesity, current evidence and potential mechanisms. *Sports Med.* 2013. 43, 243-256.

Yuan, D & Witte, P.L. Transcriptional regulation of mu and delta gene expression in bone marrow pre-B and B lymphocytes*. J. Immunol.* 1988.140, 2808-2814.

Yumuk, V., Tsigos, C., Fried, M., Schindler, K., Busetto, L., Micic, D. & Hermann, T. European Guidelines for obesity management in adults. *Obes Facts.* 2015. 8, 402-424.

Zaiss, D.M., Yang, L., Shah, P.R., Kobie, J.J., Urban, J.F. & Mosmann, T.R. Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. *Science.* 2006. 314 (5006): 1746.

Zaiss, D.M., van Loosdregt, J., Gorlani, A., Bekker, C.P., Gröne, A., Sibilia, M., van Bergen en Henegouwen, P.M., Roovers, R.C., Coffer, P.J. & Sijts, A.J. Amphiregulin enhances regulatory T cell suppressive function via the epidermal growth factor receptor. *Immunity.* 2013. 38, 275-284.

Zaiss, D.M.W., Gause, W.C., Osborne, L.C. & Artis, D. Emerging functions of Amphiregulin in orchestrating immunity, inflammation and tissue repair, *Immunity.* 2015*.* 42, 216-226.

Zaiss, D.M., Minutti, C.M. & Knipper, J.A. Immune and non-immune-mediated roles of regulatory-T cells during wound healing. *Immunology*. 2019. 10.1111/imm.13057.

Zeng, Q., Sun, X., Xiao, L., Xie, Z., Bettini, M. & Deng, T. A unique population: Adipose- resident regulatory T cells. *Front. Immunol.* 2018. 9, 2075.

Zhai, X., Qian, G., Wang, Y., Chen, X., Lu, J., Zhang, Y., Huang, Q., Wang, Q. Elevated B cell activation is associated with type 2 diabetes development in obese subjects. *Cell Physiol Biochem.* 2016. 38, 1257- 1266.

Zhang, J., Tokui, Y., Yamagata, K., Kozawa, J., Sayama, K., Iwahashi, H., Okita, K., Miuchi, M., Konya, H., Hamaguchi, T., Namba, M., Shimomura, I. & Miyagawa, J.I. Continuous stimulation of human glucagon-like peptide-1(7-36) amide in a mouse model (NOD) delays onset of autoimmune type 1 diabetes. *Diabetologia.* 2007. 50,1900-1909.

Zhao, Y., Lin, L., Li, J., Xiao, Z., Chen, B., Wan, L., Li, M., Wu, X., Cho, C.H. & Shen, J. $CD4^{+}$T cells in obesity and obesity- associated diseases. *Cellular Immunology*. 2018. 332,1-6.

Zhou, X., Tang, J., Cao, H. & Fan, H. Tissue resident regulatory T cells: novel therapeutic targets for human disease. *Cellular & Molecular Immunology.* 2015. 12, 543-552.

Acknowledgements

I take this opportunity to thank everyone who directly or indirectly made this work possible. Thanks to God Almighty for all the wondrous works in my life. Specifically, I would like to thank Prof. Dr. Matthias Tschöp for giving me an opportunity to do my PhD in his esteemed lab. I sincerely thank him for all the guidance and support provided whenever I needed.

A very special thanks to Prof. Dr. Carolin Daniel for the supervision and guidance throughout my project, for having an open door always for discussions and accepting me as part of her group. I would also like to thank Dr. Marc Walter who was always a support for me, for introducing and teaching me from the basics of flow cytometry with all patience. My heartfelt thanks to Prof. Dr. Susanna Hoffmann who was always there with helping hands and support throughout my PhD. I would also like to thank my second advisor Prof. Dr. Hannelore Daniel for all the fruitful discussions during thesis committees.

I want to thank specially Maike Becker for all the help and support in my project and discussions, Isabelle Serr, Victoria Flynn, Verena Ott, Martin Scherm, Kristina Then, Daria Opaleva, Hannah Hipp and all other members from IDF1. My special thanks to Sebastian Cucuruz, Cynthia Streise and Stefan Sachs from IDR-H with all help in animal studies. I also want to thank Dr. Christoffer Clemmenson and Dr. Brian Finan for all the scientific discussions and support and all members of the IDO Pharmacology team for their help in one way or the other.

Finally, I would like to thank my family, my husband Dr. Robby Z. Tom for his scientific inputs, never ending support and love and taking care of everything despite my late working hours, my daughter Julia for understanding and adjusting with all circumstances and my little one, Gabriella. Without your support, this would not have been possible. Most importantly, I would like to thank our parents and siblings who was always been an encouragement and pillar of support in all ups and downs of my life.

List of publications

Joseph, S.S., Becker, M., Tom, R.Z., Opaleva, D., Serr, I., Scherm, M.G, Brüning, J.C., Tschöp, M.H., Hofmann, S.M. & Daniel, C. T cell specific IL-6 receptor alpha signalling is key for regulatory T cells to control skeletal muscle function upon exercise. (*Cell Metab.* – submitted)

Quarta, C., Clemmenson, C., Zhu, Z., Yang, B., **Joseph, S.S.,** Lutter, D., Chun-Xia Yi, Graf, E., Garcia-Cacerea, C., Legutko, B., Fischer, K., Brommage, R., Zizzari, P., Franklin, B.S., Krueger, M., Koch, M., Vettorazzi, S., Li, P., Hofmann, S.M., Bakhti, M., Bastidas-Ponce, A., Lickert, H., Strom, T.M., Galius-Durner, V., Bechmann, I., Perez-Tilve, D., Tuckermann, J., Angelis, M.H., Sandoval, D., Cota, D., Latz, E., Seeley, R.J., Muller, T.D., DiMarchi, R.D., Finan, B., Tschöp, M.H. Molecular integration of incretin and glucocorticoid action reverses immunometabolic dysfunction and obesity. *Cell. Metab*. 2017. 26, 1-13.