MODULATION OF PEPITEM PATHWAY IN TYPE 2 DIABETES

By

Laleh Pezhman

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Institute of Inflammation and Ageing

College of Medical and Dental Sciences

University of Birmingham

July 2022

UNIVERSITY OF
BIRMINGHAM

University of Birmingham Research Archive

e-theses repository

This unpublished thesis/dissertation is copyright of the author and/or third parties. The intellectual property rights of the author or third parties in respect of this work are as defined by The Copyright Designs and Patents Act 1988 or as modified by any successor legislation.

Any use made of information contained in this thesis/dissertation must be in accordance with that legislation and must be properly acknowledged. Further distribution or reproduction in any format is prohibited without the permission of the copyright holder.

Abstract

Adiponectin is an anti-inflammatory and insulin-sensitising hormone produced by adipose tissue that regulates lymphocyte migration across the endothelium through the PEPITEM pathway. Impaired bioactivity of the PEPITEM pathway, due to decreased expression of adiponectin receptor 1 (AdipoR1) and AdipoR2 on B cells, has been reported in elderly and T1DM. However, the modulation of this pathway is unknown in T2DM and obesity.

Here, we first characterised the role of CDH13 (Cadherin-13), in regulating anti-inflammatory effects of adiponectin focusing on PEPITEM pathway. We showed that adiponectin strongly inhibited lymphocyte migration either by action on the lymphocytes (previously published) or directly on endothelium. We found that CDH13 and AdipoR1/R2 were expressed by endothelial cells and individual knock down of these receptors was sufficient to dampen the endothelial response and abrogated the inhibitory effects of adiponectin on lymphocyte transmigration.

To investigate the effects of hyperglycemia on leukocyte-endothelium interaction in vitro, either blood endothelial cells (BEC) or lymphocytes were exposed to high concentration of glucose (HG). HG treatment of BEC reduced the ability of endothelial cells to support lymphocyte transmigration, which was restored by insulin and metformin supplementation. In contrast, HG treatment of lymphocytes increased their adhesion and transmigration across the endothelium, suggesting activation of lymphocytes by HG treatment. Since, the expression of AdipoR1/R2 on B cells remained unchanged following HG treatment, modulation of these receptors is not a suggested mechanism for HG effects.

Given that circulating levels of adiponectin are significantly decreased in patients with obesity and T2DM, there is a distinct possibility that these individuals are unable to generate sufficient PEPITEM and therefore would benefit from replacement therapy. Using obesogenic-dietary preclinical models, we investigated the efficacy of PEPITEM to limit pancreatic islet damage and its ability to modulate systemic leukocyte trafficking. PEPITEM reduced the expansion of pancreatic islets size and limited T cells recruitment in visceral adipose tissue both prophylactically and therapeutically. In particular, therapeutic administration of PEPITEM increased the number of lymphocytes in secondary lymphatic organs, suggesting PEPITEM has the capacity to control lymphocyte egress from secondary lymphoid organs.

Our observations highlight a crucial role of CDH13 in functionality of PEPITEM pathway and indicate that HG differentially affects the endothelium and lymphocyte behaviour to support the lymphocyte recruitment. Our in vivo results, underline the importance of PEPITEM in the control of

lymphocyte transmigration during obesity-induced inflammation and offers a potential therapeutic role for PEPITEM to overcome obesity related changes which occur in early stages of disease.

To Marie Lutter and Janet Bulter

Acknowledgments

First and foremost, I am extremely grateful to my supervisors, Dr. Chimen, Dr. McGettrick and Dr. Iqbal for your invaluable advice, continuous support, and patience during my PhD study. I would also like to thank my second supervisors Dr. Hodson and Dr. Tahrani for your technical support on my study.

I would also like to thank Dr Ed Rainger for your help with the animal study.

A special thanks to Hodson's lab members specially Daniela Nasteska for helping with this project.

Thanks to every member of the LTG in particular Sophie Hopkin for your support through some of the toughest times in my project. I will not forget your kindness.

Special thanks to my desk neighbours Jenefa Begum and Adel Abo Mansour for all your supports in the lab and office.

Thanks to my mum, Heli and my Ruby for all your encouragements from distance.

Big thanks to my love Himan, I will never be able to repay the support you have given me. Thank you.

Publications

Papers arising from my thesis

Pezhman, L., A. Tahrani and M. Chimen (2021). "Dysregulation of leukocyte trafficking in type 2 diabetes: mechanisms and potential therapeutic avenues." Frontiers in cell and developmental biology 9: 624184.

Contribution to other publication during my PhD

Mansour, A. A., F. Raucci, M. Sevim, A. Saviano, J. Begum, Z. Zhi, **L. Pezhman**, S. Tull, F. Maione and A. J. Iqbal (2022). "Galectin-9 supports primary T cell transendothelial migration in a glycan and integrin dependent manner." Biomedicine & Pharmacotherapy 151: 113171.

Oral Presentations:

UKCAS Conference, The role of T-cadherin in anti-inflammatory effects of adiponectin, Birmingham UK (2019**)**

Poster Abstract

BSI Conference, The role of T-cadherin in anti-inflammatory effects of adiponectin, Liverpool UK (2019)

ICVS awayday, The role of T-cadherin in anti-inflammatory effects of adiponectin, Birmingham UK (2019**)**

UKCAS Conference, The role of T-cadherin in anti-inflammatory effects of adiponectin,

Birmingham UK (2019)

BHF accelerator symposium, The role of T-cadherin in anti-inflammatory effects of adiponectin, Birmingham UK (2019)

COVID statement

My research was severely affected by COVID due to limited access to the laboratories and blood samples, therefore I could not complete the planned experiments. My project was 100% lab-based and the amount of work contributing to the progress of my project during lockdown was very limited.

Due to lack of access to patient samples I was not able to complete the vital part of my *in vitro* research which depended on samples from patient with T2DM. Furthermore, my animal study was affected since the access to animal house, the number of animals allowed per experiment as well as duration of experiment were limited.

1. INTRODUCTION 1
1.1 An overview of the research 2
1.2 An overview of the immune system 2
1.2.1 Innate and adaptive immunity 3
1.3 Leukocytes trafficking in acute inflammation 6
1.4 Chronic inflammation 8
1.5 Diabetes 9
1.5.1 Glucose transport 10
1.5.2 Insulin synthesis 11
1.5.3 Insulin release 11
1.5.4 Insulin signaling 12
1.5.5 Insulin Resistance 12
1.5.5.1 Inflammatory mediators 13
1.5.5.2 Oxidative stress 14
1.5.5.3 Deficiencies of phosphorylation 15
1.6 Obesity related changes of immune system in T2DM 19
1.6.1 Expression of Adhesion Molecules in T2DM 19
1.6.1.1 Soluble CAMs and selectins 20
1.6.1.2 Surface expression of CAMs on the endothelium 21
1.6.2 Modulation of innate and adaptive immune cells in obesity and T2DM 22
1.6.2.1 Macrophage/Monocytes 23
1.6.2.2 T cells 24
1.6.2.3 B cells 25
1.6.3 Immunosenescence in T2DM 28
1.6.4 Therapeutic targeting of leukocyte trafficking in T2DM 28
1.6.4.1 Chemokines and cytokines inhibition 29
1.6.4.2 Anti-integrins 30
1.6.4.3 Fatty acids 32
1.6.4.4 Insulin 33
1.6.4.5 Metformin 33
1.6.4.6 Adiponectin 35
1.6.4.7 PEPITEM 37
1.7 Aims and Objectives 41

2 MATERIALS AND METHODS 43
2.1 Blood vascular Endothelial Cells (BEC) 44
2.1.1 Culture and passaging of BEC 44
2.1.2 Subculture 44
2.1.3 Cryopreservation 45
2.1.4 Seeding and treatment of BEC 45
2.2 Collection and Preparation of Whole Blood 46
2.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC) 46
2.2.2 Monocyte isolation 47
2.2.3 Peripheral Blood Lymphocytes (PBL) Isolation and Treatments 48
2.3 Static adhesion and migration assay 49
2.4 RNA Extraction 51
2.5 mRNA to cDNA conversion 51
2.6 Real-time Polymerase Chain Reaction 52
2.7 siRNA transfection 55
2.8 Protein Expression 55
2.8.1 Flow cytometry 55
2.8.1.1 Measuring AdipoRs expression on PBMC subsets. 55
2.8.1.2 Measuring total expression of CDH13 in BEC 60
2.8.1.3 Measuring surface expression of CDH13 on BEC 60
2.8.2 Immunofluorescence staining for adiponectin and CDH13 61
2.8.3 Confocal microscopy 62
2.9 Animal experiments 64
2.9.1 Mice 64
2.9.2 Mini-pump implantation and induction of glucose intolerance 64
2.9.2.1 Mice on High Fat Diet (HFD) 64
2.9.3 Identification of leukocyte subsets in murine tissues 65
2.9.4 Processing of blood samples 65
2.9.5 Processing of spleen and lymph nodes (LNs) 65
2.9.6 Processing of adipose tissue 66
2.9.7 Processing of Peritoneal Lavage 66
2.9.8 Staining leukocyte subsets for flow cytometry 66
2.9.9 Processing mice pancreas tissue 75

2.9.9.1 Paraffin embedding and sectioning of the pancreas 75
2.9.9.2 Deparaffinisation and staining of pancreas for insulin and glucagon 76
2.9.9.3 Slide scanner 77
2.10 Statistical analysis 77
3 THE ROLE OF CDH13 (CADHERIN-13) IN ANTI-INFLAMMATORY EFFECTS OF ADIPONECTIN 78
3.1 Introduction 79
3.2 Results 80
3.2.1 Evaluation of AdipoR1/R2 on expression on PBMC subsets 80
3.2.2 Effect of adiponectin on lymphocyte migration through inflamed endothelium 80
3.2.3 Expression of adiponectin receptors on endothelial cells 81
3.2.4 Endothelial responses to adiponectin 82
3.2.5 The effect of adiponectin receptors knock-down on PEPITEM pathway 89
3.2.6 Evaluating Expression of CDH13 by BEC 94
3.3 Discussion 97
3.3.1 Expression of AdipoR1/R2 in immunity 97
3.3.2 Measurement of lymphocyte transmigration *in vitro* 98
3.3.3 Evidence on immunomodulatory effects of adiponectin 99
3.3.4 Anti-inflammatory effects of adiponectin acting on BEC 101
3.3.5 Adiponectin-CDH13 interaction 103
3.3.6 Limitation 105
3.3.7 Conclusion 106
4 AN EXAMINATION OF IMMUNOMODULATORY EFFECT OF HIGH GLUCOSE, METFORMIN AND, INSULIN 107
4.1 Introduction 108
4.2 Results 108
4.2.1 Effect of HG on PBL adhesion and migration 108
4.2.2 Effect of Metformin on PBL adhesion and migration 109
1.1.1 The effect of metformin or high glucose treatment on monocyte or neutrophil recruitment to inflamed endothelium 112
4.2.3 Combined effect of high glucose and metformin treatment on lymphocyte recruitment to inflamed endothelium 112
4.2.4 Effect of Insulin on PBL adhesion and migration 116

4.2.5 Combined effect of high glucose and insulin treatment on lymphocyte recruitment to inflamed endothelium 116
4.2.6 Effects of high glucose or metformin on the expression of AdipoR1/2 on PBMC subsets 119
4.2.7 Combined effect of high glucose and metformin on adiponectin receptors expression by PBMCs subsets 119
4.2.8 Combined effect of high glucose and insulin on adiponectin receptors expression by PBMCs subsets 119
4.2.9 Effect of high glucose, metformin, or insulin treatment on expression of endothelial adhesion molecule 124
4.3 Discussion 128
4.3.1 Evidence of High Glucose actions on endothelial cells and mechanisms involved 128
4.3.2 Evidence of High Glucose actions on leukocytes and mechanisms involved 134
4.3.3 Limitations 137
4.3.4 Conclusion 137
5 Immunomodulatory effects of PEPITEM in HFD-induced glucose intolerance mice 138
5.1 Introduction 139
5.2 Results 140
5.2.1 The effect of PEPITEM on PBLs migration across the endothelium in healthy controls .. 140
5.2.2 Immunomodulatory effects of PEPITEM in 6 week-HFD mice 140
5.2.2.1 Effect of PEPITEM administration on body weight and glucose tolerance in 6 week- HFD mice 142
5.2.2.2 The effect of PEPITEM on pancreatic beta cell mass in 6-week HFD- mice 142
5.2.2.3 Quantification of leukocytes in 6-week HFD-mice treated with PEPITEM vs PBS 143
5.2.3 Immunomodulatory effects of PEPITEM in mice on HFD for 12 weeks 149
5.2.3.1 Effect of PEPITEM administration on body weight and glucose tolerance in 12-week HFD- mice 149
5.2.3.2 The effect of PEPITEM on pancreatic beta cell mass in 12-week HFD- mice 151
5.2.3.3 Quantification of leukocytes in 12-week HFD-mice treated with PEPITEM vs PBS 151
5.3 Discussion 161
5.3.1 Evidence on obesity related metabolic changes 161
5.3.2 Evidence on PEPITEMs actions in inflammation 164
5.3.3 Obesity related changes in leukocyte trafficking in adipose tissue 166
5.3.4 Obesity related changes in leukocyte trafficking in secondary lymphatic organs 170

5.3.5 Limitations 171
5.3.6 Conclusion 172
6 GENERAL DISCUSSION 173
6.1 Summary of findings 174
1.2 Future directions 178
7 References 183
8 Appendix 200
8.1 Published review 201

Table of Figures

Figure 1.1 Mechanism of insulin secretion from beta cells of pancreas 16

Figure 1.2 Insulin signal transduction pathway 17

Figure 1.3 Obesity-related dysregulation of leukocyte trafficking in T2DM 26

Figure 1.4 Signal transduction by adiponectin receptors 38

Figure 1.5 PEPITEM pathway 39

Figure 2.1 Representative image of the static adhesion and migration assay 48

Figure 2.2 Gating strategy to identify PBMC subsets 56

Figure 2.3 Frequency of PBMC subsets in healthy donors 57

Figure 2.4 Representative plots of gating strategy for identifying BEC 61

Figure 2.5 Gating strategy used to identify the T-cell subsets in spleen 67

Figure 2.6 Gating strategy for B-cell and B-cell isotype panel in spleen 68

Figure 2.7 Gating strategy for macrophage panel in spleen 69

Figure 3.1 Representative histograms of AdipoRs expression on PBMCs 81

Figure 3.2 Expression of adiponectin receptors on PBMC subsets 82

Figure 3.3 Effect of adiponectin on lymphocyte migration through inflamed adult vs juvenile endothelium in static conditions 83

Figure 3.4 Gene expression of adiponectin receptors on BEC 84

Figure 3.5 Protein expression of adiponectin receptors on BEC 85

Figure 3.6 The effect of adiponectin treatment of BEC on lymphocyte recruitment to inflamed endothelium 86

Figure 3.7 PCR confirmation of adiponectin receptors knockdown on BEC 88

Figure 3.8 Flow cytometry confirmation of adiponectin receptors knock down on BEC 89

Figure 3.9 Effect of CDH13 knockdown on the ability of adiponectin to inhibit lymphocyte recruitment to inflamed endothelium 90

Figure 3.10 Effect of AdipoR1 or R2 knockdown on the ability of adiponectin to inhibit lymphocyte recruitment to inflamed endothelium. 91

Figure 3.11 Effects of adiponectin treatment on CDH13 expression in stimulated endothelial cells. 93

Figure 3.12 Confocal microscopy imaging of the localization of CDH13 94

and adiponectin in BEC
Figure 4.1 The effect of high glucose treatment on lymphocyte recruitment to inflamed endothelium 108
Figure 4.2 The effect of metformin treatment on lymphocyte recruitment to inflamed endothelium. 109
Figure 4.3 The effect of metformin or high glucose treatment on monocyte recruitment to inflamed endothelium. 111
Figure 4.4 The effect of metformin or high glucose treatment on neutrophil recruitment to inflamed endothelium 112
Figure 4.5 Combined effect of high glucose and metformin treatment on lymphocyte recruitment to inflamed endothelium 113
Figure 4.6 The effect of insulin treatment on lymphocyte recruitment to inflamed endothelium 115
Figure 4.7 Combined effect of high glucose and insulin treatment on lymphocyte recruitment to inflamed endothelium. 116
Figure 4.8 Combined effect of high glucose and insulin on adiponectin receptors expression by PBMCs subsets 121
Figure 4.9 Effect of high glucose, metformin, or insulin treatment on surface expression of endothelial adhesion molecule 124
Figure 4.10 Effect of high glucose, metformin, or insulin treatment on gene expression of endothelial adhesion molecule. 125
Figure 5.1 Effect of PEPITEM on lymphocyte migration through inflamed endothelium in static conditions. 139
Figure 5.2 Effect of PEPITEM administration on body weight and glucose tolerance in HFD- mice treated with PEPITEM vs PBS. 142
Figure 5.3 Representative image of pancreatic islets in mice pancreas. 143
Figure 5.4 Quantification of pancreas islets number and area in HFD-mice treated with PEPITEM vs PBS. 144
Figure 5.5 Quantification of leukocyte subsets in HFD-mice treated with 146

PEPITEM vs PBS.

Figure 5.6 Effect of PEPITEM administration on body weight and glucose tolerance in HFD-mice treated with PEPITEM vs PBS 148

Figure 5.7 Representative image of pancreatic islets in mice pancreas 151

Figure 5.8 Quantification of pancreas islets number and area in HFD-mice treated with PEPITEM vs PBS 152

Figure 5.9 Quantification of leukocyte subsets in blood of HFD-mice treated with PEPITEM vs PBS 154

Figure 5.10 Quantification of leukocyte subsets in visceral adipose tissue of HFD-mice treated with PEPITEM vs PBS 155

Figure 5.11 Quantification of leukocyte subsets in spleen of HFD-mice treated with PEPITEM vs PBS 156

Figure 5.12 Quantification of leukocyte subsets in LN of HFD-mice treated with PEPITEM vs PBS. 157

Figure 5.13 Quantification of macrophages in peritoneal lavage of HFD-mice treated with PEPITEM 158

Table of tables

Table 1.1 Innate vs adaptive immune cells and the markers they express 5

Table 2.2 TaqMan gene expression assays used for real-time qPCR 51

Table 2.2 Screening of housekeeping genes 52

Table 2. 3 Sequences of siRNA pool used for BEC 55

Table 2.4 Human antibodies used for adiponectin receptors on PBMC subsets 55

Table 2.5 Enzyme cocktail concentration and final volume per 0.8 g of adipose tissue 66

Table 2.6 Anti-mouse antibodies used in T cell, T cell isotype, B cell, B cell isotype, and macrophage panel for flow cytometry. 70

Table 4-1 Effect of high glucose and metformin treatment on Adiponectin receptors expression by PBMCs subsets. 119

Table 4.2 Combined effect of high glucose and metformin on adiponectin receptors expression by PBMCs subsets. 120

Table 5.1 Quantification of leukocyte subsets in 6-week HFD-PEPITEM vs HFD-PBS mice 145

Table 5.2 Quantification of leukocyte subsets in 12- week HFD-mice treated with PBS vs PEPITEM 153

Abbreviations

ACE: Angiotensin-converting enzyme

AdipoR1: Adiponectin receptor 1

AdipoR2: Adiponectin receptor 2

AGEs: Advanced glycosylation end products

AMPK: Adenosin mono phsphate kinase

APCs: Antigen Presenting Cells

ATP: adenosine triphosphate

BEC: Blood vascular Endothelial Cells

BSA: Bovine serum albumin

CAMs: Cell adhesion molecules

CD3: Cluster of differentiation 3

CDH13: Cadherin-13

CM: Central memory

DCs: Dendritic cells

DHA: Docosahexaenoic acid

ECGS: Endothelial Cell Growth Supplement

EDTA: Ethylenediaminetetraacetic acid

EM: Effector memory

EPA: Eicosatetraenoic acid

ESL-1: E-selectin ligand-1

FCS: Fetal Calf Serum

G6P: Glucose-6-phosphate

GLUT: Glucose transporters

GPCRs: G-protein-coupled receptors

HDBEC: Human Dermal endothelial cells

HFD: High Fat Diet

HG: High glucose

HUVEC: Human umbilical endothelial cells

ICAM-1: intercellular adhesion molecule 1

IFN-γ: interferon gamma

IGF: insulin-like growth factors

IL-1β: interleukin-beta

IPGTT: intraperitoneal glucose tolerance tests

IR: insulin receptor

IRS: insulin receptor substrate

JAMs: junctional adhesion molecules

KLRL1: killer cell lectin-like receptor subfamily G member 1

LFA-1: lymphocyte function-associated antigen-1

LKB: 1liver kinase B1

LN: lymph node

MCP-1: monocyte chemoattractant protein-1

NF-κB: Nuclear factor kappa B

NKs: natural killer cells

PBL: Peripheral Blood Lymphocytes

PBMC: Peripheral Blood Mononuclear Cells

PD-1: programmed cell death 1

PDK-1: phosphoinositide-dependent kinase-1

PEPITEM: PEPtide Inhibitor of Trans-endothelial Migration

PGD2: prostaglandin- D2

PI3K; phosphoinositide 3-kinase

PKB: protein kinase B

PPARγ: peroxisome proliferator-activated receptor

PSGL-1: P-selectin glycoprotein ligand 1

PUFA: polyunsaturated fatty acid

RA: rheumatoid arthirits

ROS: reactive oxygen species

S1P: Sphingosine-1-Phosphate

sCAMs: Soluble form of CAMs

SLO: secondary lymphoid organs

STZ: streptozotocin

SVF: stroma vascular fraction

T reg: Regulatory T cells

T1DM: Type 1 diabetes

T2DM: Type 2 Diabetes Mellitus

Th17: T helper 17

TNF-α: tumor necrose factor alpha

Tyr-K: tyrosine kinase

VCAM-1: vascular adhesion molecule 1

VLA-4: very late antigen 4

Wt: wild type

1. INTRODUCTION

Part of this chapter (Section's 1.1, 1.3 and 1.6) is based on Pezhman et al, 2021 see Appendix

1.1 An overview of the research

This thesis explores how hyperglycemia and obesity modulates the PEPITEM pathway and trafficking of leukocyte in different vascular bed. The following introductory chapter will encompass the biology of the immune system and the trafficking of innate and adaptive immune cells in health and obesity induced Type 2 Diabetes Mellitus (T2DM). We particularly highlight the obesity-related accumulation of leukocytes in the adipose tissue leading to insulin resistance and beta cell dysfunction and resulting in hyperglycemia and consequent changes of adhesion and migratory behavior of leukocytes in different vascular beds. Subsequently we discuss how potential therapeutic targeting of leukocyte trafficking could be an efficient way to control inflammation, as well as diabetes and its vascular complications.

1.2 An overview of the immune system

The immune system is a complex network of cells and proteins that is distributed throughout the entire human body and defends the body against pathogens (Sorice et al., 2014). This system is compromised in various types of diseases related to the immune system and chronic inflammatory disease (Sorice et al., 2014). Therefore, understanding of its biological structures provides various predictions for disease causes and treatments.

The immune system is composed of leukocytes, tissues, and organs, lymphatic circulation as well as the blood vascular system (Rehfeld et al., 2017). The immune system can distinguish pathogens from our own cells and enables us to respond to threats and eliminate or neutralise pathogens (bacteria, viruses, parasites), molecules (bacterial proteins and toxins) and own abnormal cells (Kindt et al., 2007). The lymphatic vessels carry fluid called lymph, which is formed when the plasma escapes into intercellular spaces in the tissues under blood pressure, and

drains into lymphatic vessels and finally returns to the blood (Olszewski, 2003). The lymphoid organs are connected via extensive network of blood vessels as well as lymphatic vessels (Olszewski, 2003). The lymphatic organs are composed of primary lymphoid organs (e.g., thymus, bone marrow) and secondary lymphoid organs (SLO), including spleen, lymph nodes (LNs), tonsils (Rehfeld et al., 2017). The leukocytes are created and matured in primary lymphoid organs and are activated in SLO (Rehfeld et al., 2017). SLO are essential for the initiation of adaptive immunity as explained in below section (Grasso et al., 2021)

1.2.1 Innate and adaptive immunity

The immune system can be divided into innate and adaptive immune system (Iwasaki and Medzhitov, 2010). The main differences between innate and adaptive immune cells, the marker they express, and their duties are summarised in Table 1.1. The innate immune system includes macrophages, monocytes, dendritic cells (DCs), neutrophils, eosinophils, and basophils and provides an immediate defense in response to an insult (Stögerer and Stäger, 2020). Macrophages and neutrophils response to invading pathogens by phagocytosis of microbes and/or release of both pro-inflammatory and antimicrobial mediators and are crucial to handle the common bacterial infections (Stögerer and Stäger, 2020). However, due to less specificity they cannot always fully recognise and eliminate the infectious organisms (Stögerer and Stäger, 2020). Dendritic cells function as a link between innate and adaptive immunity by presenting antigens to lymphocytes in SLO and mediating T cells polarization into effector cells.

The adaptive immune system responds to stimuli slower, however, more effectively and specifically compared to the innate immune system (Cooper and Alder, 2006). B cells and T cells

play a central role in adaptive immunity (Cooper and Alder, 2006). Upon maturation in thymus, T cells express the T cell receptor cluster of differentiation 3 (CD3), and either the co-receptor cluster of differentiation 4 (CD4) or cluster of differentiation 8 (CD8) (Owen et al., 2007). They enter into circulation as naïve T cells ($CD62L^+$, $CD44^-$), which are not strong enough to respond to antigen, but are able to enter to SLO from the bloodstream (Saravia et al., 2019), where their activation happens through recognising antigens presented by Antigen Presenting Cells (APCs) which are transported to SLO through afferent lymph (Jensen, 2007). Following activation, T cells differentiate into central memory (CM, $CD62L^+$, $CD44^+$) and effector cells (EM, $CD62L^-$, $CD44^+$) (Ley and Kansas,2004). Regulatory T cells (T reg) are another subset of T cells that express the surface marker CD4 CD25 (Sakaguchi et al., 1995), but a more specifically transcription factor forkhead box P3 (FoxP3), (Hori et al.,2003), which have immunosuppressive functions and are important in maintaining immune homeostasis of an organism in inflammation (Li et al., 2020)

B cells play a fundamental role in adaptive immunity, by producing antibody and serving as APCs (Crotty, 2015). Activation of B cells happens at SLO when the B cell binds to an antigen (Yuseff et al., 2013) which results in B cells proliferation, differentiation and production of antibodies (Crotty, 2015) CD19, is the earliest and most specific markers of B cells (Crotty, 2015).

Overall, when the immune system recognises antigens attacks them by inducing an immune response which develops inflammation. Understanding the mechanisms of inflammation would allow us to identify the key steps and manipulate them to control diseases.

Table 1 .1 Innate vs adaptive immune cells and the markers they express.

Innate (non-specific)		**Adaptive (specific)**		
Line of Defense	First	Second		
Timeline	Immediate response (0 -96 hours)	Long term (>96 hours)		
Cell subsets	Natural killer cells (**H**: $CD56^+$) Macrophages (**H**:$CD14^+$), (**M**:$F4/80^+$) Neutrophils (**M**:$LY6G^+$) dendritic cells (**M**:$CD11C^+$) Mast cells Basophils Eosinophils (**M**:SIGLEC F^+)	T cells: ($CD3^+$)	($CD4^+$)	Naive: ($CD62L^+$, $CD44^-$) CM: ($CD62L^+$, $CD44^-$) EM: ($CD62L^+$, $CD44^-$) T reg: $CD4^+$ $CD25^+$
			($CD8^+$)	Naive: ($CD62L^+$, $CD44^-$) CM: ($CD62L^+$, $CD44^-$) EM: ($CD62L^+$, $CD44^-$)
		B cells: ($CD19^+$) Age associated B cells: ($CD19^+CD93^-$/$CD43^-CD23^-CD21^-$)		
Antigen Dependency	Independent	Dependant		
Soluble factors	Cytokines, complement, Acute phase proteins	Cytokines, antibodies		

H:Human, **M**:Mouse

1.3 Leukocytes trafficking in acute inflammation

Inflammation is a protective response against harmful stimuli such as injuries, infections, and toxins, which requires the trafficking of leukocytes from the blood stream to the site of inflammation within damaged tissues (Wright and Cooper, 2014). This process is similar for all leukocyte subsets described above, and critical for elimination of harmful stimuli and for tissue repair. It is tightly regulated by a variety of mediators such as cell adhesion molecules (CAMs), cytokines and chemokines (Wright and Cooper, 2014).

Recruitment of leukocytes is triggered by the local release of inflammatory mediators such as histamine, tumor necrose factor alpha (TNF-α) and interferon gamma (IFN-γ) in inflamed tissue (Liu et al., 2004; Zhang et al., 2011; Muller, 2013). These mediators induce the expression of specific adhesion molecules, and chemokines, which are expressed or presented at the surface of endothelial cells and bind to integrins or chemokine receptors located at the surface of leukocytes (Zhang et al., 2011) Adhesion molecules interaction with their ligands on leukocytes results in their tethering and rolling from rapidly flowing blood (Kansas, 1996; Harjunpää et al., 2019). Selectins are transmembrane calcium-dependent lectins that consist of three functionally different adhesion molecules expressed by endothelial cells (E-selectin), platelets and endothelial cells (P-selectin) and leukocytes (L-selectin), (McEver, 2015). L-selectin and P-selectin mediate fast rolling of leukocytes through sequential interactions with their ligands. However, slow rolling events are mediated by E-selectin on endothelial cells binding to E-selectin ligand-1 (ESL-1) or P-selectin glycoprotein ligand 1 (PSGL-1) on leukocytes (Yago et al., 2010).

Leukocyte arrest and migration on the endothelium depends on chemokines (Luu et al., 2000). The shift from fast rolling to slow rolling allows G-protein-coupled receptors (GPCRs) on

leukocytes to bind to the chemokines expressed on the surface of endothelial cells, which induces leukocyte integrin activation and enables leukocyte firm adhesion to the vessel wall (Giagulli et al., 2004; Rutledge and Muller, 2020).

Integrins are a family of transmembrane receptors expressed by leukocytes. Both VLA-4 (very late antigen 4,[3] α4β1-integrin, CD49d/CD29) and lymphocyte function-associated antigen-1 (LFA-1) integrins have been found to play a role in leukocyte arrest (Uotila et al., 2014). VLA-4 and LFA-1 are expressed on leukocytes at a resting low affinity state (Uotila et al., 2014). Conformational changes are required for integrins to switch from a low affinity state to an active high affinity state enabling them to bind to their ligands expressed on the endothelium (Kim et al., 2003; Ley et al., 2007; Fan and Ley, 2015). The activation of integrins is mediated by chemokines and results in integrin extension and changes in the cytoskeleton of leukocytes, enabling tight adhesion on the vessel wall (Rutledge and Muller, 2020). LFA-1 and VLA-4 on leukocytes binding to their respective ligand intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) on endothelial cells to mediate leukocyte firm adhesion (Hyduk et al., 2007; Kuwano et al., 2010), and induces intracellular signals to allow leukocyte arrest (Giagulli et al., 2006; Kummer and Ebnet, 2018). Leukocytes then extend lamellipodia and prepare for trans endothelial migration (Rutledge and Muller, 2020).Additional downstream signals, such as prostaglandin- D2 (PGD2) signals, are required for shape change and dynamic transmigration of activated leukocytes (Ahmed et al., 2011). LFA-1 interaction with ICAM-1 expressed by the inflamed endothelium is required for the transition of leukocytes from tight adhesion to crawling along the vessel wall (leukocyte locomotion) to then find a suitable site for trans endothelial migration (Schenkel et al., 2004).

Leukocytes either transmigrate (diapedesis) through the endothelial junctions (paracellular route), or through the body of the endothelial cells (transcellular route) (Ley et al., 2007). The most common of these ways is paracellular migration, where leukocytes migrate between gaps in cells via tight junctions and use endothelial cell adhesion molecules such as PECAM-1, junctional adhesion molecules (JAMs) and vascular endothelial (VE)-cadherin (Ley, 2007; Muller, 2011). The less common way, is transcellular migration (Carman and Springer, 2004), is where leukocytes migrate directly through the endothelial cells via formation of vesiculovacuolar organelles, creating intracellular endothelial cell channels (Dvorak and Feng, 2001). To reach inflamed tissues, leukocytes need to migrate through the basement membrane (Mutgan et al., 2020). Finally, .following a successful diapedesis, leukocytes utilize a chemokine gradient to migrate toward the site of infection upon their entry into inflamed tissues (Ley et al., 2007).

1.4 Chronic inflammation

Persistent acute inflammation which is not resolved can result in chronic inflammation (Luster et al., 2005). Chronic inflammation is accompanied by excessive trafficking of leukocytes into the site of inflammation and release of local inflammatory mediators that continuously recruit the leukocytes and activate them (Luster et al., 2005). This process can lead to tissue damage and subsequent development of a variety of chronic inflammatory diseases such as type 1 diabetes (T1DM) and, rheumatoid arthirits (RA) (Luster et al., 2005). The etiology of chronic inflammation is not fully understood. However the imbalance between pro-inflammatory and anti-inflammatory processes is speculated to be the most important mechanism involved in progression of chronic inflammation (Kubo and Motomura, 2012). T2DM is identified as a chronic inflammatory disease,

in which homeostasis of glucose is dysregulated as result of persistent inflammation. Therefore, the section 1.5 will focus on pathophysiology of diabetes, and dysregulation of glucose levels in T2DM.

1.5 Diabetes

Diabetes or diabetes mellitus is a chronic metabolic disease which is characterised by hyperglycemia resulting from either impaired insulin production, or decreased sensitivity of cells to insulin (Leahy, 2005). There are two types of diabetes that correspond to these defects respectively: insulin dependent (type 1; T1DM), and non-insulin dependent (type 2; T2DM) diabetes. Some T2DM subjects may also develop inadequate production of insulin, as seen in T1DM patients, as their disease progresses (Bluestone et al., 2010).

In 2017, nearly 462 million subjects were identified with T2DM, which is predicted to increase to 7079 individuals per 100,000 by 2030 (Oguntibeju, 2019), indicating a global increase. Most cases of T2DM are diagnosed over the age of 40 years old (Leahy, 2005). The chances of developing T2DM depends on a combination of risk factors, including the genes and lifestyle (Herder and Roden, 2011). T2DM is more likely to develop in obese individuals with sedentary life style, aged over 45, have a family history of diabetes or are of African American descent (Melmed et al., 2015). Although the risk factors such as age or family history are not controllable, lifestyle risk factors such as diet and physical activity are manageable (Melmed et al., 2015).

1.5.1 Glucose transport

Glucose is a simple carbohydrate with the molecular formula of $C_6H_{12}O_6$ (Shendurse and Khedkar, 2016). Glucose is the main source of energy in body and is stored as glycogen in animals (Brown and Ransom, 2007) d-glucose is natural and metabolically active occurring form of glucose, while l-glucose is an organic isoform and can be synthesised in laboratory and is of lesser importance (Zhang et al., 2021).

Glucose is a big sugar and is not able to enter the cell, thus requires a specific transporter (Hall and Hall, 2020). Glucose either enter the cell in a sodium dependent manner via Na+-coupled glucose transporters, or enter via glucose transporters (GLUT) (Scheepers et al., 2004). GLUTs are proteins that facilitate a bidirectional glucose transport through the plasma membrane based on concentration gradient of glucose without consuming energy (Garde et al., 2022). There are 14 isoforms of GLUTs in human (GLUT-1 to GLUT-14). However, GLUT-1-4 are the most important in glucose control (Adekola et al., 2012). While GLUT 1–3 function independently to insulin (Hall and Hall, 2020) function, expression also localization of GLUT-4 is exclusively insulin dependent (Moraes-Vieira et al., 2016). GLUT-4 is mainly expressed on insulin target tissues such as adipose tissue, cardiomyocytes, and skeletal muscle cells and is able to translocate to cell membrane in response to insulin and facilitate the glucose uptake (Huang and Czech, 2007). A kinase is attached to GLUT-4 and phosphorylates the glucose to make Glucose-6-phosphate (G6P) in response to insulin (Salani et al., 2013). G6P does not bind to GLUT, thus trapped within the cell (Salani et al., 2013). Therefore GLUT-4 plays a crucial role in glucose homeostasis by facilitating the glucose uptake from blood (Huang and Czech, 2007).

1.5.2 Insulin synthesis

Insulin is a 51 amino acid-protein hormone, which is secreted by pancreatic beta cells in response to nutrient stimuli such as glucose and amino acids, normalising them to physiologic levels (Campbell and Newgard, 2021).

Insulin is composed of α and β chain and three 3 disulfide bridges (Østergaard et al., 2020) and belongs to a family of peptide hormones with high degree of structural similarity that includes relaxin, insulin-like growth factors (IGF) I and II, and other insulin-like peptides (Lamson et al., 1991) Insulin is produced as preproinsulin and is then cleaved to proinsulin and stored in secretory granules (Liu et al., 2014). Mature insulin is generated from proinsulin by proteases that reside in the same storage vesicles and is released from storage vesicles into circulation via exocytosis (Liu et al., 2014).

1.5.3 Insulin release

As illustrated in Figure 1.1, elevated plasma glucose stimulates the insulin secretion by a sequence of events in beta cells (Arora et al., 2021). Indeed, glucose is rapidly phosphorylated to G6P by glucokinase activity after entering into the beta cells via GLUT-2 (Rutter et al., 2015). G6P increase the ATP levels in beta cells which induce ATP-sensitive K^+ channels to shut down and depolarization of cell membrane and subsequent opening of the voltage-gated Ca2+ channels (Rutter et al., 2015**).** Increased cytosolic calcium levels stimulates the insulin exocytosis from beta cells into the blood circulation (Arora et al., 2021). Insulin remains in circulation for 5 to 8 minutes

(Koeppen and Stanton, 2017) and exerts its effects through mediating the glucose disposal into target tissues (Zierath et al., 2000)

1.5.4 Insulin signaling

As shown in Figure 1.2-A, the insulin receptor (IR) comprises two α and two β subunits, forming an α2β2 heterotetramer (Hubbard, 2013)**.**The two α subunits are extracellular, whereas the two β subunits begin on the extracellular side of the membrane and then traverse the membrane into the cytoplasmic region (Hubbard, 2013). The β subunits contain the cytoplasmic tyrosine kinase domains. A single molecule of insulin binds asymmetrically to the ectodomain, triggering a conformational change that is transmitted to the cytoplasmic kinase domains, which activates the tyrosine kinase (Tyr-K) domain by facilitating tyrosine phosphorylation in the juxtamembrane region of IR (Y1158, Y1162, and Y1163 residues), which in turn creates recruitment sites for insulin receptor substrate (IRS) family of proteins (1.2-B), (Hubbard, 2013). Phosphorylated IRS activates numerous signalling partners such as phosphoinositide 3-kinase (PI3K), protein kinase B (PKB or AKT) and phosphoinositide-dependent kinase-1 (PDK-1) (Hall and Hall, 2020). The signaling processes enable translocation of GLUT4 to the cell surface, where it contributes to glucose uptake particularly into in muscle and fat cells (Koeppen and Stanton, 2017; Hall and Hall, 2020). Following entry, the glucose is either processed for energy or cell growth (Koeppen and Stanton, 2017).

1.5.5 Insulin Resistance

Insulin resistance occurs when insulin-target cells, such as skeletal muscle and fat, fail to properly respond to physiologic levels of insulin (Yaribeygi et al., 2019).Thereby, the cells cannot take up glucose and hyperglycemia arises. Insulin resistance is a main pathologic feature of metabolic disorders, such as T2DM and obesity and since many enzymes and mediators are involved in insulin signal transduction, any change in function or expression of these factors may influence the normal insulin signaling leading to insulin resistance in target tissues (Wondmkun, 2020).

Insulin resistance in T2DM is explained by several different hypothesised mechanisms, mostly at the cellular level through post-receptor defects in insulin signaling as discussed below.

1.5.5.1 Inflammatory mediators

Inflammatory mediators such as obesity-induced cytokines, IL-6, TNF-α, and IL-1β, are associated with insulin resistance (Zand et al., 2017). In particular, TNF-α is upregulated in obesity and impairs insulin signaling by reducing GLUT-4 expression, thus affecting the glucose uptake resulting in hyperglycemia (Peraldi and Spiegelman, 1998).

It has also been shown that prolonged, but not acute, treatment of 3T3-L1 adipocytes with interleukin-1 beta (IL-1β) slightly decreased the gene expression of Glut-4 and strongly suppressed its translocation to the cell surface in response to insulin. This effect was attributed to ability of IL-1β to reduce the gene expression of IRS in 3T3-L1 adipocytes, demonstrating that IL-1β can modulate the insulin signaling at post-receptor levels (Jager et al., 2007). Later, Stienstra R et al, showed that IL-1β strongly reduced the expression of the insulin sensitivity genes, such as

peroxisome proliferator-activated receptor (PPARγ), adiponectin and most importantly the GLUT-4, both in adipose tissue of diet-induced and genetically induced obese animal models (Stienstra et al., 2010) This fact was also reported by Gao et al, where they found reduced gene and protein expression of GLUT-4 and other genes involved in glucose metabolism and insulin signaling, such as PPARα and IRS-1, in human macrophage-conditioned medium adipocytes treated with IL-1β, and this was reversed by blocking IL-1β activity using a neutralizing antibody (Gao et al., 2014), suggesting IL-1β blocker as a beneficial candidate to prevent the obesity-associated insulin resistance and inflammation in human adipose tissue.

1.5.5.2 Oxidative stress

Oxidative stress is defined by excess endogenous oxidative species, such as reactive oxygen species (ROS), which are produced at low physiological levels mostly in the mitochondria (Schieber and Chandel, 2014). However, they are increased in many chronic diseases and in T2DM (Hurrle and Hsu, 2017). Indeed, in conditions like hyperglycemia and hyperinsulinemia, which exist in T2DM, the ROS production is increased by malfunctioning mitochondria (Campa et al., 2015). This in turn signals the trans-Golgi network downstream, and instead of the plasma membrane, GLUT-4 is translocated into lysosomes for degradation; thus results in elevated glucose levels via decreased glucose uptake by insulin responding cell (Ma et al., 2014). Furthermore, this mitochondrial dysfunction appears to be effected by free fatty acids, which are abundant in obesity (Boucher et al., 2014). In fact, a study done in adipocytes showed that when these cells were exposed to either high glucose (HG) or free fatty acids levels the endogenous mitochondrial ROS production increased in them, which altered the insulin transduction pathway

by upregulating the p38 MAPK expression and downregulating the IRS-1 and Akt (Boucher et al., 2014). This pathway switch reduce the translocation of GLUT-4 to cell membrane and resulting in impaired glucose control (Boucher et al., 2014). Indeed, increased mitochondrial ROS production was shown to be associated with increase in Ser307 phosphorylation of IRS-1 and decrease in insulin-stimulated tyrosine phosphorylation of IRS-1, all of which are believed to be the molecular basis of insulin resistance (Nishikawa et al., 2007)

. Taken together, these studies suggest the importance of oxidative stress in pathophysiology of obesity and T2DM, and the potential of antioxidants as treatments to improve the metabolic dysfunction.

1.5.5.3 Deficiencies of phosphorylation

As discussed in section 1.5.4 binding of insulin to its receptor activates the intrinsic tyrosine kinase activity of the receptor, which phosphorylates Tyr residues of target proteins such as the IRS (Boura-Halfon and Zick, 2009). Decreased phosphorylation of IRS and IR in response to insulin has been reported in skeletal muscle of obese subjects and patients with T2DM and was associated with impaired insulin-stimulated glucose uptake in these individuals (Goodyear et al., 1995; Pratipanawatr et al., 2001) Furthermore, reduced phosphorylation of Akt, a key regulator of GLUT-4 translocation was also shown in adipocytes of T2DM patients compared to healthy individuals (Smith et al., 1999). The IR and the IRS proteins are targets for feedback control mechanisms with phosphorylation of IRS proteins on Ser residues being a key step in these feedback control processes. Many of the insulin-stimulated Ser/Thr kinases that are downstream effectors of IRS proteins serve as negative modulator of IRS proteins function. Interestingly, it is

becoming apparent that inducers of insulin resistance such as TNF-α, FFA, and cellular stress make use of similar mechanisms by activating a set of IRS kinases that phosphorylate the IRS proteins and inhibit their function (Boura-Halfon and Zick, 2009)

In response to insulin resistance, pancreatic islets initially increase their cell mass and insulin secretion, but this is unable to fully compensate the situation, resulting in development of T2DM (Kahn et al., 2006) and its common chronic vascular complications, including diabetic nephropathy, neuropathy, and retinopathy (Fowler, 2008). Therefore, understanding the mechanisms involved in insulin resistance can lead the development of new therapeutic agents.

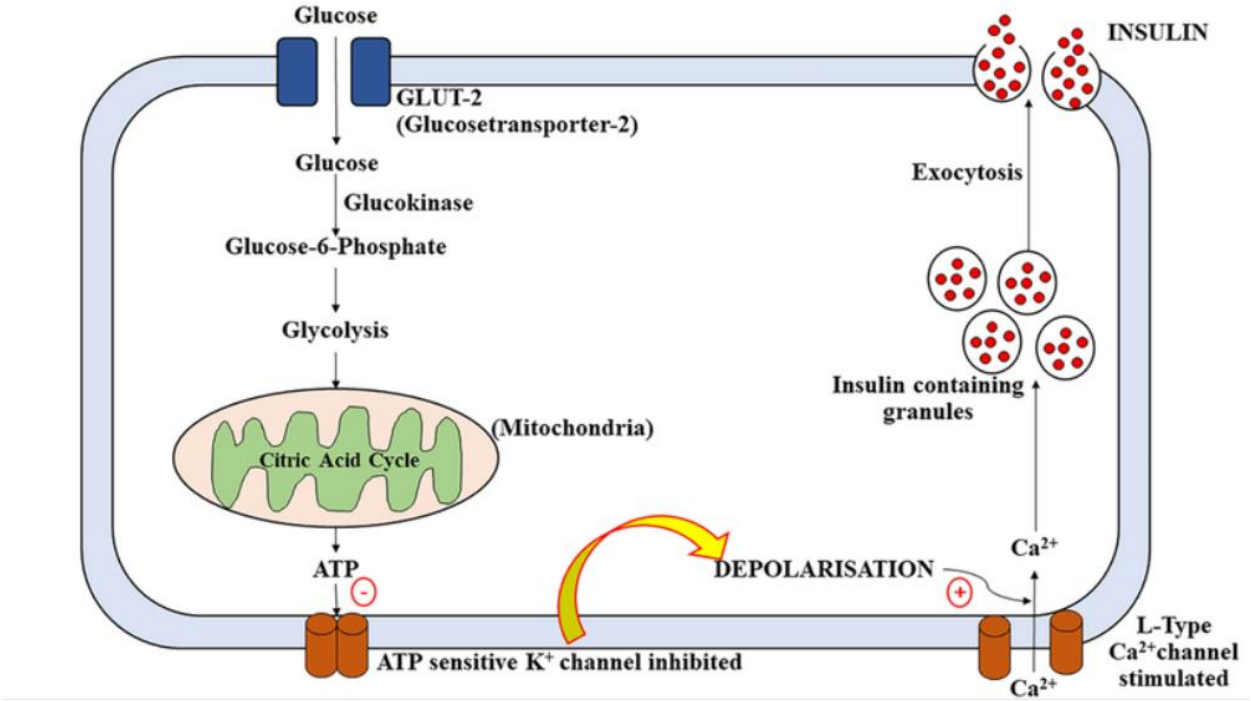


Figure 1.1 Mechanism of insulin secretion from beta cells of pancreas. Glucose entering the beta cells via GLUT-2 stimulates the insulin secretion. Once entered, glucose is phosphorylated to G6P by glucokinase activity. G6P enters the glycolysis cycle and increase the ATP levels in beta cells, which result in inhibition of the ATP-sensitive K+ channels and consequent depolarization of beta cell membranes. This opens the voltage-gated Ca2+ channels and increases intracellular calcium levels which induces exocytosis of storage vesicles containing insulin/proinsulin into circulation. Taken from (Arora et al., 2021).

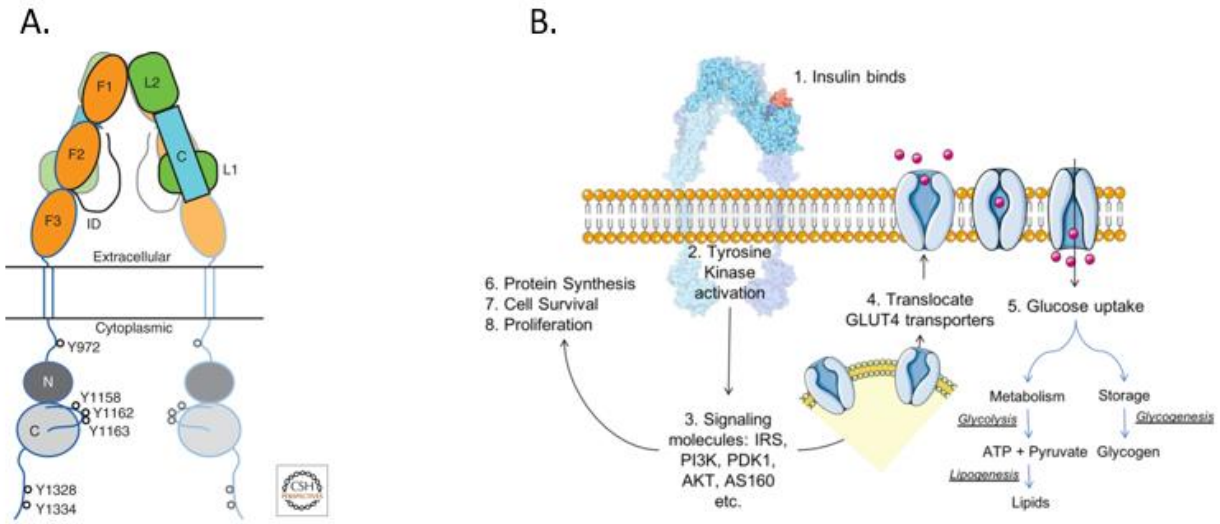


Figure 1.1 Insulin signal transduction pathway

A. The α subunits (extracellular) comprise two leucine-rich domains (L1 and L2) with an intervening cysteine-rich domain (C), one intact fibronectin type III (FnIII) domain (F1), a partial FnIII domain (F2), and a long insert domain (ID) that contains the site of furin cleavage (L1-C-L2-F1-F2-ID). The β subunits (extracellular and cytoplasmic) contain the completion of the second FnIII domain (F2), followed by an intact FnIII domain (F3), a transmembrane helix, and a cytoplasmic tyrosine kinase domain (N and C lobes). The major sites of tyrosine autophosphorylation in the cytoplasmic domain are indicated (Y1158, Y1162, and Y1163). B-1. The insulin receptor (colored blue) binds insulin (colored red) and (2) activate the tyrosine kinase catalytic domain positioned within the cell. (3) The tyrosine kinase, in turn, activates various other proteins such as IRS, PI3K, PDK1 and AKT in a signaling cascade leading to (4) translocation of GLUT4 to the cell surface and (5) subsequent glucose uptake or (6-8) insulin's various anabolic functions. Taken from (Hubbard, 2013) and (Siddle 2011).

1.6 Obesity related changes of immune system in T2DM

Obesity, particularly visceral adiposity, is a major risk factor for T2DM (Donath and Shoelson, 2011) and the adipose tissue of patients with T2DM and/or obese patients appears to be a major site of inflammation (Lontchi-Yimagou et al., 2013). Both innate and adaptive immune cells present in the adipose tissue have critical roles in the regulation of metabolic homeostasis (Lontchi-Yimagou et al., 2013). Dysregulated trafficking of leukocytes is observed in the adipose tissue from obese individuals/animals, where there is a shift in subtypes of recruited cells from an anti-inflammatory to a pro-inflammatory profile in obesity (Esser et al., 2014). This switch to a production of pro-inflammatory cytokines leads to the development of systemic low-grade inflammation, which results in impaired insulin signaling, beta cell dysfunction and subsequent insulin resistance (Esser et al., 2014). Hyperglycemia arises as the pancreas fails to compensate insulin resistance (Donath and Shoelson, 2011), resulting in oxidative stress and systemic inflammation through the production of inflammatory cytokines (Noshita et al., 2002; Dimayuga et al., 2007) and chemokines, cumulating in endothelial dysfunction and vascular complications (Nguyen et al., 2012) as summarised in Figure 1.3 (Pezhman et al., 2021). Following sections will more describe the modulation of leukocytes subsets trafficking in concept of obesity and T2DM.

1.6.1 Expression of Adhesion Molecules in T2DM

CAMs are glycoproteins expressed on the surface of various cell types, such as endothelial cells and leukocytes (Galkina and Ley, 2007). Upon inflammation, vascular endothelial cells express or up-regulate the expression of CAMs that increase the attachment of leukocytes to the

endothelium (Cook-Mills et al., 2011). Selectins, ICAM-1 and VCAM-1 are the major CAMs responsible for leukocyte adhesion (Huo and Ley, 2001). Soluble form of CAMs (sCAMs) in the circulation indirectly reflects the rate of endothelial expression and activation of CAMs since they are shed from the surface of endothelial cells and lymphocyte after being activated (Abe et al., 1998). Elevated expression and activity of CAMs are therefore indicative of inflammation and endothelial dysfunction (Cook-Mills et al., 2011).

1.6.1.1 Soluble CAMs and selectins

The baseline levels of circulating E-selectin, ICAM-1, and VCAM-1 were found higher in patients with T2DM compared to healthy controls (Meigs et al., 2004). A study on 150 Japanese patients with T2DM showed that higher serum concentrations of sVCAM-1 and sE-selectin were positively correlated with fasting plasma glucose levels, and negatively correlated with insulin sensitivity (Matsumoto et al., 2002). Higher levels of circulating CAMs in T2DM are linked to an increased production of advanced glycosylation end products (AGEs) and oxidative stress occurring under hyperglycemic conditions (Hadi and Al Suwaidi, 2007). The interaction of AGEs with the vessel wall components increases the generation of reactive oxygen species, which results in an increase in the surface expression of adhesion molecules on activated endothelial cells (Wen et al., 2002; Basta et al., 2004; Farhangkhoee et al., 2006).

Hyperinsulinemia can also directly affect the surface expression of adhesion molecules on endothelial cells from healthy volunteers and from patients with non-insulin-dependent diabetes *in vitro* (Okouchi et al., 2002). Culture of human umbilical endothelial cells (HUVEC) in insulin-

rich medium (over 50 micro Unit/ml) for 24 hours increased the surface expression of PECAM-1, but not of ICAM-1, P-selectin or E-selectin (Okouchi et al., 2002).This was clearly associated with an increase in neutrophil transmigration across the insulin treated HUVEC as the recruitment of neutrophils isolated from both healthy control and patients with T2DM, were increased through the endothelium compared to not treated HUVEC; However, this recruitment was greater in neutrophils isolate from patient with T2DM; suggesting that high insulin conditions promote vascular injury and therefore T2DM-associated macro- and micro-vascular complications.

However, a recent study on 58 patients with T2DM on insulin therapy and displaying microvascular complications, found lower levels of serum sICAM-1 in these patients irrespective of the type of diabetic complication when compared to age-matched healthy controls (Hocaoglu-Emre et al., 2017). Since the participants with T2DM were also receiving angiotensin-converting enzyme (ACE)-inhibitor agents, decreased levels of sICAM-1 might have resulted from the combination of insulin and ACE-inhibitor therapies. Indeed, both insulin and ACE inhibitors can individually inhibit CAMs surface expression on endothelial cells, as well as their circulating levels (Drexler et al., 1995; Aljada et al., 2000). Overall, the conflicting evidence about the levels of sCAMs in the circulation suggests that different CAMs may play different roles in the different stages of vascular complications in T2DM (Hocaoglu-Emre et al., 2017).

1.6.1.2 Surface expression of CAMs on the endothelium

Variations in the expression of adhesion molecules on the vasculature play a role in microangiopathy in patients with T2DM by enhancing leukocyte adhesion in the vasculature and

causing capillary obstruction (McLeod et al., 1995). This has been observed in different vascular beds and is linked to a majority of complications, such as diabetic nephropathy (Gu et al., 2013) and retinopathy (McLeod et al., 1995). A study on 40 patients with T2DM showed a significant increase in the expression of ICAM-1 and VCAM-1 on vascular endothelial cells from the conjunctiva in comparison with conjunctiva from healthy controls (Khalfaoui et al., 2008). Confocal microscopy imaging of retina vessels in streptozotocin (STZ)-induced hyperglycemic mice reported increased VCAM-1 protein levels after 8 weeks, and this was synchronized with the expression of the inflammatory cytokines TNFα, IL-6 and IL-1β in the retina (Gustavsson et al., 2010). However, not many studies have performed in depth study of leukocyte recruitment in patients and in mice models to determine how defects in expression of adhesion molecules can modulate leukocyte trafficking.

1.6.2 Modulation of innate and adaptive immune cells in obesity and T2DM

Nowadays, the pathogenesis of T2DM is considered to be linked to both innate and adaptive immune factors that are recognised as important etiological components in the development of insulin resistance (Goldberg, 2009). Although earlier studies focused on the role of innate immunity (macrophages) as the major cause of chronic low-grade inflammation in T2DM, the adaptive immune system also plays a role in progression of T2DM (Zhou et al., 2018).

1.6.2.1 Macrophage/Monocytes

In recent years, it has been reported that macrophages play key roles in the progression of T2DM (Gordon and Martinez, 2010). Monocytes derived from the bone marrow remain in the circulatory system for 1–2 days before they migrate into peripheral tissues, where they turn into fully mature resident macrophages (Martinez-Pomares et al., 2003) with either a M1 pro-inflammatory or M2 anti-inflammatory phenotype in response to local factors (Mosser, 2003). In a recent *in vivo* study, an acute decrease in number of circulating monocytes was synchronized with a concomitant infiltration of macrophages into visceral adipose tissue at the early stages of high fat diet (HFD)-induced diabetes, therefore demonstrating dynamic changes in blood monocyte trafficking in preclinical disease (Liu et al., 2020). Higher levels of F4/80 and CD11c mRNA expression in adipose tissue of HFD fed mice, representative of a M1 polarization, were attributed to an elevated gene expression of ICAM-1, VCAM-1, CCL2 and CXCL14 in this tissue, all of which facilitate macrophage trafficking (Brake et al., 2006; Kawanishi et al., 2010). The polarization of M1 macrophages was shown to be associated with elevated serum and adipose tissue levels of IL-6 and TNF-α, higher fasting plasma glucose levels and insulin resistance in HFD-induced T2DM rats (Shanaki et al., 2020). The long-term impact of diabetes on the function of macrophages was elicited using a 5-month-old db/db mice model of T2DM and demonstrated a reduced ability of peritoneal macrophages to polarization toward a M2 phenotype (Liu et al., 2012). Furthermore, *in vitro* assessment of db/db peritoneal macrophages highlighted their decreased capacity to adhere to plastic surfaces, along with a decreased ability to phagocytose compared to wild-type (wt) peritoneal macrophages (Liu et al., 2012). These observations validate that diabetes and hyperglycemia is associated with immune cells dysfunction, which can affect

their migratory behavior and predispose these patients to an increased susceptibility to infections (Liu et al., 2012).

1.6.2.2 T cells

Evidence shows that T cells accumulation in adipose tissue of HFD mice happens after infiltration of B cells and is quickly followed by accumulation of macrophages leading to insulin resistance (Duffaut et al., 2009). Nishimura et al. (2009) found larger numbers of infiltrated $CD8^+$ effector T cells, along with reduced numbers of $CD4^+$ helper and lower number of Tregs in visceral adipose tissue of HFD fed mice compared to control mice. In these conditions, a majority of $CD8^+$ T cells infiltrate the adipose tissue thereby promoting recruitment and activation of macrophages. Studies in the lymphocyte deficient $RAG2^{-/-}$ knockout mice provide strong evidence for the role of lymphocytes in HFD-mediated adipose tissue inflammation (Duffaut et al., 2009). Unexpectedly, lymphocyte-deficient animals displayed striking accumulation of macrophages and NK (NK) cells in the adipose tissue compared to wt mice (Duffaut et al., 2009). This accumulation of NK cells highlights an overreaction of the innate immunity in absence of the adaptive immune system. Indeed, NK cells have potent cytotoxic effector functions and produce chemokines and cytokines that can recruit macrophages (Duffaut et al., 2009). The exaggerated recruitment of macrophages in lymphocyte-deficient mice demonstrates that early lymphocyte infiltration could be considered a protective process to decrease adipose tissue inflammation and suggests adipose tissue as a site of dynamic innate and adaptive immune system during diet-induced obesity and insulin resistance (Duffaut et al., 2009).

Furthermore, the depletion of Tregs using diphtheria toxin led to an induction of genes encoding inflammatory cytokines, such as TNF-α and IL-6, in visceral adipose tissue and enhanced levels of fasting insulin in C57BL/6 mice (Feuerer et al., 2009). Conversely, increasing the quantity of Tregs in the adipose tissue, improved insulin resistance and glucose tolerance (Feuerer et al., 2009). Since the gene expression of IL-10 was increased in the adipose tissue of Tregs–enriched animals, the metabolic changes observed can be attributed to IL- 10 synthesis by Tregs in the adipose tissue of these mice (Feuerer et al., 2009). These findings suggest a strong therapeutic potential for Tregs to suppress inflammation and improve insulin action in obesity and T2DM. It is therefore key to understand how Tregs migratory capacity and infiltration in the adipose tissue could be manipulated in patients. In addition, further studies are required to fully characterize the involvement of different T cell subsets and characterize the intricate balance between innate and adaptive immune regulations in concept of T2DM.

1.6.2.3 B cells

B cells have been shown to play important roles in many chronic inflammatory and autoimmune conditions, but over the last 15 years their role in obesity-associated insulin resistance has been revealed (Yanaba et al., 2008; Mariño and Grey, 2012). B cells infiltrate the white adipose tissue in diet-induced obesity models and contribute to insulin resistance through secreting inflammatory cytokines, producing pathogenic antibodies and the activation of T cells and macrophages (Winer et al., 2011). B cells from T2DM subjects secrete a proinflammatory cytokine profile (IL-8 and IL-17), and are unable to secrete the potent anti-inflammatory cytokine IL-10 (Jagannathan et al., 2010). Similarly they also switch antibody production from IgM to IgG, which

can activate T cells and macrophages (Winer et al., 2009). The importance of B cells in adipose tissue inflammation has been studied in B cell null mice on HFD (DeFuria et al., 2013). Following 15 weeks of HFD, serum glucose levels were unchanged in B cell null mice while fasting glucose levels increased in the control wild-type group (DeFuria et al., 2013). Attenuated inflammation in visceral adipose tissue of B cell null mice was shown by a reduced number of M1 macrophages and decreased T helper 17 (Th17) numbers compared to wild-type control (DeFuria et al., 2013). Transfer of B cells from HFD mice into B cell null mice worsened metabolic parameters, implicating the B cells as important pathogenic mediators of insulin resistance (DeFuria et al., 2013). Therefore, B cell manipulation represents a novel approach to the treatment of obesity-related insulin resistance and potentially to the prevention of T2DM.

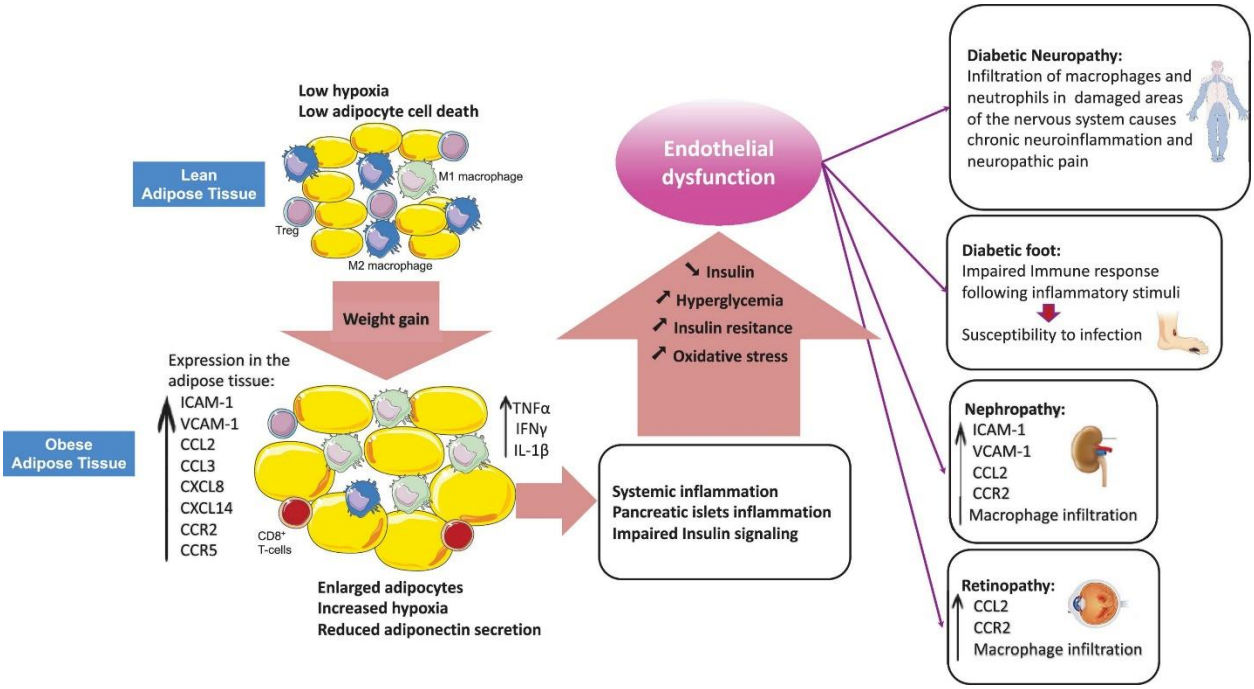


Figure 1.3 Obesity-related dysregulation of leukocyte trafficking in T2DM Adipose tissue from lean individuals is characterized by increased numbers of anti-inflammatory M2 macrophages and higher presence of regulatory T cells (Tregs). Obesity induces adipose tissue enrichment of cytotoxic $CD8^+$ T-cells and pro-inflammatory M1 macrophages with a shift from an anti-inflammatory to a pro-inflammatory state. In obesity, the imbalance among leukocytes results in production of inflammatory cytokines (TNFα, IFNγ, IL-1β) and chemokines which promote systemic inflammation and peripheral insulin resistance as well as beta-cell dysfunction. Hyperglycemia occurs as a result of beta-cells failure to compensate for insulin resistance. Hyperglycemia exacerbates systemic inflammation by inducing pro-inflammatory cytokines production and modulating leukocyte trafficking through alteration of the expression of adhesion molecules, chemokines, and chemokine receptors in different vascular beds, which ultimately results in vascular complications. Taken from (Pezhman et al., 2021)

1.6.3 Immunosenescence in T2DM

The immune system is identified as one of the most important regulators to maintain the physiological homeostasis in body (Aiello et al., 2019). However, with aging, immune function is compromised through a phenomenon called immunosenescence, which results in altered immune response to foreign pathogens (e.g., reduced ability to respond to new antigens and excessive inflammatory responses) (Akbar and Fletcher, 2005; Fulop et al., 2018; Aiello et al., 2019). Immunosenescence occurs in both innate and adaptive immune cells and is closely associated with development and progression of inflammatory disease, such as visceral obesity (Shirakawa et al., 2016) and T2DM (Lau et al., 2019). Indeed, Shirakawa et al, demonstrated an HFD-induced accumulation of $CD4^+$ T cells expressing programmed cell death 1 (PD-1) and CD153 in the adipose tissue of obese mice (Shirakawa et al., 2016). Furthermore $CD8^+$ T cells that expressing killer cell lectin-like receptor subfamily G member 1 (KLRG1, end-stage differentiation marker), (Akbar and Fletcher, 2005) were reported higher in HFD mice compared with those fed a normal diet (Kiran et al., 2021). Moreover, the infiltration of age associated B cells (identified as $CD19^+CD93^-/CD43^-CD23^-CD21^-$) were also reported in visceral adipose tissue of obese mice (Frasca et al., 2017). Thus, collectively these studies suggesting senescent immune cells as a new therapeutic target to control the inflammation.

1.6.4 Therapeutic targeting of leukocyte trafficking in T2DM

Given that T2DM is a chronic inflammatory disorder, characterizing and targeting possible inflammatory pathways could be efficient to prevent and control diabetes and its vascular complications.

1.6.4.1 Chemokines and cytokines inhibition

Chemokine and cytokine pathways are known to govern the trafficking of leukocytes into peripheral tissues (Yao et al., 2014). Potential antagonists targeting chemokine receptors and drugs blocking inflammatory cytokines have been developed and tested in many inflammatory conditions, but little is known about their efficacy in obesity or T2DM and associated complications. In this section, we will discuss some of these targets.

CCL2 and CCL5 are key mediators of monocyte recruitment induced by high glucose levels via their receptor CCR2 and CCR5 (Nunemaker et al., 2014). Oral administration of RO5234444, a CCR2 antagonist, to *db/db* mice, reduced infiltration of monocytes in the glomerulus, resulted in preservation of glomeruli podocytes numbers and reduced albuminuria (Sayyed et al., 2011). Similarly, intraperitoneal administration of TAK-779, a dual inhibitor of chemokine receptors CCR2 and CCR5, reduced macrophage infiltration and expression of ICAM-1 in the retinas of STZ-diabetic mice (Monickaraj et al., 2020). These studies clearly indicate that blocking key recruitment chemokine receptors, such as CCR2 and/or CCR5, is a promising therapeutic avenue to ameliorate common diabetic complications, such as nephropathy and retinopathy. Those compounds are currently pursued in human clinical trials as well as others such as INCB8761/PF-413630, which are a new series of CCR2 antagonists that are orally bioavailable (Xue et al., 2011).

Due to their pro-inflammatory nature, IL-1β and TNFα actions have been widely studied in many inflammatory conditions and their blockade resulted in improvements in T2DM-related conditions, such as chronic kidney disease (Lei et al., 2019) and pancreatic islet inflammation (Zha et al., 2016). However, it remains unsure whether targeting these cytokines effects leukocyte infiltration in peripheral tissue in T2DM. Circulating levels of IL-1β are higher in T2DM (Reinehr

et al., 2016) and macrophages are the primary source of IL-1β in obesity-induced inflammation (Gao et al., 2014). Administration of LY2189102, a neutralizing IL-1β antibody, in patients with T2DM improved glycemic control and demonstrated significant anti-inflammatory effects by lowering circulating IL-6 levels when compared with placebo treatment (Sloan-Lancaster et al., 2013). Moreover, islets from IL-1β-deficient mice exposed to high glucose *in vitro*, produced lower IL-6 and chemokines compared to wild-type islets (Ehses et al., 2009). Thus, efficacy of IL-1β blocking on inflammatory biomarkers and glycemic control raises the possibility of its use as a treatment in T2DM and other inflammatory conditions.

Although, anti-TNFα is an approved medication for some patients with rheumatoid arthritis (Katsumata et al., 2019), the evidence showing the efficacy of this drug in T2DM is conflicting. Indeed, anti-TNFα treatment using recombinant soluble TNFα receptor-immuno-globulin G increased insulin sensitivity in obese rodents (Hotamisligil et al., 1993). However, treatment with Ro 45-2081, a TNFα antagonist (recombinant fusion protein that consists of the soluble TNF-receptor linked to the Fc portion of human IgG1), had no effect on blood glucose levels and insulin-mediated glucose uptake in patients with T2DM (Paquot et al., 2000). The inefficiency of Ro 45-2081 to control blood glucose in patients with T2DM suggest that in addition its endocrine action, TNFα may also act through an autocrine or paracrine route. Consequently, sole neutralization of circulating TNFα might not be enough to observe improvements in insulin actions (Paquot et al., 2000).

1.6.4.2 Anti-integrins

The importance of integrins in leukocyte adhesion and arresting clearly offer potential therapeutical avenues in relapsing inflammatory conditions, such as rheumatoid arthritis (von Andrian and Engelhardt, 2003) and multiple sclerosis (Chaudhuri and Behan, 2003). Neutralization of integrins has been tested in murine experimental models of diabetes, particularly for the treatment and/or prevention of diabetic complications (Barouch et al., 2000; Iliaki et al., 2009; Miyachi et al., 2017). However, data showing efficacy in human clinical trials are not available in the context of T2DM.

The expression of CD11a, CD11b, and CD18 integrins is increased on the surface of neutrophils from STZ-induced diabetic rats (Barouch et al., 2000). This increase was associated with an enhanced adhesion of diabetic neutrophils to rat endothelial cell monolayers *in vitro.* Pretreatment of leukocytes with either anti-CD11b or anti-CD18 antibodies significantly lowered the proportion of adherent diabetic neutrophils (Barouch et al., 2000). In the same study, systemic administration of anti-CD18 $F(ab')_2$ fragments to STZ-induced diabetic rats significantly decreased diabetic retinal leukostasis (Barouch et al., 2000). Furthermore, intraperitoneal administration of anti-α_4 integrin neutralizing antibody to a STZ-induced rat model of diabetes retinopathy attenuated leukocyte adhesion to the retina and suppressed TNFα expression and nuclear factor kappa B (NF-κB) activity in the retina of treated rats (Iliaki et al., 2009). Notably, blockage of VLA-4, using a neutralizing antibody, in HFD-fed mice attenuated myeloid cell accumulation in the liver and improved systemic glucose tolerance in treated mice compared to control HFD-fed mice (Miyachi et al., 2017). Overall, all these animal data identify the integrins as functional adhesive molecule in diabetic complications and provides a potential target for the prevention and/or treatment of the disease. A humanized anti–VLA-4 mAb (natalizumab) was clinically beneficial in the treatment of Crohn's disease (Lew and Stoffel, 2003), and rheumatoid

arthritis (von Andrian and Engelhardt, 2003). However, VLA-4 also has key regulatory roles in immune response, including the formation of the immune synapse (Mittelbrunn et al., 2004) and the differentiation of Th1 T cells (Isobe et al., 1997). Obviously, the long-term administration of anti-integrins to patients with inflammatory conditions may have undesirable or unexpected effects and more studies are required to fully understand their action.

1.6.4.3 Fatty acids

Long-chain *n*−3 polyunsaturated fatty acid (*n*−3 PUFA), such as eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), found in marine fish oils, are able to down-regulate the activity of NF-κB directly and therefore reduce the production of inflammatory cytokine (Xu et al., 2001). n-3 PUFA can also control leukocyte recruitment during inflammation and display immunomodulatory properties (Yates et al., 2011). Inclusion of *n*−3 PUFA in HFD prevented macrophage infiltration into the adipose tissue of *db/db* mice (Todoric et al., 2006). Interestingly, *n*−3 PUFA efficiently prevented the HFD-induced downregulation of adiponectin circulating levels normally observed in *db/db* (Todoric et al., 2006). These data therefore suggest that the beneficial effects of *n*−3 PUFA on diabetes could be mediated by their effect on adipose tissue inflammation, which could in turn contribute to improving insulin sensitivity (Oliver et al., 2010). However, much of the human evidence examining the beneficial effects of n-3 PUFA in patients with T2DM have limited follow-up periods (Vessby et al., 2001). As a result, it is unclear whether n-3 PUFA supplementation has long lasting beneficial effects and the exact mechanisms of n-3 PUFA action and how they modulate the adipose tissue, macrophages and even T cells remain unknown.

1.6.4.4 Insulin

Several studies suggest a direct anti-inflammatory action of insulin irrespectively of its glucose modulating capacity as mentioned here in previous sections (Hyun et al., 2011). Insulin was found to attenuates the activity of NF-κB and monocyte chemoattractant protein-1 (MCP-1) on human aortic endothelial cells *in vitro* (Aljada et al., 2001). Insulin infusion in individuals with obesity reduced plasma sICAM-1 and CCL2 levels (Dandona et al., 2001). In addition, treatment of human monocytes with insulin *in vitro* promoted the secretion of IL-8 (CXCL8), a potent chemoattractant for neutrophils (Dandona et al., 2001). All together, these findings suggest a possible regulatory effects of insulin on leukocyte trafficking via regulating chemokine and cytokine secretion, as well as modulating adhesion molecules shedding. However, more studies are required to clarify the effect of insulin on leukocyte migration by including *in vitro* models taking into account the high glucose levels observed in some patients with T2DM, their associated complications and BMI to accurately determine the mechanism of insulin action and lift the controversy in this field.

1.6.4.5 Metformin

Despite the fact that metformin is prescribed as the first-line drug for treatment of T2DM for over 50 years (Saisho, 2015), the mechanisms underlying the plasma glucose level-lowering effects of metformin are not fully understood (Foretz et al., 2019). Nuclear magnetic resonance spectroscopy data from patients with T2DM revealed that metformin treatment for 3 weeks suppressed the hepatic gluconeogenesis rate and decreased the production of endogenous glucose in these patients (Hundal et al., 2000). Moreover, insulin sensitising effects of metformin were shown by increased systemic glucose uptake in patients with T2DM , which happened mainly in

skeletal muscles (Stumvoll et al., 1995). Most importantly, the glucose lowering effects of metformin was attributed to its ability to activate the adenosin mono phsphate kinase (AMPK) in primary hepatocytes of rats in an *in vivo* sudy (Zhou et al., 2001), which resulted in the inhibition of mitochondrial respiratory-chain complex 1 by metformin, and a subsequent decreased energy charge in these cells that supressed gluconeogenesis and inhibited the glucose production (Zhou et al., 2001). The importance of AMPK in mediating the effects of metformin were further highlighted when Shaw et al, showed that deletion of an upstream kinase of AMPK, liver kinase B1 (LKB1) suppressed AMPK activation in liver of adult mice and caused sever hyperglycemia (Shaw et al., 2005). On the other hand, an *in vivo* study by Foretz et al. reported that even in the mice lacking hepatic AMPK, metformin was able to exert its glucose-lowering effects compared to wt control mice, suggesting AMPK-independent capacity of metformin in controlling glucose levels (Foretz et al., 2010).

Some human studies have reported beneficial effects for metformin by decreasing the systemic inflammatory cytokines, such as serum levels of TNF-α and IL-6 as well as plasma sVCAM levels in individulas with T2DM (Abbasi et al., 2004; Fidan et al., 2011; Evia-Viscarra et al., 2012; Jing et al., 2018). Indeed, most of the evidence supporting anti-inflammatory effects of metformin have been obtained from *in vivo* and *in vitro* studies in which metformin was used higher than its theraputic concentrations (Jing et al., 2018). For example, it has been showed that administration of metformin for 7 weeks to C57BL/6 male mice following 11 weeks of HFD feeding decreased the systemic level of the inflammatory cytokines IL-6 and TNF-α, as well as reduce the gene expression of adipose tissue *CD11c and MCP-1,* which are typical markers for inflammatory M1 macrophage (Jing et al., 2018). Furthermore their *in vitro* experiments supported the fact that the

anti inflammatory effects of metformin was mediated through activation of AMPK pathway. In fact, their flow cytometry data showed that treatment of palmitate-stimulated bone marrow drived macrophages with metformin and AICAR (an AMPK activator) reduced the proportion of M1 macrophages and increased the proportion of M2 macrophages, which highlights the role of AMPK pathway in improving the fat inflammation by modulating macrophage polarization (Jing et al., 2018). Since AMPK is also one of the downstream signalling for adiponectin (Section 1.8.6), this may suggest similar biologic capacities for metformin and adiponectin in controling the inflammation. However, unlike adiponectin there is no study showing any impact of metformin on lymphocytes behaviour; Indeed metformin is a cation with low membrane permeability and is not taken up significantly by cells lacking transporters of the organic cation transporter family (OCT) (Hawley et al., 2010). It has been shown that OCT is not expressed by T cells , thus no obvious effects of metformin on T cells behaviour is expected in concept of T cells trafficking (Vara-Ciruelos et al., 2019).

.

1.6.4.6 Adiponectin

Adiponectin is an adipokine secreted exclusively by adipose tissue and is found at 5–15 μg/mL serumic levels in human (Hada et al., 2007). Adiponectin is well characterised as an insulin-sensitising hormone, as well as an anti-inflammatory adipokine (Ruan and Dong, 2016) and previous studies has shown the involvment of adiponectin in both metabolic and immune pathways (Kadowaki et al., 2006; Ouedraogo et al., 2007). Circulating levels of adiponectin are decreased in patients with obesity and T2DM (Hotta et al., 2000; Engeli et al., 2003).

Moreover, downregulation of adiponectin receptors was reported in insulin target organs, such as skeletal muscle and liver in obesity and T2DM (Civitarese et al., 2004; Tsuchida et al., 2004). In addition, adiponectin is also a regulator of adhesion molecules on endothelial cells (Ouchi et al., 1999). Treating endothelial cells with adiponectin inhibited the expression of adhesion molecules such as VCAM-1, E-selectin, and ICAM-1 *in vitro* (Ouchi et al., 1999) and *in vivo* (Ouedraogo et al., 2007) suggesting that dysregulation of adiponectin may be relevant to obesity-linked endothelial dysfunction in these individuals (Cho et al., 2002; Katsiki et al., 2017).

Adiponectin circulates as either its globular domain or full-length homo-complexes with different molecular weights including: a trimer (low MW; LMW); a hexamer (medium MW; MMW) or an oligomer (high MW; HMW), (Kadowaki et al., 2006). .

As shown in Figure 1.4 three adiponectin receptors have been identified: adiponectin receptor 1 (AdipoR1), AdipoR2 and CDH13 (cadherin-13; T-cadherin), (Takeuchi et al., 2007). Unlike AdipoR1/R2 which are seven-transmembrane proteins with both intracellular and extracellular domains, CDH13 only has a large extracellular domain (Figure 1.3) which lacks a cytoplasmic domain (Ranscht and Dours-Zimmermann, 1991) AdipoR1 and AdipoR2 serve as receptors for globular and full-length adiponectin respectively (Takeuchi et al., 2007). However, CDH13 does only bindes to full-length HMW adiponectin with no affinity to globular or trimeric adiponectin (Kadowaki and Yamauchi, 2011) Binding to AdipoR1/2, adiponectin activates downstream signalling through several pathways, including AMPK, MAPK p38 and PPARα which all induce the adiponectin metabolic functions (Kadowaki and Yamauchi, 2011). However, among these pathways, adiponectin stimulated AMPK has been directly implicated in insulin-sensitizing function of adiponectin in liver and muscle (Kadowaki and Yamauchi, 2011)

Despite lacking intracellular domain, many studies have suggested that CDH13 directly participates in cellular signal cascades (Takeuchi et al., 2000; Huang et al., 2003) through binding to yet unidentified transmembrane receptor (Takeuchi et al., 2007) Indeed, CDH13 and adiponectin were shown colocalized in atherosclerotic lesions of human aorta, emphasizing on CDH13 as a major receptor for adiponectin in the vasculature (Ivanov et al., 2001). Both CDH13 and adiponectin are shown to protect vascular endothelial cells from apoptosis, which suggests CDH13 and adiponectin may have overlapping biological properties (Takeuchi et al., 2007). However, the functional relevance of CDH13 in mediating the effects of adiponectin is currently unclear. Moreover, potential signalling mechanisms downstream of adiponectin CDH13 interactions have not yet been fully explored.

1.6.4.7 PEPITEM

As demonstrated in previous section, there is growing evidence to support the anti-inflammatory capacity of adiponectin and its role in glucose homeostasis (Ruan and Dong, 2016; Katsiki et al., 2017). It is also known that adiponectin is able to regulate T cell trafficking during inflammation in a novel pathway characterised in our laboratory a few years ago (Chimen et al., 2015). As shown in Figure 1.5, during inflammation T cells need to migrate across endothelial layer to enter inflamed tissue. In fact, inflammatory cytokines, such as TNF-α and IFN-γ, activate the endothelial cells at the site of inflammation and cause lymphocyte capture from blood (Chimen et al., 2015). The chemokine signals induced by cytokines, stimulate the activation of the β_1- and β_2-integrin, as well as the surface expression of SIP receptors on T cells, which cause lymphocytes migration through endothelium via PGD_2 support (Chimen et al., 2015). However, in presence of adiponectin which is shown in the second half of the Figure 1. 5, adiponectin induces the release

of the novel immunopeptide (PEPtide Inhibitor of Trans-endothelial Migration; PEPITEM) by B cells via signaling through the adiponectin receptors (AdipoR1/2). PEPITEM induces the release of sphingosine-1-phosphate (S1P) by endothelial cells via binding with endothelial cadherin-15 (CDH15). In turn, S1P inhibits T cell transmigration by preventing integrin activation (Chimen et al., 2015). The Adiponectin-PEPITEM pathway is dysregulated in inflammatory conditions, such as T1DM, RA and in older adults. Dysregulation of this pathway is caused by a lack of AdipoR1/2 expression on B cells in these patient groups leading to insufficient secretion of PEPITEM and consequently allowing aberrant T cell trafficking resulting in chronic inflammatory responses (Chimen et al., 2015).

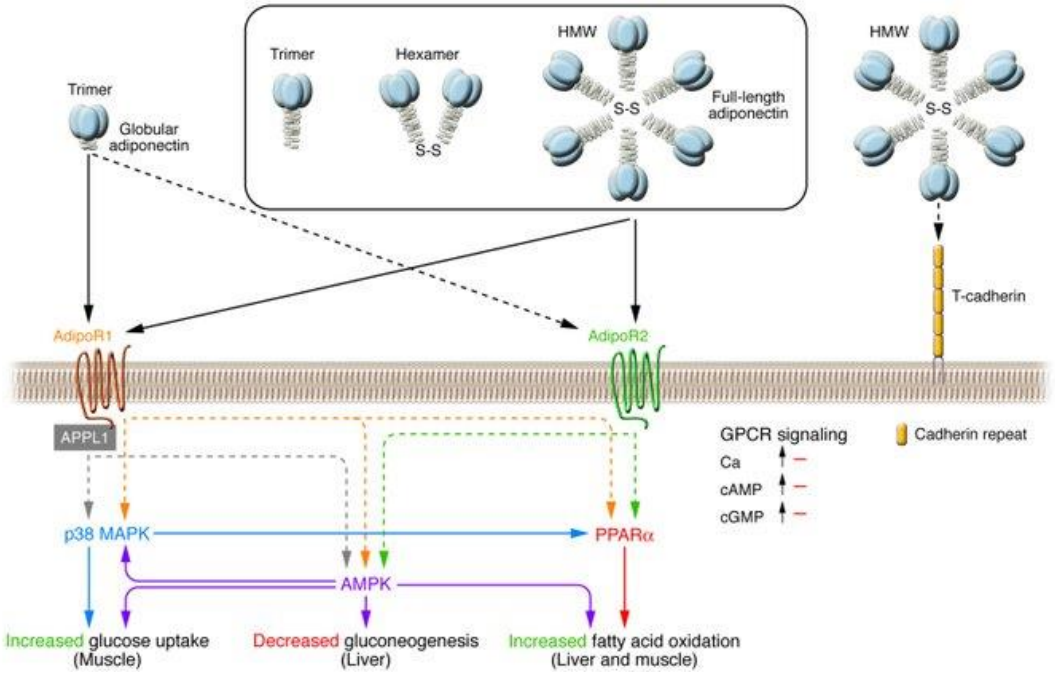


Figure 1.4 Signal transduction by adiponectin receptors Globular adiponectin which exists as a trimer has a higher affinity to AdipoR1 and lower affinity to AdipoR2 (shown by dots) whereas full-length adiponectin which exists as three multimers with different molecular weight (MW): (LMW trimer, MMW hexamer, and HMW multimer) binds to both AdipoR1/R2 with higher affinity to AdipoR2. Among adiponectin multimers CDH13 is only able to bind to HMW multimer. AdipoR1/R2 activate sets of downstream signaling molecules such as PPARα, AMPK, and p38 MAPK which induce adiponectin biological functions for glucose homeostasis in muscles and liver. The signal transduction of CDH13 is not clear. Taken from (Kadowaki et al., 2006)

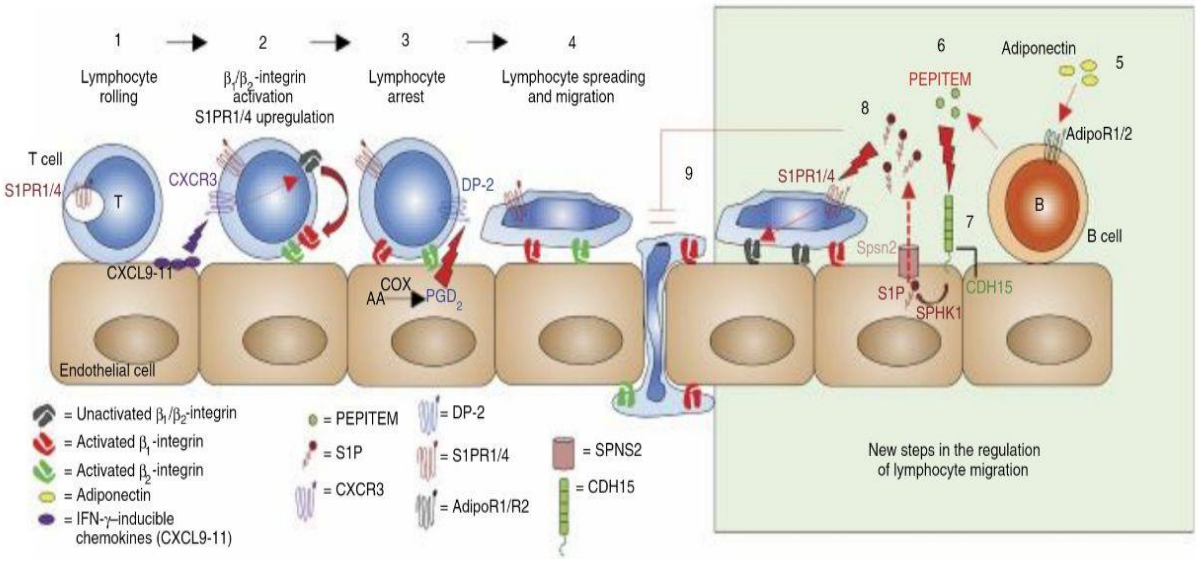


Figure 1.5 PEPITEM pathway

(1) Endothelial cells stimulated with pro-inflammatory cytokines such as TNF-α and IFN-γ recruit flowing T cells in the blood. (2) β1- and β2-integrins are activated by chemokine signals from CXCL9−11 presented on the endothelial surface, which interact with CXCR3 on T cells and lead to T cell arrest. (3) At the same time, chemokine stimulation induces S1PR1/4 surface expression on T cells. T cell spreading and migration is supported by PGD2, generated through the metabolism of arachidonic acid by cyclooxygenases, which operates through the PGD2 receptor, DP-2. (4) T cells spread and migrate across and through vascular endothelial cells. (5) Simultaneously, adherent B cells are simulated by circulating adiponectin through the adipoR1/2 receptors, (6) resulting in the release of PEPITEM from B cells. (7) PEPITEM binds to CDH15 on endothelial cells, stimulating the release of S1P from endothelial cells. (8) S1P binds to S1P receptors (S1P1/4) on recruited T cells, (9) which regulates the activation of the β2-integrin LFA-1 and inhibits trans-endothelial T cell migration. Taken from (Chimen et al., 2015).

1.7 Aims and Objectives

With the knowledge of reduced AdipoR1/R2 expression on B cells, reduced capacity of adiponectin to inhibit T cell trans-endothelial migration and impaired PEPITEM pathway in patients with chronic inflammatory disease compared to age-matched healthy controls, we aimed to investigate whether the PEPITEM pathway is also impaired in T2DM and mechanisms underlying this loss in both T2DM.

As a noted receptor to adiponectin, we also aimed to clarify the role of CDH13 in PEPITEM pathway.

- We hypothesise that CDH13 play a role in PEPITEM pathway.
- We hypothesise that B cells under hyperglycemic condition in T2DM are deficient for the AdipoRs and cannot produce PEPITEM and the homeostatic mechanism (PEPITEM) controlling T cell trafficking during inflammation, is suppressed in T2DM.
- We also hypothesise that inefficiency of homeostatic PEPITEM pathway affects pancreatic beta cells and insulin secretion and impaired PEPITEM pathway plays a role in obesity induced inflammation in T2DM.

Therefore, the project had the following aims:

1) To assess the role of CDH13 in anti-inflammatory effects of adiponectin *in vitro*.
2) To examine the effect of hyperglycemia, insulin and metformin on leukocyte-endothelium interaction *in vitro*.

3) To investigate PEPITEM effects on disease onset and glucose homeostasis in a mouse model of T2DM, and the impact this has on leukocyte trafficking into different vascular beds.

2 MATERIALS AND METHODS

2.1 Blood vascular Endothelial Cells (BEC)

2.1.1 Culture and passaging of BEC

Cryopreserved human dermal blood endothelial cells (PromoCell, Heidelberg, Germany, catalogue number #C-12225) were purchased at passage 2, thawed at 37°C and cultured in 25cm^2 flasks (T25, BD Falcon) using BEC growth media (PromoCell; catalogue number #C-222110) supplemented with Fetal Calf Serum (FCS; 5%), Endothelial Cell Growth Supplement (ECGS; 0.4%) and Epidermal Growth Factor (EGF; 10 ng/ml; all in one pack, PromoCell; catalogue number #C-39220). BEC were incubated at 37 °C and 5% CO_2 for 3 days to reach to 90-95% confluency and expanded through four passages.

2.1.2 Subculture

Briefly, the media was aspirated, and cells were washed with 0.5mM ethylenediaminetetraacetic acid, (EDTA; Sigma-Aldrich, catalogue number # E8008) for 30 seconds. EDTA was aspirated and cells were incubated with trypsin (Trypsin 0.05%-EDTA 0.53 mM; Gibco, Thermofisher, UK, catalogue number #25300054) for 2 minutes at room temperature. Phase-contrast microscopy (Zeiss Primovert, Carl Zeiss limited, Birmingham, UK) was used to visualise cells to ensure they had all detached. Trypsin was neutralised by the addition of medium 199 (M199, Gibco Life Technologies, catalogue number #31150-022) supplemented with 20% foetal bovine serum (FBS, Sigma-Aldrich; Paisley, UK, catalogue number #16000). The cell suspension was then centrifuged at 400g for 5 minutes, and the remaining cell pellet was re-suspended in 1ml BEC growth media. Cells were counted using cellometer Auto T4 (Nexcelom Bioscience Ltd, Manchester, UK) and were seeded into 2x T25 flasks containing 5ml BEC growth

media at 37°C and 5% CO_2. At 90% confluency, cells were passaged as described splitting 1 in 3 until passage 4 (P_4).

2.1.3 Cryopreservation

Passage 4 BEC at 90% confluency were trypsinised as described above, and the pellet re-suspended in 1.5mL cryo-SFM (PromoCell, catalogue number #29910) and split in 3x cryovials (~10^6 cells/vial), (Gem Scientific, Bradford, UK, catalogue number #123261). Cryovials were cooled to -80°C using a Mr. Frosty™ freezing container (Thermofisher, catalogue number # 51001-0001) filled with fresh isopropanaol (propan-2-ol) for 24 hours, before being stored in liquid nitrogen.

2.1.4 Seeding and treatment of BEC

Cryopreserved BEC vial was brought out of the liquid nitrogen, placed on dry and defrosted at 37°C in water bath. BEC were subcultured as in section 1.1.2 and re-suspended in $2x10^5$ cells/ml of BEC growth media and seeded into 12 well plates (BD Falcon; catalogue number #353043) at $2x10^5$ cells per well to ensure a confluent monolayer formed in 24 hours. BEC were incubated at 37 °C and 5% CO_2 for 24 hours prior to use.

To cytokine stimulate, BEC were treated with 100U/ml recombinant human TNF-α (R&D Systems, Oxford, UK, catalogue number #210-TA-010) and 10ng/ml IFN-γ (Peprotech, London,

UK, catalogue number #300-02) for 24 hours prior to the experiment to model inflammation (Chimen et al., 2015).

For HG treatment of BEC, D-glucose (Sigma-Aldrich, catalogue number, #G7021-1KG**)** was added to the BEC media to make a final concentration of 30mM/L (Crabtree et al., 2008; Yamagata et al., 2012). BEC were treated in HG media supplemented with or without 100U/ml TNF-α and 100U/ml IFN-γ for 24 hours prior to the experiment.

In some experiments, BEC were treated with metformin hydrochloride at 20, 200 and 2000 μM/L (Merck, Darmstadt, Germany, catalogue number #1115-70-4) for 24 hours during the cytokine treatment to assess the dose-dependent effects of metformin concentrations (Isoda et al., 2006). For combined treatment of BEC with HG and metformin, BEC were stimulated with TNF-α/IFN-γ in HG media for 24 hours, followed by 4 hours metformin treatment at therapeutically relevant concentrations (20 and 200 μM/L) in HG media supplemented with TNF-α/IFN-γ.

For insulin treatment, BEC were treated with insulin at 10^7M/l (Merck, catalogue number # 91077C-100MG**)** during cytokine treatment for 24 hours (Ding et al., 2000; Kuboki et al., 2000; De Nigris et al., 2015). For combined treatment of BEC with HG and insulin, BEC were stimulated with TNF-α/IFN-γ in HG media for 24 hours, followed by 24 hours insulin treatment at 10^7M/l in HG media supplemented with TNF-α/IFN-γ.

2.2 Collection and Preparation of Whole Blood

2.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Venous blood samples were collected from healthy individuals following written and informed consent, into EDTA coated tubes (BD Vacutainer; Greiner Bio-One, Stonehouse, UK;

catalogue number #455045) to prevent coagulation. PBMC were isolated from whole blood as previously described (Rainger et al. 1996). Briefly, whole blood was layered on a double density Histopaque gradient, Histopaque 1119 (Sigma-Aldrich, Poole, UK, catalogue number #11191) and Histopaque 1077 (Sigma-Aldrich, catalogue number, #10771) and centrifuged at 800g for 45 minutes at room temperature. The PBMC layer sediments lies just beneath the plasma, while the neutrophils sediment 2cm above the hematocrit (Munir and McGettrick, 2015). The PBMC layer and neutrophils were isolated separately and washed twice in phosphate buffered saline with calcium and magnesium (PBS+; Sigma-Aldrich, #14040141) at 400g for 6 minutes at room temperature. After the second wash, the PBMC or neutrophil pellet was re-suspended in 1ml M199 supplemented with 0.15% BSA (Bovine Albumin Fraction V, 7.5% solution; Sigma-Aldrich; catalogue number #15260037; M199-BSA) and cell counts were determined with the cellometer Auto T4. In some experiments leukocyte cones were used to supply the PBMCs due to COVID restrictions. Briefly, after collecting from blood centre, leukocyte cone content was transferred to a falcon 50 and diluted with PBS+ up to 50 ml and layered on a double density Histopaque gradient. Blood was processed as described above.

2.2.2 Monocyte isolation

Monocytes were isolated from PBMCs using EasySep monocyte Human Isolation Kit (StemCell Technologies, EasySep, Cambridge, UK, catalogue number, #19059) according to the manufacturer's protocol. A suspension of PBMC ($5x10^7$ cells/ml) were prepared in MACS buffer (PBS+, supplemented with 0.1mM EDTA and 0.6% bovine serum albumin) and added to 14ml polystyrene round-bottom tube (EasySep, catalogue number, #38008). Cells were incubated with

50µl/ml antibody cocktail (StemCell Technologies, EasySep, catalogue number #19359C) for 10 minutes at room temperature. Magnetic beads (StemCell Technologies, EasySep, catalogue number #19550) were added to the cells at 75µl/ml and left for 5 minutes at room temperature. Cells were topped up to 5ml of MACS buffer and placed into the magnet (The Big Easy, EasySep, catalogue number, #18001) and left for 5 minutes at room temperature. All other lymphocytes were adhered to the walls of the tube and the enriched monocyte suspension were sorted by negative selection into a new 15ml tube, washed at 400g, counted and resuspended (1×10^6 cells /ml) in M199-BSA prior to the experiment.

2.2.3 Peripheral Blood Lymphocytes (PBL) Isolation and Treatments

The PBMCs were resuspended in M199-BSA in a T25 flask and incubated at 37 °C for 45 minutes to plate the monocytes onto plastic. The surface of T25 flask was washed five times with a 6 ml aliquot of 37°C M199-BSA. The cell suspension containing PBL were then centrifuged at 400g for 5 minutes at room temperature. PBL were resuspended with 1 ml of M199-BSA, counted and resuspended at 1x 10^6 cell/ml. In some experiments, PBL were treated with 10µg/ml adiponectin (Enzo Life Sciences, Exeter, UK; catalogue number, #ALX-522-063-C050) or 20ng/ml PEPITEM (SVTEQGAELSNEER, Alta Bioscience, Birmingham, UK; catalogue number, #M3231A) (Chimen et al., 2015).

To treat PBL with adiponectin, 1×10^6 PBL were transferred to 1.5 ml Eppendorf (Sigma-Aldrich), centrifuged at 400g for 6 minutes at room temperature, aspirated and then resuspended in 100µl of M199-BSA supplemented with 10µg/ml of adiponectin and incubated for 1 hour on

shaker at room temperature. After 1 hour, 900µl of M199-BSA was added to the Eppendorf, centrifuged at 400g for 6 minutes, aspirated and resuspended in 1 ml M199-BSA.

To treat PBL with PEPITEM, 1×10^6 PBL were resuspended in M199-BSA supplemented with 20ng/ml PEPITEM immediately before the assay was performed (no incubation).

In some of the glucose experiments, PBL were isolated a day before the assay and cultured into the 12 well plate at $5x10^6$ cells/ml in M199-BSA for 24 hours in presence or absence of 30mM D-glucose (Crabtree et al., 2008; Yamagata et al., 2012), 20, 200 and 2000 µM metformin (Isoda et al., 2006) or 10^7M/L insulin (Ding et al., 2000; Kuboki et al., 2000; De Nigris et al., 2015). For combined treatment, PBL were isolated a day before the assay and cultured in M199-BSA with HG, followed by metformin treatment (20 and 200µM) for 4 hours or with insulin treatment at 10^7M/L for 1 hour prior to the experiments.

2.3 Static adhesion and migration assay

Static adhesion and migration assays were performed as previously described (McGettrick et al. 2009). Cytokine-stimulated BEC were twice washed with M199-BSA to remove residual cytokines and non-adhered cells. Treated or untreated PBL ($1x10^6$ cells/well) were immediately added to BEC and allowed to adhere and migrate for 6 min 30 seconds at 37 °C on BEC. Immediately after, BEC were twice washed with M199-BSA to remove non-adherent PBL. Cells were fixed with 2% glutaraldehyde (Sigma-Aldrich, catalogue number, #G5882) diluted in PBS+. Digitised images of 10 random fields were acquired using a phase-contrast microscopy (Olympus IX71; Southend-on-Sea, UK) and analysed using Image-Pro Plus software (version 6.0, Media Cybernetics). Phase bright round lymphocytes on the surface of the BEC monolayer were

classified as adherent. Phase dark lymphocytes were classified as transmigrated, and typically appeared to be spread out under the endothelial cell monolayer (Figure 2.1) The numbers of adherent and transmigrated cells were counted in 10 images per well, averaged and converted to cells per mm2 and multiplied by the known surface area of the endothelial cell monolayer to calculate the total number adherent. The percentage of adherent lymphocytes that had transmigrated was also calculated.

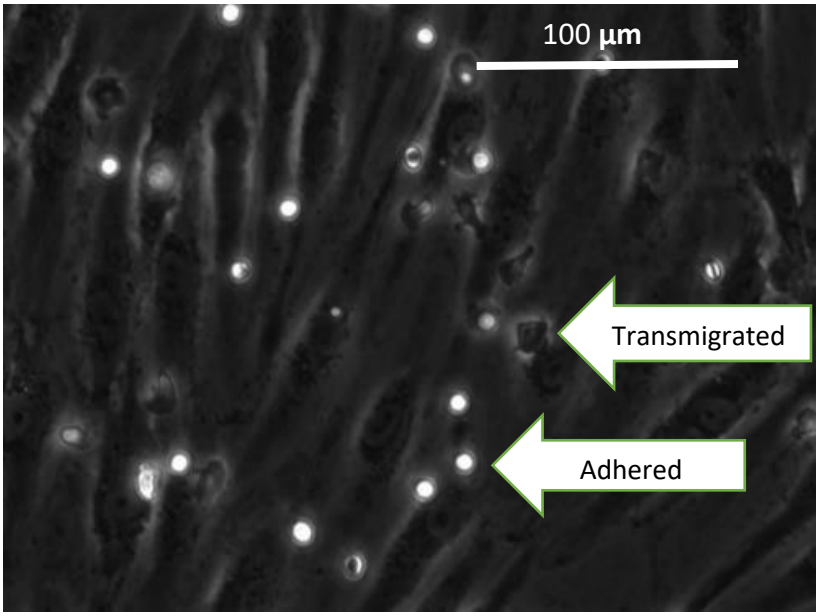


Figure 2.1 Representative image of the static adhesion and migration assay. Representative micrograph image of PBL adhesion and migration through inflamed endothelium observed using phase contrast microscopy. Firmly adherent phase bright PBL remain circular and are on top of the endothelium. The transmigrated phase dark PBL appear flattened and irregular shaped underneath the endothelial cell layer. Scale bar = 100 µm.

2.4 RNA Extraction

Total mRNA was extracted from cells using the RNasy Minikit (Qiagen, Manchester, UK; catalogue number #74104) according to the manufacturer's guidance. Briefly, BEC were lysed by adding 350µl RLT buffer directly to the wells and collected in a 1.5mL Eppendorf tube. Ethanol (70%, in distilled water) was added to the suspension and the sample was vortexed for 60 seconds to disrupt cell membranes. The sample was transferred to a spin column for centrifugation at 8000g. The supernatant was discarded and RW1 buffer was added to the collection tube (to efficiently remove biomolecules such as carbohydrates, proteins, fatty acids etc…) before being centrifuged for 30 seconds at 8000g. The supernatant was discarded and RPE buffer was added to the collection tube to remove traces of salts before centrifugation for 30 seconds at 8000g. The supernatant was discarded and RPE buffer was added to the collection tube before centrifugation for 2 minutes at 8000g. The spin column was transfered into a new 2ml collection tube and dried by centrifugation for 2 min at 10000g. mRNA was eluted from the spin column by adding 32µL RNase-free dH_2O directly to the filter and centrifuged at 8000g for 1 min. The RNase-free dH_2O flow-through was collected and re-added to the spin column for another centrifugation to increase RNA yield. RNA concentration was measured using Nanodrop spectrofluorometer (LabTech). The acceptable average of A230/260 and A260/280 ratios for RNA were 2.0-2.2. Purified RNA was stored at -80°C for <72 hours before being converted to cDNA.

2.5 mRNA to cDNA conversion

Using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK; catalogue number, #4368814) the 1µg of extracted RNA (from section 2.4) was convert to cDNA following the user guide. A mixture volume of 20µl containing: 3.2µl RNase free H_20, 2µl

RT buffer, 0.8µl dNTP mix (100mM), 2µl RT random primers, 1µl RNaseOUT, 1µl reverse transcriptase and 1µg of total RNA placed in a thermocycler at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, with a final hold at 4°C. cDNA was kept at -20°C before qPCR use.

2.6 Real-time Polymerase Chain Reaction

To replicate all the cDNA (from section 2.5) TaqMan multiplex master mix (Applied Biosciences; catalogue number #4461881) was applied and the gene of interest was identified using TaqMan probes (Thermofisher, catalogue number #4331182). All probes were labelled with FAM, except for the human housekeeping: GAPDH, 18s and HPRT which were labelled with VIC.

Mixture of: 5.5µl of Taqman multiplex master mix, 3.9µl of RNase free H_20, 0.6µl of TaqMan probes, and 0.5µl of cDNA was used for each reaction. PCR was conducted using the 7900HT Fast Real-Time PCR System with 384-Well Block Module (Applied Biosystems, catalogue number #4329001). qPCR employed 40 cycles of: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute, with the final holding at 4°C. Each sample was run in technical duplicates and the mean cycle threshold (Ct) of the duplicates was calculated. Only values with standard deviation (SD) <1 were used for further analysis. To compare gene expression, the cycle threshold (Ct) was recorded and compared to the house keeping control to work out ΔCt (ΔCt = Ct target gene – Ct reference gene). Results are expressed as relative expression to the endogenous control ($2^{-\Delta Ct}$), (Rieu and Powers, 2009). Three housekeeping genes were screened using unstimulated and TNF-α/IFN-γ treated BEC (Table 2.2) and the one with the least variation in the mean cycle threshold (Ct) of the duplicates (GAPDH) was chosen to be used in the experiments (Table 2.2)**.**

Table 2.1 TaqMan gene expression assays used for real-time qPCR

Primers	Primer Sequence	Supplier
AdipoR1	Hs00360422_m1	Thermofisher
AdipoR2	Hs00226105_m1	Thermofisher
CDH13	HS01004531-ml	Thermofisher
ICAM 1	Hs00164932_m1	Thermofisher
VCAM1	Hs01003372_m1	Thermofisher
E selectin	Hs00174057_m1	Thermofisher
HPRT	Hs02800695_m1	Thermofisher
GAPDH	Hs02786624_g1	Thermofisher
18s	Hs03003631_g1	Thermofisher

Table 2.2 Screening of housekeeping genes

	18S			GAPDH			HPRT		
	Ct1	Ct2	SD	Ct1	Ct2	SD	Ct1	Ct2	SD
1	7.39534	7.475779	0.056878	17.9573	18.15742	0.141504	23.553	23.41926	0.09457
2	8.375191	8.286764	0.062527	18.92881	18.58469	0.243332	24.33584	24.26452	0.050431
3	10.54198	9.87243	0.473442	19.3426	19.2474	0.067312	25.14752	25.15101	0.002469
4	7.363573	7.328754	0.024621	18.57716	18.57806	0.000641	24.16162	24.09489	0.047187
5	11.85918	11.69684	0.114793	18.62675	18.51483	0.079142	24.67466	24.74695	0.051118
6	11.98841	11.9651	0.016488	18.09298	18.19844	0.074576	23.95423	23.98115	0.019035
7	10.5744	10.49496	0.056175	18.50626	17.85165	0.462885	24.40052	24.72341	0.228318
8	8.296925	8.357039	0.042507	18.66192	18.7611	0.070134	24.48141	24.64232	0.113781
9	7.640275	7.668687	0.020091	18.77945	18.0606	0.508307	23.65369	23.79125	0.097272
10	6.549867	6.423791	0.08915	18.25302	18.24798	0.003563	24.30474	24.37836	0.052053
11	9.153357	9.210382	0.040322	18.05619	18.23701	0.127857	23.97283	23.6052	0.259956
12	12.35238	12.28226	0.049585	18.69194	18.60107	0.064261	24.67972	24.64138	0.027112

Housekeeping genes: 18s, GAPDH and HPRT. Ct: cycle threshold, SD: Standard Deviation

1-6 untreated BEC samples, 7-12 cytokine treated BEC samples.

2.7 siRNA transfection

BEC were plated at 150,000 cell/well in a 12 well plate for 24 hours. The AdipoR1 (Catalogue number # GS51094), AdipoR2 (catalogue number # GS79602), CDH13 (catalogue number # GS1012) and control siRNA (catalogue number # 1027310) (all from Qiagen) FlexiTube siRNAs (Table 2. 3) were added at a final concentration of 50nM to 83.75µl of Optimem media (Gibco Life Technologies, catalogue number # 51985). 1.5µl of RNAi Lipofectamine (Invitrogen, catalogue number #13778) was mixed with 13.5µl of Optimem and these were incubated 10 minutes at room temperature. 15µl of Lipofectamine mix was added to each siRNA mixture, gently mixed, and incubated for a further 10 minutes. BEC were washed twice with PBS and 400µl of Optimem was added to the Lipofectamine siRNA duplexes. After gentle agitation, the mix was added on the BEC and incubated for 4 hours at 37 °C and 5% CO_2 (Chimen et al., 2015). After aspirating the BEC media, siRNA-lipofectamine complex media was added to the respective wells and cells were incubated. Subsequently, BEC were washed twice with PBS before BEC growth media was added to the wells and cells incubated at 37 °C and 5% CO_2 for 48 hours before use. After 48 hours BEC were stimulated with TNF-α and IFN-γ for 24 hours before being analysed by real time PCR, flow cytometry or incorporation into a transmigration assay.

2.8 Protein Expression

2.8.1 Flow cytometry

2.8.1.1 Measuring AdipoRs expression on PBMC subsets.

Flow cytometry was used to measure the AdipoRs surface expression in PBMC populations. PBMCs were transferred to FACs tubes (BD Falcon, catalogue number #352063) at 0.5 x 10^6 cells/tube. FC receptors were blocked in all tubes using FcR Blocking agent (Miltenyi

Biotec, Germany, catalogue number #130-091-765) at a 1:50 dilution (Chimen et al., 2015). PBMCs were incubated with Rabbit anti-human adipoR1 (Pheonix Pharmaceuticals, Karlsruhe, Germany, catalogue number #G-001-44) or anti-AdipoR2 (Pheonix Pharmaceuticals, catalogue number #G-001-23) antibodies at 4°C at 1/100 dilution for 20 minutes(Chimen et al., 2015), then washed with PBS at 400g for 5 minutes at room temperature. Isotype tubes received no AdipoR antibodies. PBMCs were stained with goat-anti rabbit Alexa Fluor 488 secondary antibody (FITC; Invitrogen, catalogue number #A11008) at 1/250 (Chimen et al., 2015) and a panel of antibodies (Table 2.4) for 30 min at 4 °C. Single stains and secondary only were included as controls for compensation and non-specific antibody staining. Zombie Aqua Fixable Viability Kit was used to test the viability of cells. PBMCs were washed with PBS at 400g for 5 minutes at room temperature and fixed with 400 µl/tube of 2% formaldehyde (TAAB Laboratories Equipment Ltd; catalogue number #F003) diluted in PBS+ for 20 min at 4 °C. Then the cells were washed with PBS+ at 400g for 5 minutes at room temperature and re-suspended in 300 µl PBS+, before being kept in the dark at 4^{o}C until ready for analysis. Flow cytometry was performed using a Cyan flow cytometer (Beckmann Coulter, USA) with Summit software and then analyzed using FlowJo software (TreeStar, Ashland, OR). PBMC population was identified using forward vs. side scatter (FSC vs SSC), (Figure 2.2-A). Single cells were identified by using FSC vs Pulse Width (Figure 2.2-B). Viable cells were identified using a Zombie Aqua Viability kit (Figure 2.2-C). B cells and T cells were identified by using CD19 vs CD3 (Figure 2.2-D). Monocytes were identified using CD14 vs CD3 (Figure 2.2-E). NKs, NK cells and NK T-cells were identified using CD56 vs CD3(Figure 2.2-F). $CD8^{+}$ and CD4T-cells were gated on T cells using CD8 vs CD4 (Figure 2.2-G). The percentage of PBMC subset of healthy donors is shown in Figure 2.3. AdipoR1 and AdipoR2 expression on PBMCs was based on the secondary only.

Table 2. 3 Sequences of siRNA pool used for BEC

	ON-TARGETplus SMART pool (Qiagen)			
Target	**siRNA1**	**siRNA2**	**siRNA3**	**siRNA4**
AdipoR1	**GACAAGAGCAGGCGUGUUC**	**GGCUAAAGGACAACGACUA**	**CCUUUAUGCUGCUCGAAUU**	**GAGAAGGGCAAACGGGUAA**
AdipoR2	**GGAGUGAGGUACAAGACGA**	**GAGAAGAACUUGAACGUUU**	**CAGACUUGCUCUUCGGUCA**	**GAAGGUCGGUGGCGAGUGA**
CDH13	**GGAAAGGUGUGUUUGCAGU**	**GAAAUCUCCUUCACGCUGA**	**GAUGGGGAAUUGAUGGUUA**	**CGACGAGGACUUUGUGCUA**

Table 2.4 Human antibodies used for adiponectin receptors on PBMC subsets

Antibody	Fluorophore	Concentration	Clone	Catalogue number	Supplier
CD56 Mouse / IgG1, kappa	PE	1:100	CMSSB	12-0567-42	eBiosciences (Paisley, UK)
CD3 Mouse / IgG2a, kappa	PerCpCy5.5	1:100	OKT3	45-0037-42	Invitrogen
CD19 Mouse / IgG1, kappa	PECy7	1:50	HIB19	25-0199-42	Invitrogen
CD8 Mouse / IgG2a, kappa	PB	1:50	OKT8	48-0086-42	eBiosciences
CD14 Mouse / IgG1, kappa	APC	1:50	61D3	17-0149-42	Invitrogen
CD4 Mouse / IgG1, kappa	APCCy7	1:50	RPA-T4	56-0049-42	Invitrogen
Goat serum		1:500	N/A	A-11008	Invitrogen
Zombie Aqua dye	BV510	1:100		77143	BioLegend (Highgate, UK)
CD4 FITC Mouse / IgG2b, kappa	FITC	1:50	OKT4	11-0048-42	eBiosciences

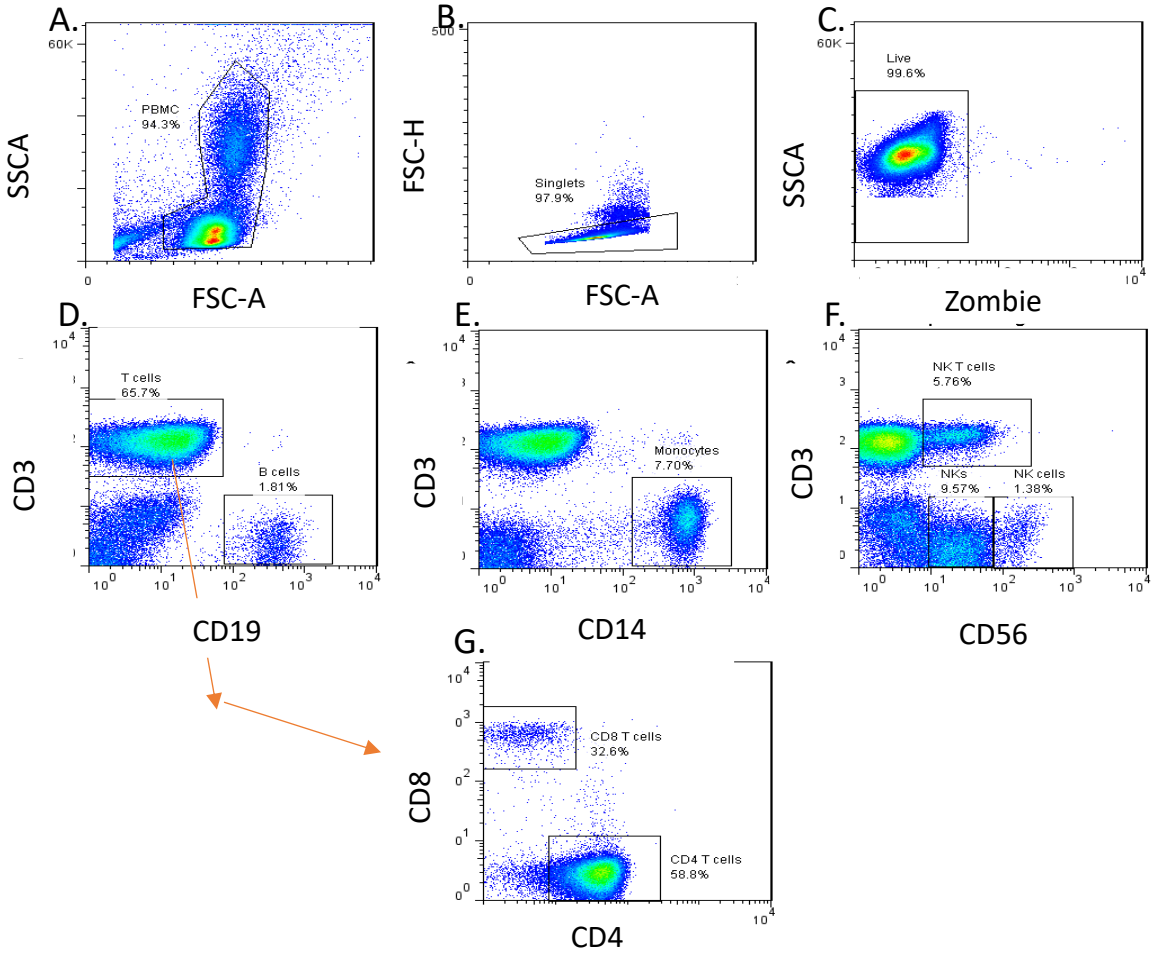


Figure 2.2 Gating strategy to identify PBMC subsets.
(A) PBMC population was identified using forward vs. side scatter (FSC vs SSC). **(B)** Single cells were identified by using FSC vs Pulse Width. **(C)** Viable cells were identified using a Zombie Aqua Viability kit. **(D)** T cells ($CD3^+$) and B-cells ($CD19^+$) were identified by using CD3 and CD19 respectively. **(E)** Monocytes ($CD14^+$) were identified using CD3-CD14 gating. **(F)** NKs, NK cells and NK T-cells were identified using CD3 vs CD56. **(G)** $CD8^+$ and $CD4^+$ T cells were gated on T cells identified by CD8 or CD4, respectively.

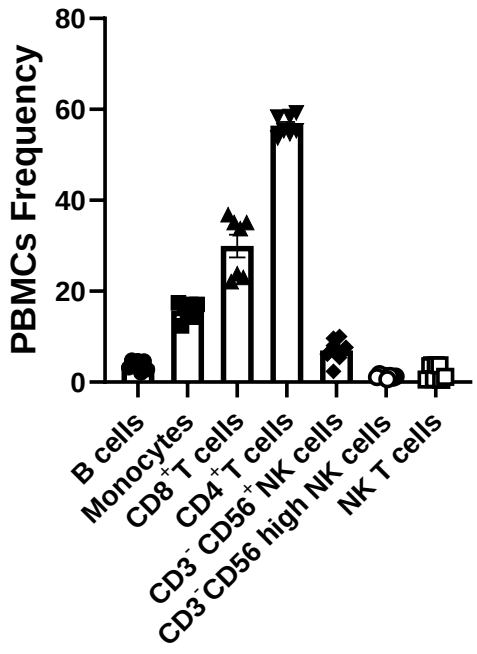


Figure 2.3 Frequency of PBMC subsets in healthy donors. PBMCs were isolated from whole blood and stained with antibodies against $CD19^+$, $CD14^+$, $CD8^+$, CD4+ and $CD56^+$ to distinguish B cells, monocytes, CD8+ T cells, CD4+ T cells, NK cells and NK T cells using flow cytometry and quantified as frequency of PBMC subsets. Data are shown as mean±SEM, n=7 independent experiments using 7 different blood donors.

2.8.1.2 Measuring total expression of CDH13 in BEC

BEC were cultured in 24 well plates at 0.1 x 10_6 cell/well. BEC were stimulated with TNF-α/IFN-γ for 24 hours prior to staining. In some experiments BEC were treated with 10μg adiponectin for 24 hours. To measure the total expression of CDH13, cultured BEC were washed twice with warmed PBS+ before being treated with 200μl accutase (Sigma-Aldrich, catalogue number #A6964) to detach the cells. Cells were washed in 800 μl PBS+ and transferred to individual FACs tubes. Tubes were filled with PBS+ and centrifuged at 400g for 6 minutes at room temperature. After discarding the supernatant, the eBioscience intracellular fixation and permeabilisation kit (Ebioscience, catalogue number #88-8824-00) was used according to manufacturer's instructions. Briefly, BEC were left in fixation buffer for 60 minutes room temperature, then washed once with PBS+ and twice with permeabilisation buffer at 400g 4 °C. Primary rabbit anti-human CDH13 antibody (Abcam, Waltham, US, clone: 39241, catalogue number #ab36905) was added at 1/200 (Wang et al., 2014) to the fixed and permeabilised cells and incubated for 20 minutes at 4 °C in dark. BEC were washed once with PBS+ and twice with permeabilisation buffer at 400g 4 °C. An Alexa Flour 647 conjugated goat anti- rabbit antibody (Jakson ImmunoResearch, Cambridge, UK catalogue number 111-605-008) was incubated at 1/300 (Wang et al., 2014) with the BEC for 20 minutes in dark at 4 °C. BEC were washed with PBS at 400g and resuspended with 300 μl of PBS and stored at 4 °C until the day of analysing.

2.8.1.3 Measuring surface expression of CDH13 on BEC

BEC were cultured and treated as described in section 1.3.1.2. To measure the surface expression of CDH13, cultured BEC were washed twice with PBS+. Primary rabbit anti-human CDH13 antibody was applied to the BEC at 1/200 for 20 minutes at 4 °C in dark. Isotype well received no primary antibody. BEC were washed twice with PBS+ for 5 minutes at 4°C. Alexa

Fluor 647 conjugated goat anti- rabbit antibody was incubated at 1/300 with the BEC for 20 min in dark at 4 °C. BEC were washed twice with PBS+ for 5 minutes at 4°C, treated with accutase as described above and transferred to FACs tubes. The cells were then washed with PBS+ at 400g for 6 minutes. After discarding the supernatant, the cells were fixed with 400 µl/tube of 2% formaldehyde diluted in PBS+ and re-suspended in 300 µl PBS+ and kept in the dark at 4ºC until ready for analysis. Flow cytometry was performed using Cyan flow cytometer. BEC population were identified using forward vs. side scatter (FSC vs SSC), for total (Figure 2.4-A) and surface staining (Figure 2.4-B), single cells were identified by using FSC vs Pulse Width (Figure 2.4-C). CDH13 expression on BEC was based on secondary only.

2.8.2 Immunofluorescence staining for adiponectin and CDH13

Human BEC, were cultured 50 000 per slide in µ-ibidi chamber slides (vThistle Scientific, Glasgow, UK, catalogue number # 80826-G500). BEC were stimulated with TNF-α/IFN-γ for 24 hours prior to staining. In some experiments BEC were treated with adiponectin (10 µg/ml) for 24 hours. Cultured BEC were incubated and fixed with 2% formaldehyde diluted in PBS+ in ibidi slides for 10 min at 37°C. BEC were washed two times with PBS+ for 5 minutes following fixation and stored in fridge 4°C until ready to carry out immunofluorescence protocol. For Immunofluorescence staining of adiponectin and CDH13, BEC were incubated with 0.1% Triton X100 (Sigma, catalogue number # 11332481001) diluted in PBS for 30 minutes, then washed twice with PBS+ for 5 minutes and blocked with 10% FBS diluted in PBS+ for 30 minutes at room temperature in the dark. Blocking solution was aspirated and primary rabbit anti-human CDH13 (Abcam, clone: 39241, catalogue number #ab36905) and mouse anti-human adiponectin (Abcam, clone:19F1, catalogue number # ab22554) antibodies were applied at 1/200 and incubated with

BEC for 24 hours at 4°C. Antibodies were prepared in 10% FBS diluted in PBS. Following day BEC were washed twice with PBS+ for 5 minutes prior adding the secondary antibodies for 1 hour at 4°C in dark. Secondary antibody for adiponectin was an Alexa Flour 488 goat anti-mouse antibody at 1/300 (Jakson ImmunoResearch, catalogue number # 115-545-008). Secondary antibody for CDH13 was Alexa Flour 647 goat anti- rabbit antibody at 1/300. Subsequently the slides were washed twice 5 minutes with PBS+. Vectamount (Vector Laboratories, Peterborough, UK, catalogue number # H-1200-10) containing 4′,6-diamidino-2-phenylindole (DAPI) was used (1 drop/slide) to stain the nuclear. The slides were then visualized using a Zeiss LSM780 inverted laser scanning confocal microscope (Zeiss, Gottingen, Germany).

2.8.3 Confocal microscopy

Images were captured using a 63x water-immersion objective with excitation at 488nm and 580nm. The laser power and acquisition parameters and were kept constant for every experiment. At least 5 digital images were taken for each slide. Three separate channels were used to avoid overlap in emission detection from each fluorochrome. Images were processed on ZEN (Black Edition, version 2.2) software and were stored as digital arrays. Digital arrays were quantified using ImageJ 1.53k. The average fluorescence of CDH13 and adiponectin was calculated by measuring the field intensity of the red and green channels divided by the number of cells within the field. The background fluorescence, for each experiment, was calculated by measuring the field intensity of the red and green channels from digital arrays of control cells (secondary only). This value was subtracted from the field intensity of the red and green channels from treated cells. The blue channel was used to count the number of cells in each digital array. At least 4 arrays for each culture condition were analysed to calculate the average fluorescence of CDH13 and adiponectin.

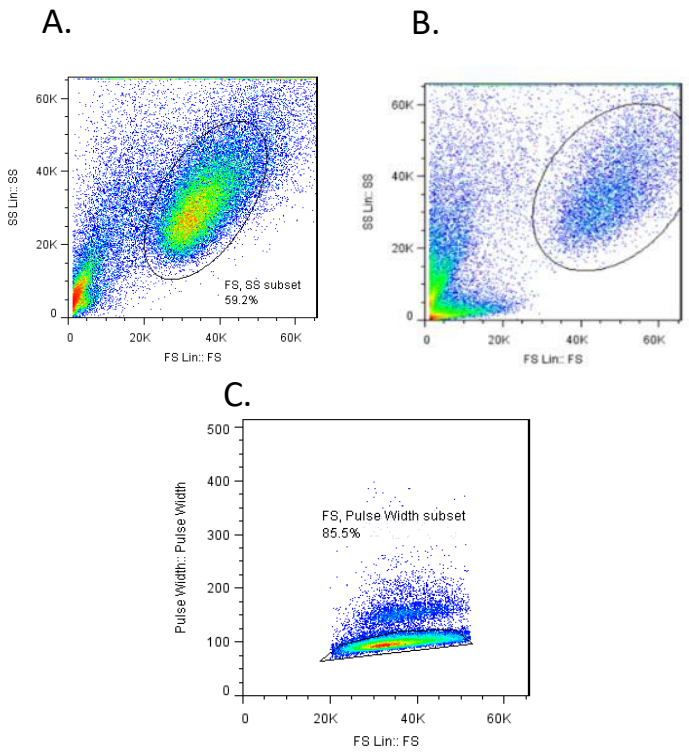


Figure 2.4 Representative plots of gating strategy for identifying BEC.
BECs were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ for 24 hours. BEC population were identified using forward vs. side scatter (FSC vs SSC), for **(A)** total and **(B)** surface staining, **(C)** single cells were identified by using FSC vs Pulse Width.

2.9 Animal experiments

2.9.1 Mice

C57BL/6 wt mice were purchased from Charles River and maintained under controlled conditions in a 12-hour light/12-hour dark cycle. Mice received food/water ad libitum as stated by the UK Home Office Regulations.

2.9.2 Mini-pump implantation and induction of glucose intolerance

2.9.2.1 Mice on High Fat Diet (HFD)

Minipumps (Charles River Ltd, Kent, UK, Alzet pumps 2006) were loaded with PEPITEM (3.5mg/ml) or PBS and implanted subcutaneously in 6-weeks old male C57Bl/6 wt mice. Briefly, mice were anaesthetised, and the skin was shaved and cut using surgical scissors over mini pump implantation site to create a subcutaneous pocket under the back skin of the mice. Osmotic minipumps were placed either at day 0 or 6 weeks into the feeding protocol. The skin wound was cleaned and closed using two surgical clips. Mice were monitored in cages and allowed to recover for 1 week before their normal chow diet was changed to high fat diet containing 60% fat (Research Diets INC, Newb Brunswick, USA catalogue number # D12492) for 6 or 12 weeks. Body weight was checked at the start and once per week until the end of the experimental period. For intraperitoneal glucose tolerance tests (IPGTT) at 3 and 6 weeks of HFD for the 6-week experiment, or 8 and 12 weeks of HFD for the 12-week experiment, the mice were fasted for 4-6 hours prior to intraperitoneal injection of glucose (2 g/kg body weight). Blood samples were taken from the tail vein at 0, 15, 30, 60, 90 and 120 min after glucose injection. Glucose was measured using a Contour XT glucometer (Bayer, Germany) and expressed as mmol/l.

2.9.3 Identification of leukocyte subsets in murine tissues

After 6 or 12 weeks on HFD, mice were scarified via cardiac puncture under terminal anesthesia and tissues were collected. Blood was collected into Eppendorf's coated with EDTA anti-coagulant (Sarstedt Ltd, Leicester, UK, catalogue number # 41.1395.005) and gently inverted. The peritoneum was lavaged with 5ml of 5mM EDTA (Sigma, catalogue number # #03690) in PBS and collected in falcon tubes, the spleen and inguinal LNs were collected in bijoux tubes containing PBS. Both visceral (gonadal fat pads) and subcutaneous fat (abdominal) tissue was collected in RPMI (Gibco™ RPMI 1640 Medium, catalogue number # 11530586) +2% BSA.

2.9.4 Processing of blood samples

To stain for leukocyte subsets, 3ml of red blood cell lysis buffer was added per 300 µl of blood and incubated at room temperature for 10 minutes, washed twice with 15ml PBS+ at 400g at room temperature. Pellet was resuspended in 500 µl MACS buffer and transferred into propylene tube (Falcon, catalogue number #352052) to be stained for leukocytes subsets.

2.9.5 Processing of spleen and lymph nodes (LNs)

After weighing, the spleen and LN were crushed on a 40µM filter and lysed with 3ml formulated RBC lysis buffer for 10 minutes at room temperature. After washing twice with PBS for 5 minutes at 400g, room temperature, the pellet was resuspended processed in the same way as we processed blood.

2.9.6 Processing of adipose tissue

After weighing the tissue, an enzyme cocktail was prepared per 0.8 g of fat tissue as described in Table 2.5. After cutting the tissue in tiny pieces, tissue was transferred into the enzyme cocktail and placed in a water bath at 37°C for 30 min. Using a 70μM filter, tissue was filtered into fresh 5ml RPMI+ 2% FBS and washed at 400g for 10 minutes. The oily floating adipocytes were aspirated, and the stroma vascular fraction (SVF) was resuspended in 3ml red blood cell lysis buffer for 10 minutes at room temperature, washed and resuspend in 500μl of RPMI+ 2% FBS and added to propylene tubes for staining.

2.9.7 Processing of Peritoneal Lavage

Peritoneal cells were centrifuged at 400g for 5 minutes at room temperature, supernatant was collected and stored at -80°C for future analysis, and cells were resuspended in 1ml MACS buffer. Peritoneal cells were centrifuged at 400g for 5 minutes and resuspended in 500μl MACS buffer. 100μl were added to each propylene tube.

2.9.8 Staining leukocyte subsets for flow cytometry

After adding the cells to propylene tube all tissues were stained for (Figure 2.5) T-cell and T-cell isotype, (Figure 2.6) B-cell and B-cell isotype and (Figure 2.7) macrophage panel. After adding 2μl mouse FcR blocker per tube cells were incubated with the subset markers (Table 2.6) for 20 minutes at 4°C. Single stains were included only for spleen (Table 2.6) and used for

compensation of all other tissues. Cells were then washed with PBS and fixed with 2% formaldehyde diluted in PBS+ for 10 min at 4°C, washed and resuspended in 300µl PBS for flow cytometry analysis. 20µl/tube CountBright (Invitrogen, cat Catalogue number #36950) beads were added to all Samples before acquisition on the Fortessa-X20 (BD Bioscience). Samples analysed using FlowJo (V-10.2.6) software. Figure 2.5-8 represent the gating strategy used to identify the leukocytes subsets.

Leukocyte populations were identified, using forward/side scatter and single cells were gated using FSC-A vs FSC-H. Viable cells were gated using zombie aqua in all panels which is only shown in Figure 2.5-A, B and C. $CD45^+$ cells were gated on live cells using CD45 antibody (Figure 2.5-D, Figure 2.6-A, Figure 2.7-A).

T cells and B cells were gated on $CD45^+$ cells using CD3 and CD19 antibodies (Figure 2.5-E and Figure 2.6-B). T-cell subsets identified gating on T-cells using CD4 and CD8 antibodies (Figure 2.5-F). Naive, memory (CM) and effector memory cells (EM) were gated on both $CD4^+$(Figure 2.5-G) and $CD8^+$ (Figure 2.5-H) populations using CD62L vs CD44. Tregs were identified by gating on CD4+ T-cells using CD4 vs CD25(Figure 2.5-I). Senescent T-cells gated on T-cells using KLRG1 antibody (Figure 2.5-J) based on isotype control (Figure 2.5-K).

In B-cell panel, age associated B-cells were gated on $CD93^-$ $CD43^-$ cells (Figure 2.6-D) using isotype control (Figure 2.6-F).

In macrophage panel, eosinophils were gated on $CD45^+$ cells using F4/80 vs SIGLEC F (Figure 2.7-B), macrophage/monocyte and DCs were identified using F4/80 vs CD11C (Figure 2.7-C), neutrophils were gated on leukocytes using SSC-A vs LY6G (Figure 2.7-D).

Table 2.5. Enzyme cocktail concentration and final volume per 0.8 g of adipose tissue.

Enzyme	**dilution**	**Final concentration**	**Volume to add per sample**
Collagenase P (Sigma-Aldrich, catalogue number # 11213857001)	1:500	200 ug/ml	10 µl
DNase (Merck, catalogue number #04716728001)	1:100	100 ug/ml	50 µl
Collagenase Dispase (Merck, catalogue number # 10269638001**)**	1:125	800 ug/ml	40 µl
RPMI 2% FBS			5 ml

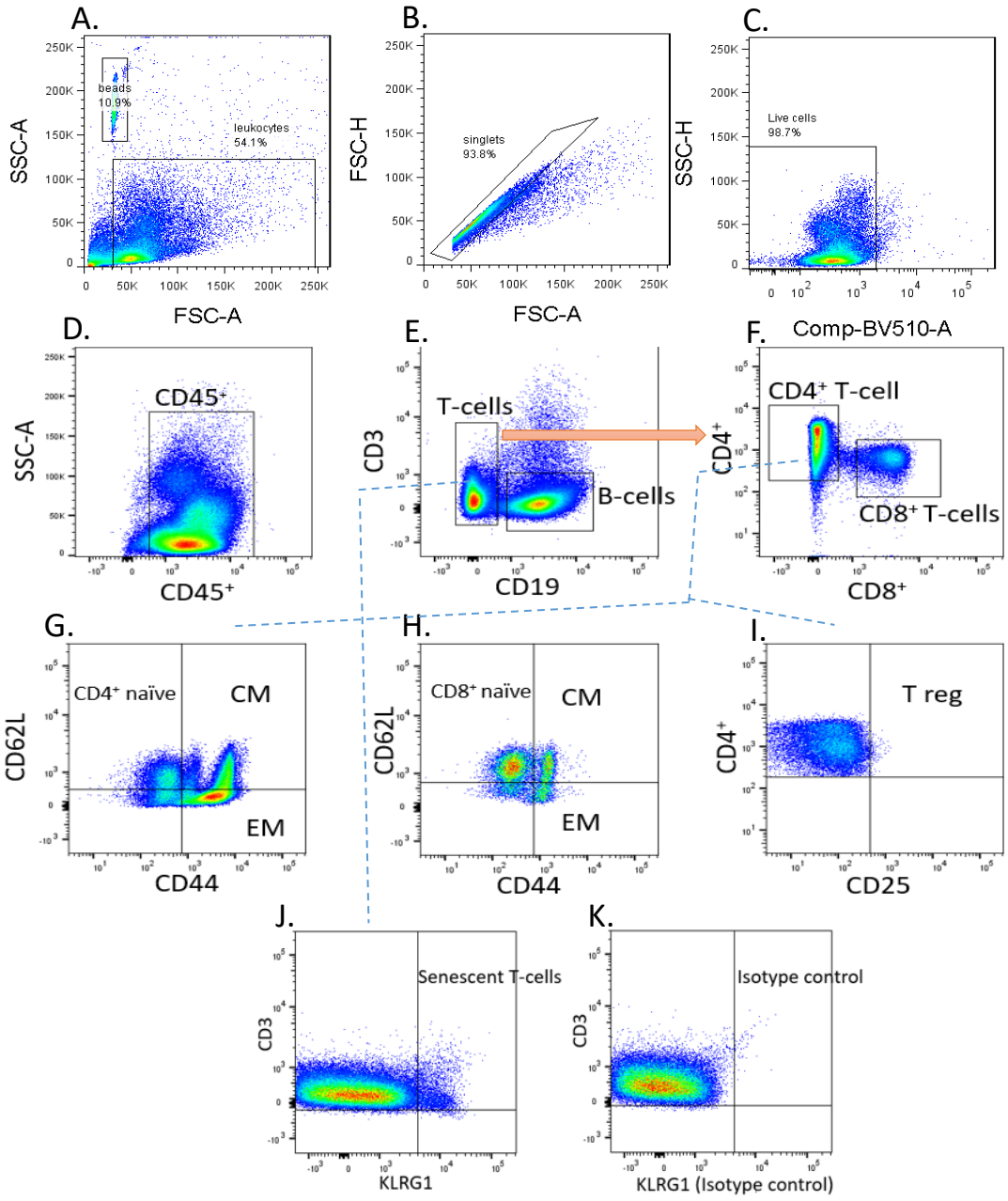


Figure 2.5 Gating strategy used to identify the T-cell subsets in spleen. (A) Leukocyte populations were identified using FSC-A vs SSC-A, **(B)** single cells were identified using FSC-A vs FSC-H. **(C)**Viable cells were gated using BV510-A vs SSC-A and **(D)** $CD45^+$ cells gated on live cells using CD45 antibody. **(E)** T-cells were gated using CD3 vs CD19 antibodies. **(F)** T-cell subsets identified gating on T-cells using CD4 and CD8 antibodies. Naive, central memory (CM) and effector memory cells (EM) were gated on both **(G)** $CD4^+$ and **(H)** $CD8^+$ populations using CD62L vs CD44. **(I)** Tregs were identified by gating on CD4+ T-cells using CD4 vs CD25. **(J)** Senescent T-cells gated on T-cells using KLRG1 antibody based on **(K)** isotype control.

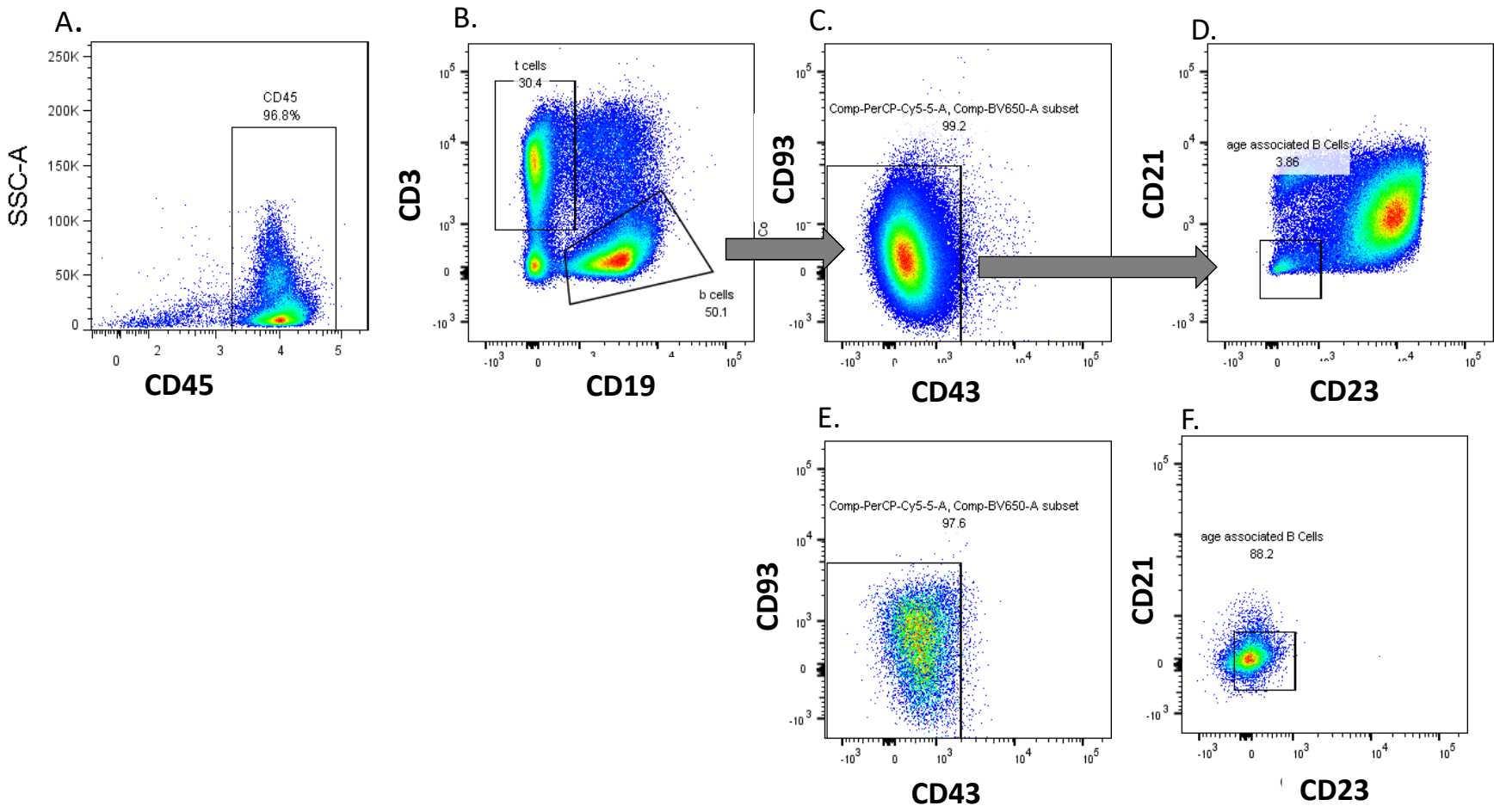


Figure 2.6 Gating strategy for B cell and B cell isotype panel in spleen.

(A) $CD45^+$ cells were gated on live cells, **(B)** B-cells were gated on $CD45^+$ cells using CD3 vs CD19, **(C)** $CD93^-$ $CD43^-$ cells were identified using **(E)** isotype control, **(D)** age associated B cells were gated on $CD93^-$ $CD43^-$ cells using **(F)** isotype control.

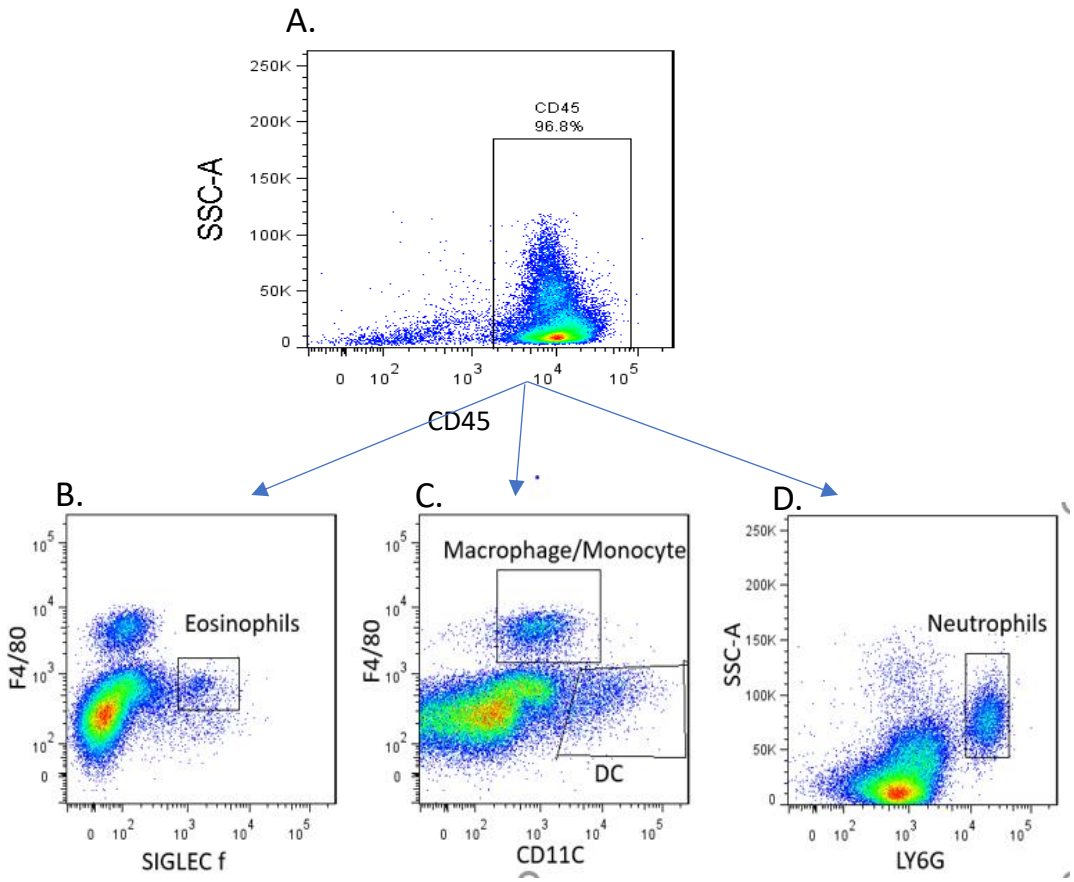


Figure 2.7 Gating strategy for macrophage panel in spleen. (A) $CD45^+$ cells were gated on live cells, **(B)** eosinophils were gated on $CD45^+$ cells using F4/80 vs SIGLEC F, **(C)** macrophage/monocyte and DCs were identified using F4/80 vs CD11C, **(D)** neutrophils were gated on $CD45^+$ cells using SSC-A vs LY6G.

Table 2.6. Anti-mouse antibodies used in T cell, T cell isotype, B cell, B cell isotype, and macrophage panel for flow cytometry.

Antibody	Fluorophore	Concentration	Clone	Catalogue number	Supplier	Panel
CD45.2 (0.2 mg/ml), Mouse (SJL) IgG2a, κ	BV605	1:100	104	109841	Biolegend	T-cell, B-cell, T-cell isotype, B-cell isotype
CD3 (0.2 mg/mL), Armenian hamster / IgG	PECy7	1:50	145- 2c11	25-0031-82	Thermofisher	T-cell, B-cell, T-cell isotype, B-cell isotype
CD4 (0.2 mg/mL) Rat / IgG2b, kappa	eFluor 450	1:100	GK1.5	48-0041-82	Thermofisher	T-cell
CD8 (0.2 mg/mL) Rat / IgG2b	PE-Texas Red	1:200	5H10	MCD0817	Thermofisher	T-cell
CD44(0.5 mg/mL) Rat / IgG2b, kappa	FITC	1:500	lM7	11-0441-82	Thermofisher	T-cell
CD25 (0.2 mg/mL) Rat / IgG1, lambda	AF700	1:50	PC61.5	56-0251-82	Thermofisher	T-cell
KLRG1 (0.2 mg/mL) Syrian hamster / IgG	APC-e Fluor780	1:100	2F1	47-5893-82	Thermofisher	T-cell
CD62L (0.2 mg/m) Rat / IgG2a, kappa	PE	1:500	MEL-14	12-0621-82	Thermofisher	T-cell
CD19 (0.2 mg/mL) Rat / IgG2a, kappa	APC	1:50	1D3	17-0193-82	Thermofisher	T-cell, T-cell is type, B-cell, B-cell isotype
Zombie	BV510	1:500		77143	Biolegend	T-cell, B-cell, T-cell isotype, B-cell isotype, macrophage, compensation
Armenian hamster IgG, (0.2 mg/mL)	APC-e Fluor780	1:100	eBio299Arm	47-4888-80	Thermofisher	T-cell isotype

Antibody	Fluorophore	Concentration	Clone	Catalogue number	Supplier	Panel
CD23 (0.2 mg/ml) Rat LOU IgG2a, κ	BV421	1:100	B3B4	562929	BD	B-cell
CD93 (0.2 mg/ml) Rat SD, IgG2b, κ	BV650	1:100	AA4.1	563807	BD	B-cell
CD43 (0.2 mg/ml) Rat DA x LOU IgG2a, κ	PerCPCy5.5	1:100	S7	562865	BD	B-cell
CD21/35 (0.2 mg/ml) Rat SD IgG2b, κ	PE	1:100	7G6	552957	BD	B-cell
Rat IgG2b	PE	1:100	eB149/ 10H5	12-4031-81	Thermofisher	B-cell isotype
Rat IgG2ak	BV421	1:100	R35-95	562602	BD	B-cell isotype
Rat IgG2bk	BV650	1:100	R35-38	563233	BD	B-cell isotype
Rat IgG2ak	PerCPCy5.5	1:100	R35-95	550765	BD	B-cell isotype
CD45 (0.2 mg/mL) Mouse / IgG2a, κ	APCCy7	1:200	104	47-0454-82	Thermofisher	macrophage
F4/80 (0.2 mg/mL) Rat / IgG2a, κ	FITC	1:50	BM8	17-4801-82	Thermofisher	macrophage
CD11c (0.2 mg/mL) Armenian hamster / IgG	PECy7	1:50	N418	25-0114-82	Thermofisher	macrophage
Gp38 (0.2 mg/mL) Syrian hamster / IgG	PE	1:200	8.1.1	12-5381-82	Thermofisher	macrophage
Siglec F (0.2 mg/m) Rat LOU/IgG2a, κ	TR	1:50	E50-2440	562757	BD	Macrophage

Antibody	Fluorophore	Concentration	Clone	Catalogue number	Supplier	Panel
Ly6G (0.2 mg/m) Rat LEW/IgG2a, κ	APC	1:50	1A8	560599	BD	macrophage
CD3 (0.2 mg/mL) Armenian hamster / IgG	PE	1:50	145-2C11	12-0031-82	Thermofisher	Compensation
CD45 (0.2 mg/mL) Rat / IgG2b, κ	AF700	1:50	30-F11	56-0451-82	Thermofisher	Compensation
CD3 (0.2 mg/mL) Rat / IgG2b, κ	APC	1:50	17A2	17-0032-82	Thermofisher	Compensation
CD3 (2 mg/mL) Rat/IgG2b, κ	BV650	1:50	17A2	100229	Biolegend	Compensation
CD3 (0.2 mg/mL) Rat/ IgG2b	BV605	1:50	17A2	100237	Thermofisher	Compensation

2.9.9 Processing mice pancreas tissue

Isolated pancreases were fixed in 10% buffered formaldehyde (Sigma-Aldrich; Catalogue number #HT5011) for 48 hours and then placed in 70% ethanol until processing for sectioning and immunofluorescence staining of insulin and glucagon.

2.9.9.1 Paraffin embedding and sectioning of the pancreas

The fixed mice pancreas was transferred to ethanol solutions prepared in dH_2O as below:

- 50 minutes in 70% ethanol
- 50 minutes in 95% ethanol
- 50 minutes in 100% ethanol
- 30 minutes in xylene (Source Chemicals Ltd, Littleborough, UK, catalogue number # 203-396-5)
- 30 minutes in fresh xylene

The wax machine was turned on 2 hours earlier to make liquid paraffin. The tissue was transferred from xylene to running liquid paraffin for 20 minutes to get rid of excess xylene, then placed into the embedding cassette (Thermofisher, catalogue number # M485SL) containing fresh wax on ice for 30 minutes until becomes solid. The tissue blocks were kept in room temperature until sectioning (Moran et al., 2014).

Tissues were then sectioned at 2 × 5 μm each using microtome and flouted on water at 45°C using water bath. A super frost plus slide (Thermo Scientific, catalogue number # 631-9483) was

used to collect each of the tissue sections. The slides were then transferred to drier at 80°C for 1 hour and kept in room temperature before being stained (Moran et al., 2014).

2.9.9.2 Deparaffinisation and staining of pancreas for insulin and glucagon

Deparaffinisation was performed to remove the paraffin penetrated the tissue prior to the staining. Briefly, the slides transferred to histoclear (Sigma Aldrich, catalogue number # H2779) 3 times for 2 minutes with fresh histoclear each time. Then placed in 100, 95, 70% ethanol for 5 minutes each respectively. The slides then moved into dH_2O for 2 minutes, then incubated for 25 minutes in citrate buffer (10mM citric acid anhydrous, 1 L dH_2O, 0.05% Tween 20; 0.5Ml, pH to 6 with NaOH) for antigen retrieval. After, slides were placed under cold running water for 10 minutes and dried at room temperature. A pap pen (Merck, catalogue number # Z377821-1EA) was used to draw around pancreas tissue. The slides were incubated in blocking solution (PBS+; 50ml, 0.1% triton X-100 and 2% BSA) for 1 hour. The blocking buffer was removed and a mix of primary antibodies including anti-insulin rabbit antibody (Cell signalling, London, UK, clone: C27C9, catalogue number #3014) at 1:500 and anti-glucagon mouse antibody (Sigma Aldrich, clone: K79bB10, catalogue number #G 2654) at 1:2000 both diluted in blocking solution, was applied at 4°C in humidity chamber overnight (Moran et al., 2014). The day after, the slides were washed 3 x 10 minutes in blocking buffer. Secondary antibodies including goat anti-mouse IgG (H+L) Alexa fluor plus 488 (Thermofisher, catalogue number # A32723) and goat anti-rabbit IgG (H+L) Alexa fluor plus 633 (Thermofisher, catalogue number # A-21071) were diluted in blocking buffer both at 1:2000 and were applied for 2 hours at room temperature in the dark (Moran et al., 2014), which subsequently washed 3 x 10 minutes in blocking buffer. The excess liquid was eliminated from the slides and a drop of vectashield mounting medium with DAPI was applied on

each slide over the tissue. Coverslip (London LaboQuip, London, UK, catalogue number # 0111620) was added to the slides and stored at 4°C in dark until imaged using a slid scanner.

2.9.9.3 Slide scanner

Slides were imaged using a zeiss axioscan 7 slid scanner (Zeiss, Oberkochen, Germany) at 20x magnification. Three separate channels were used to avoid overlap in emission detection from each fluorochrome. Images were processed on ZEN (black edition, version 2.2) software and were stored as digital arrays. Digital arrays were analysed using ImageJ 1.53k and quantified as the average number of the islets/10000mm^2 of whole area or the average area of islets/10000mm^2 of whole area.

2.10 Statistical analysis

All data have been analysed on Prism software 8.0 (GraphPad, San Diego, USA) and is shown as means ± standard error of mean (SEM).). Normality was first tested for via the Kolmogorov-Smirnov test. For *in vitro* experiments one -way ANOVA followed by Dunnett's, or Bonferroni's multiple comparisons post-test were performed; For *in vivo* experiments, unpaired t tests were performed. P-value less than 0.05 were considered significant.

3 THE ROLE OF CDH13 (CADHERIN-13) IN ANTI-INFLAMMATORY EFFECTS OF ADIPONECTIN

3.1 Introduction

Adiponectin is known to exert its anti-inflammatory effects through binding to its receptors AdipoR1/R2, which are expressed on immune cells and endothelium (Takeuchi et al., 2007). In addition to AdipoRs, another receptor has been identified as a potential binding partner for adiponectin called CDH13 (Takeuchi et al., 2007). CDH13 is highly expressed by endothelium (Matsuda et al., 2015) and is colocalized with adiponectin in vessel walls, emphasizing CDH13 as a major receptor for adiponectin in the vasculature (Ivanov et al., 2001). As mentioned before, the presence of AdipoRs on B cell is crucial in PEPITEM pathway (Chimen et al., 2015). However, the importance of CDH13 in PEPITEM pathway is unknown. Previous studies suggest the tissue accumulation of adiponectin specifically with CDH13 but not with AdipoRs (Denzel et al., 2010). Since the AdipoRs are inseparable part of PEPITEM pathway, here we hypothesise that CDH13 presents adiponectin to the B cells to trigger the PEPITEM pathway.

In this chapter, to assess the functionality of the PEPITEM pathway *in vitro*, we started the quantification of AdipoR1/R2 expression on PBMC subsets using flow cytometry. Using static transmigration assay we measured the inhibitory capacity of adiponectin in T cell migration acting on PBLs. To explore the role of adiponectin receptors in PEPITEM pathway we first confirmed CDH13 and AdipoRs expression on BEC both at protein and mRNA levels. Then, we measured the inhibitory effect of adiponectin in T cell migration by treating the BEC with adiponectin. As we found the pathway functional, we proceeded to knock-down AdipoR1 and AdipoR2 and CDH13 in BEC and measure the pathway. To investigate the modulation of CDH13 in inflammation, also its interaction with adiponectin, we explored the BEC expression of CDH13 in presence and absence of adiponectin using flowcytometry and confocal microscopy.

3.2 Results

3.2.1 Evaluation of AdipoR1/R2 on expression on PBMC subsets

To evaluate the functionality of the PEPITEM pathway we identified the PBMC subset in healthy donors (see gating strategy in Material and Methods Figure 2.2), we aimed to measure the expression of both adiponectin receptors AdipoR1 and AdipoR2 on PBMCs using flow cytometry. AdipoR1 and AdipoR2 expressions were determined on each PBMC subsets based on secondary antibody control (absence of primary antibodies for AdipoR1/R2), (Figure 3.1A-G) and quantified at protein levels as frequency of cells expression AR1/2 or MFI on PBMC subsets (Figure 3.2A-N). We observed higher frequencies and surface expression of both receptors on B cells (Figure 3.2-A and H), monocytes (Figure 3.2-B and I) and CD3-CD56+ low NK cells (Figure 3.2-D and K) and little expression were found on CD56 high NK cells (Figure 3.2-C and J), NK T cells (Figure 3.2-E and L), $CD8^+$ T cells (Figure 3.2-F and M) and $CD4^+$ T cells (Figure 3.2-G and N), which agrees with the previous data showing higher expression of these receptors on B cell compared to T cell (Chimen et al., 2015). The surface expression of AdipoR1 was significantly higher on B cells, monocytes and CD3-CD56+ NK cells compared to AdipoR2 (Figure 3.2H, 1.2I and 3.2K). Higher expression of AdipoR1/R2 on B Cell, monocytes and NKs, suggests an important immunomodulatory capacity for adiponectin.

3.2.2 Effect of adiponectin on lymphocyte migration through inflamed endothelium

To determine whether the PEPITEM pathway is active in healthy individuals, we measured PBL recruitment across BEC stimulated with TNF-α/IFN-γ for 24 hours prior to the adhesion assay. Here we aimed to compare and optimise commercially available BEC donors based on

different ages in their ability to support PBL recruitment to levels previously published and seen within the group. Seeding with the same density, we observed a better monolayer using adult BEC (Figure 3.3-A) compared to juvenile (Figure 3.3-B) despite a similar response to adiponectin. Thus, we decided to proceed the future experiments with adult BEC. Moreover, adiponectin treatment of PBL had no effect on the level of PBL adhesion to either adult or juvenile inflamed BEC (Figure 3.3-C and 1.3-D). Adiponectin efficiently reduced the migration of PBL across the endothelium both in adult and juvenile BEC (Figure 3.3-E and 3.3-F).

3.2.3 Expression of adiponectin receptors on endothelial cells

The original adiponectin-PEPITEM story investigated the effect of pre-treating the lymphocytes with adiponectin prior to adding them to the endothelial monolayer and these data agree with this. However, as indicated endothelial cells also express adiponectin receptors and therefore could potentially respond to adiponectin, independently of the responses seen by PBL. The efficiency of adiponectin acting on BEC led us to consider the involvement of adiponectin receptor on BEC. We aimed to confirm the expression of adiponectin receptors CDH13, AdipoR1 and AdipoR2 on resting and activated BEC using qPCR and flow cytometry. All three receptors' expressions were found on BEC at both mRNA (Figure 3.4) and protein levels (Figure 3.5). We found higher gene expression of CDH13 (Figure 3. 4-A**)**, but not AdipoRs (Figure 3. 4-B) in stimulated BEC compared to resting BEC. We saw no change in frequency of CDH13 (Figure 3.5-A) and AdipoRs (Figure 3.5-C) in cytokine stimulated BEC compared to baseline. However, the surface expression (MFI) of CDH13 (Figure 3.5-B), but not AdipoRs (Figure 3. 5-D**)** were higher

in stimulated BEC compared to unstimulated, indicating a potentially important role of CDH13 in vasculature and within the adiponectin-PEPITEM pathway.

3.2.4 Endothelial responses to adiponectin

We subsequently determined whether adiponectin could influence lymphocyte migration by directly acting on BEC, we measured lymphocyte recruitment on TNF-α/IFN-γ-stimulated BEC. In static conditions, treatment of adult BEC with adiponectin for 24 hours did not alter total adhesion of PBL (Figure 3.6-A) but did significantly reduce PBL transmigration (Figure 3.6-B). Thus, indicating that endothelial response to adiponectin was sufficient to dampen PBL transmigration.

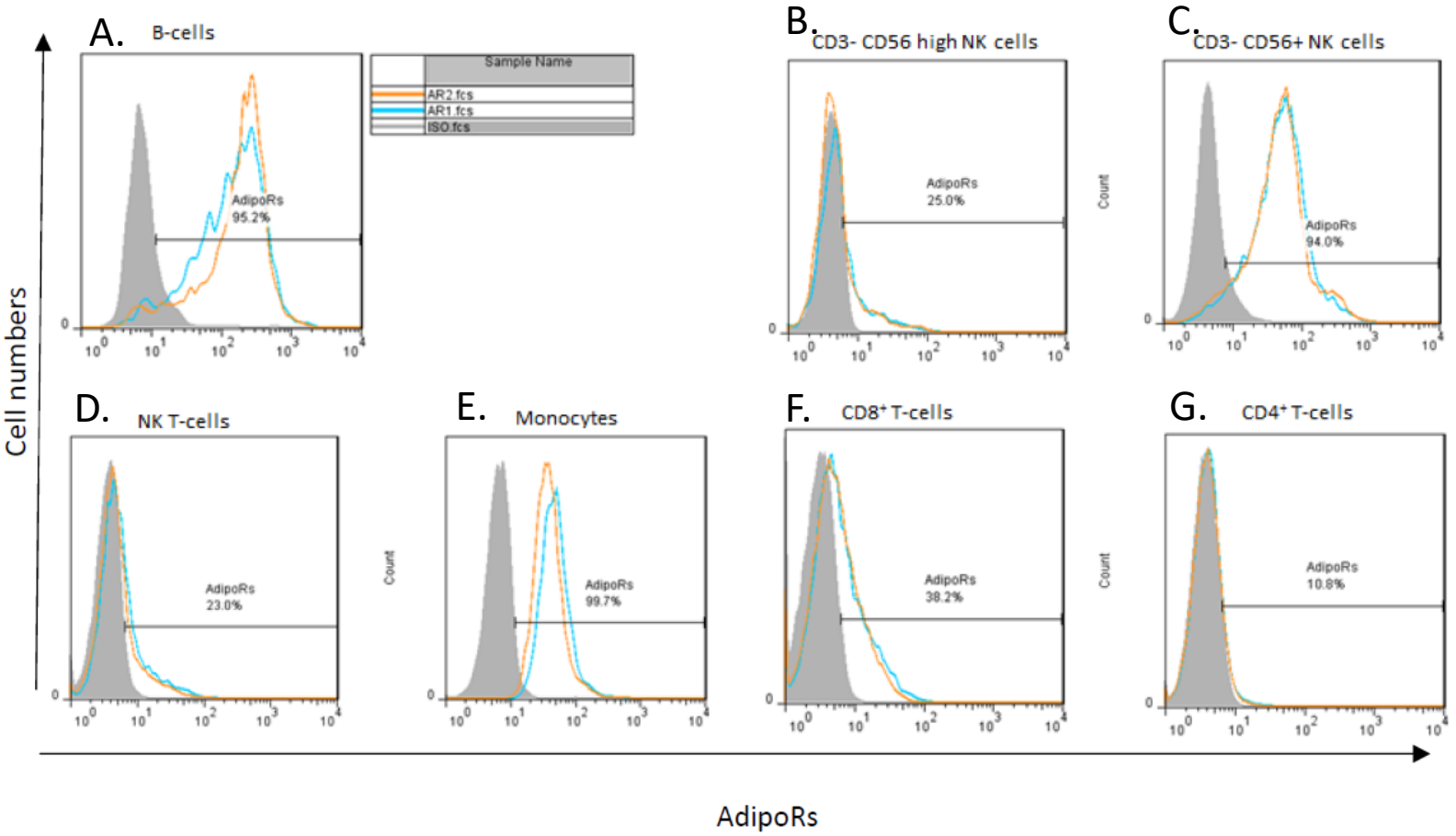


Figure 3.1 Representative histograms of AdipoRs expression on PBMCs. AdipoRs expression was determined on **(A)** B ells, **(B)** $CD3^{-}CD56$ high NK cells, **(C)** $CD3^{-}CD56$ NK cells, **(D)** NK T-cells, **(E)** monocytes, **(F)** $CD8^{+}$ T-cells and **(G)** $CD4^{+}$ T-cells, using flow cytometry based on the secondary only for each PBMC subset. Data are representative of 1 donor, for n=6 different blood donors analysed in Figure 3.2.

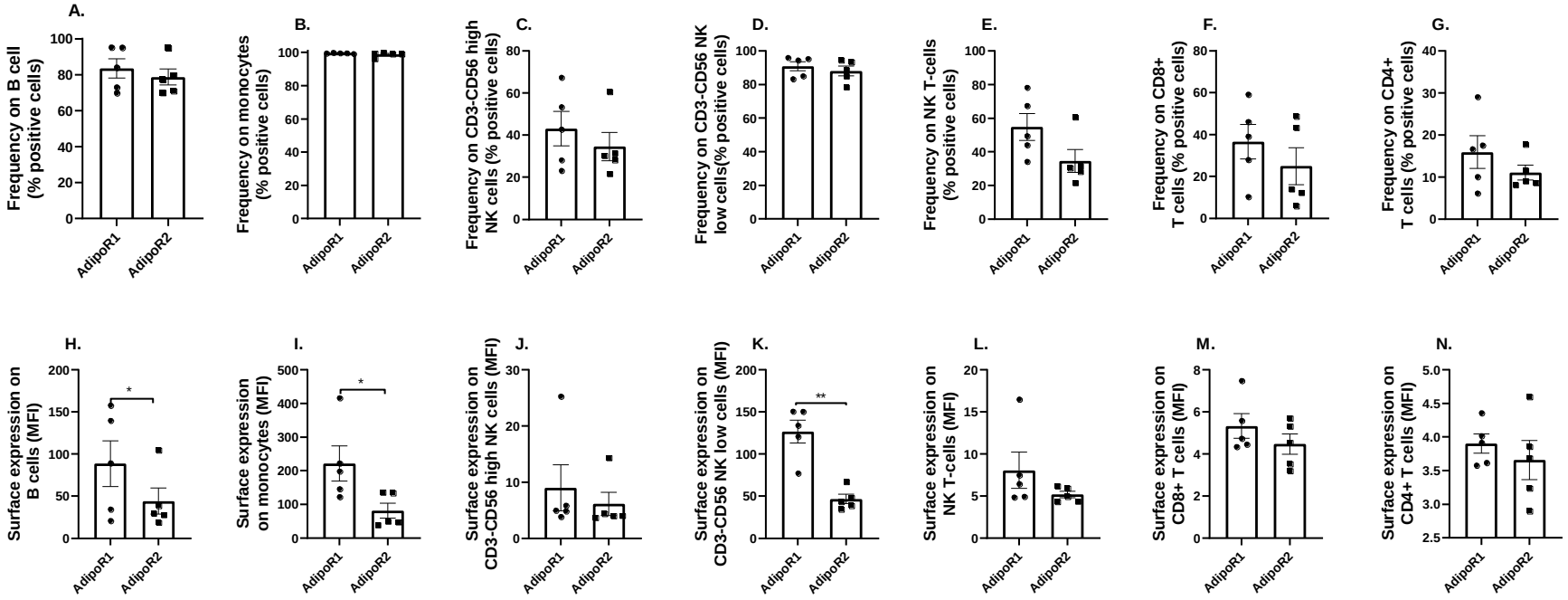


Figure 3.2 Expression of adiponectin receptors on PBMC subsets. PBMCs were isolated from whole blood and stained with respective antibodies for PBMC subsets. AdipoRs expressions were measured using flow cytometry and quantified as (A-G) frequency of cells expression AR1/2 or (H-N) MFI on (A and H) B-cells, (B and I) monocytes, (C and J) $CD3^{-}CD56$ high NK cells, (D and K) $CD3^{-}CD56$ NK low cells, (E and L) NK T-cells, (F and M) $CD8^{+}$ T-cells and (G and N) $CD4^{+}$ T-cells. Data are mean ± SEM from n=5 independent experiments using 6 different blood donors. * =$p<0.05$ and ** = $p<0.01$ by paired t-test.

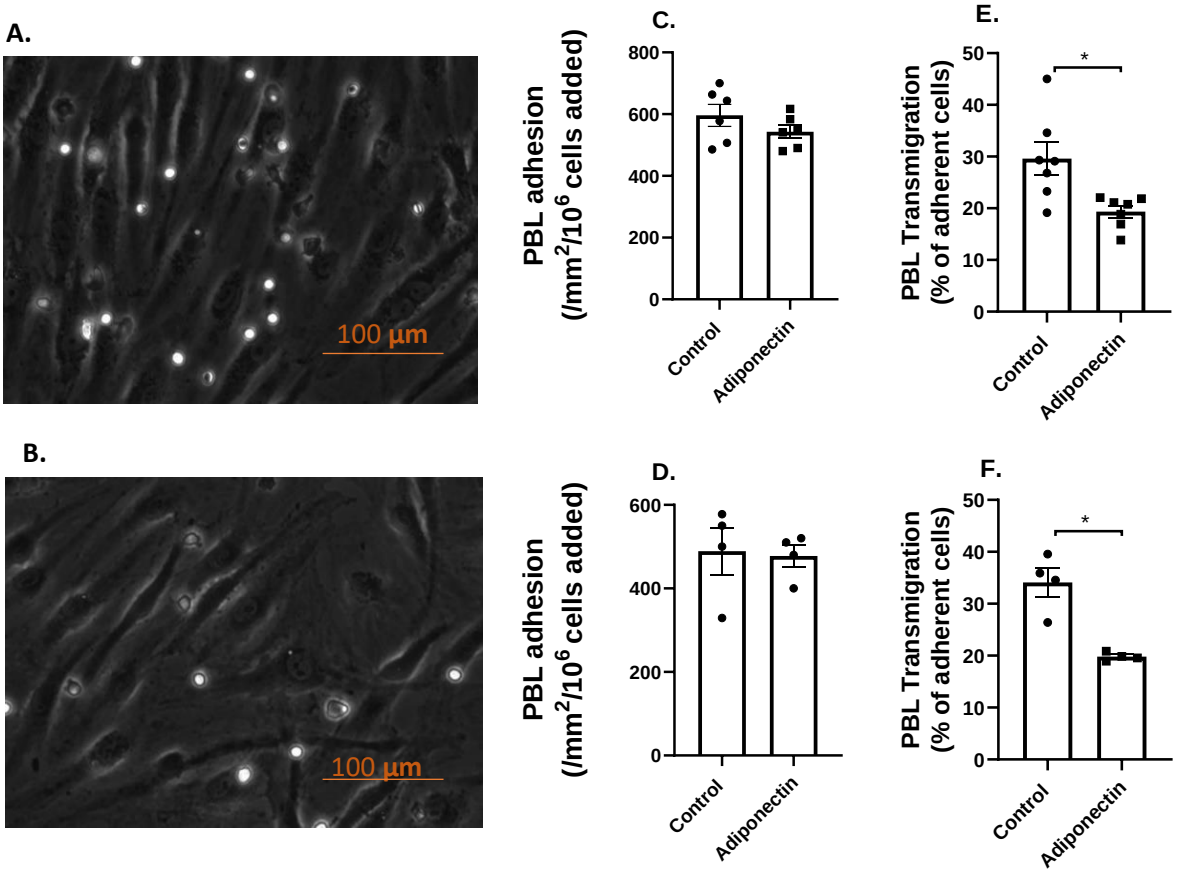


Figure 3.3 Effect of adiponectin on lymphocyte migration through inflamed adult vs juvenile endothelium in static conditions. BEC were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ for 24 hours. PBLs were isolated, left untreated (control) or treated with adiponectin at 10µg/ml l. PBLs (1 × 10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired from **(A)** adult or **(B)** juvenile BEC and analysed offline. The number of adherent PBLs **(C)** to adult or **(D)** juvenile BEC expressed as the number of cells/mm^2/10^6 cells added**.** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated through **(E)** adult or **(F)** juvenile BEC. Data are shown as mean ± SEM for n = 4-6 independent experiments using 2 different BEC donors and 4-6 different lymphocyte donors. *= $p<0.05$ compared to control, by paired t-test. Scale bar=100 µm.

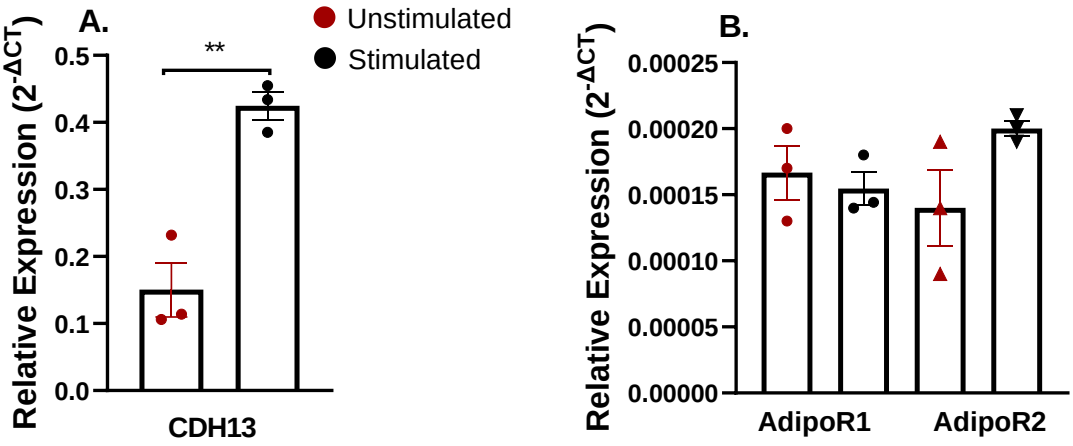


Figure 3.4 Gene expression of adiponectin receptors on BEC. BEC were cultured and left untreated or treated with TNF-α/IFN-γ for 24 hours. **(A)** CDH13 and **(B)** AdipoR1/R2 gene expression were measured using qPCR and quantified as relative expression to the endogenous untreated control. Data are mean ± SEM from n=3 independent experiments using one BEC donor. ** = $p<0.01$ by paired t-test.

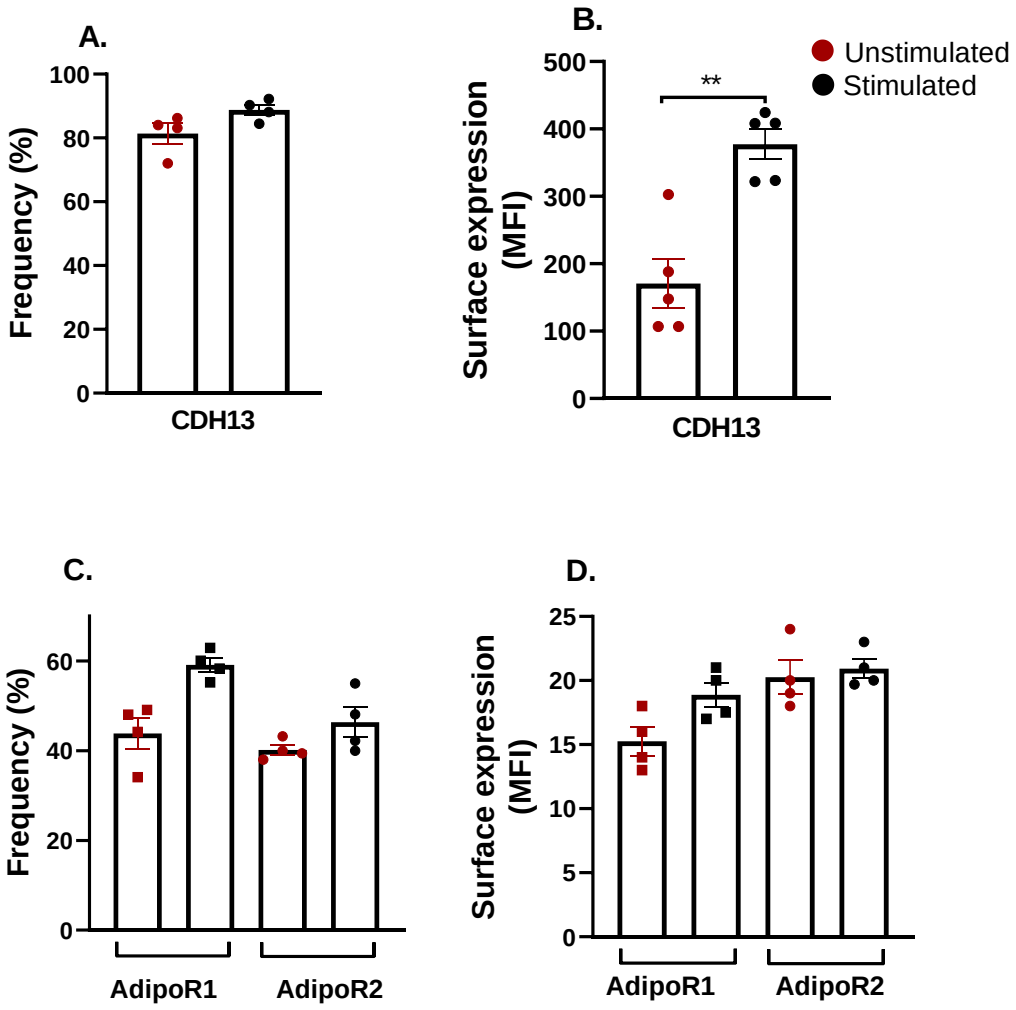


Figure 3.5 Protein expression of adiponectin receptors on BEC. BEC were cultured and left untreated or treated with TNF-α/IFN-γ for 24 hours. CDH13, AdipoR1 and AdipoR2 expression were measured using flow cytometry and quantified as **(A and C)** frequency of cells expressing the receptors and **(B and D)** MFI. Data are mean ± SEM from n=4 independent experiments using one BEC donor. ** = $p<0.01$ by paired t-test.

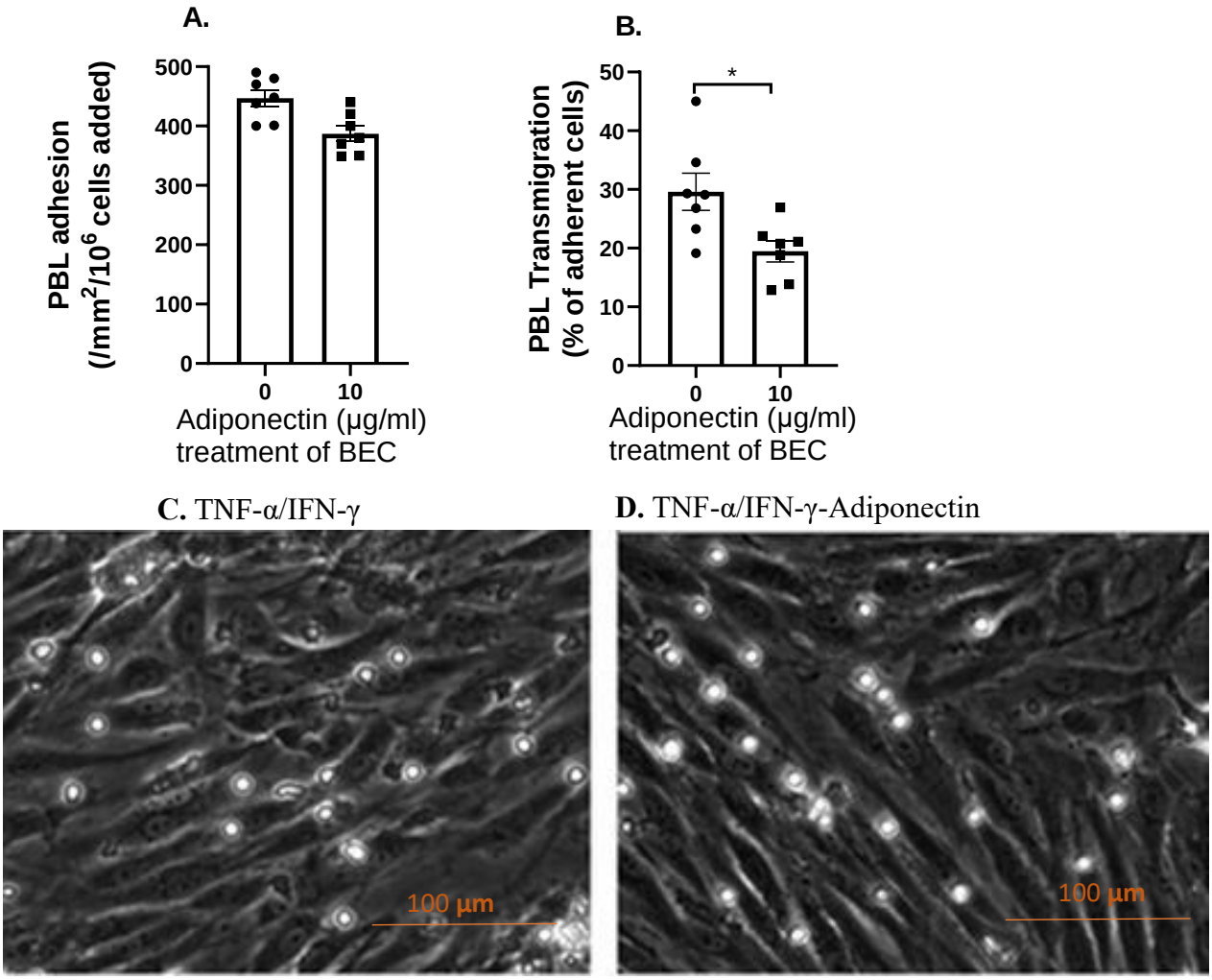


Figure 3.6 The effect of adiponectin treatment of BEC on lymphocyte recruitment to inflamed endothelium. Adult BECs were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ in the absence or presence of adiponectin at 10 µg/ml, for 24 hours. PBL were isolated and (1×10^6 cells/ml) added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. Representative images of transmigration assay **(C)** in absence or **(D)** presence of adiponectin. Data are shown as mean ± SEM for n = 7 independent experiments using 2 BEC donors and 7 different lymphocyte donors. *= $p<0.05$ by paired t-test compared to no adiponectin treated BEC. Scale bar=100 µm

3.2.5 The effect of adiponectin receptors knock-down on PEPITEM pathway

When we observed the efficiency of adiponectin to reduce lymphocyte transmigration through acting directly on BEC, we wondered whether the adiponectin receptors which we showed the expression on BEC, are involved in this effect. Thereby, we tried to knockdown these receptors individually and measure the functional consequence using the adhesion assay. Knockdown of each receptor was confirmed both with qPCR (Figure 3.7) and flow cytometry (Figure 3.8). The gene expression of receptors (expressed as fold change to no siRNA) was significantly reduced by using siRNA targeting CDH13 (Figure 3.7-A) AdipoR1 (Figure 3.7-B) and AdipoR2 (Figure 3.7-C) when compared to the no siRNA control BEC. A similar observation was made at the protein level using flow cytometry. The endothelial cells population were identified using forward vs. side scatter (Figure 3.8-A). Single cells were identified (Figure 3.8-B) before expression of adiponectin receptors was measured by flow cytometry based on the secondary only (Figure 3.8-C and 3.8-D) and quantified as MFI. The total protein expression of receptors was significantly reduced by using siRNA targeting CDH13 (Figure 3.8-E) AdipoR1 (Figure 3.8-F) and AdipoR2 (Figure 3.8-G) when compared to the no siRNA control BEC. After confirmation of knockdown of the potential receptors for adiponectin, we explored if adiponectin was still able to signal through the endothelial to modulate lymphocyte trafficking. The knockdown of CDH13 had no effect on PBL adhesion (Figure 3.9-A), however, it decreased the inhibitory effects of adiponectin such that adiponectin was no longer able to suppress PBL migration (Figure 3.9-B). Moreover, our data showed that individual knockdown of AdipoR1 and AdipoR2 reduced the efficiency of adiponectin to inhibit PBL transmigration compared to BEC transfected with control siRNA (Figure 3.10). These data collectively suggest the ability of these receptors to individually influence PBL migration and thus potentially impact on the PEPITEM pathway.

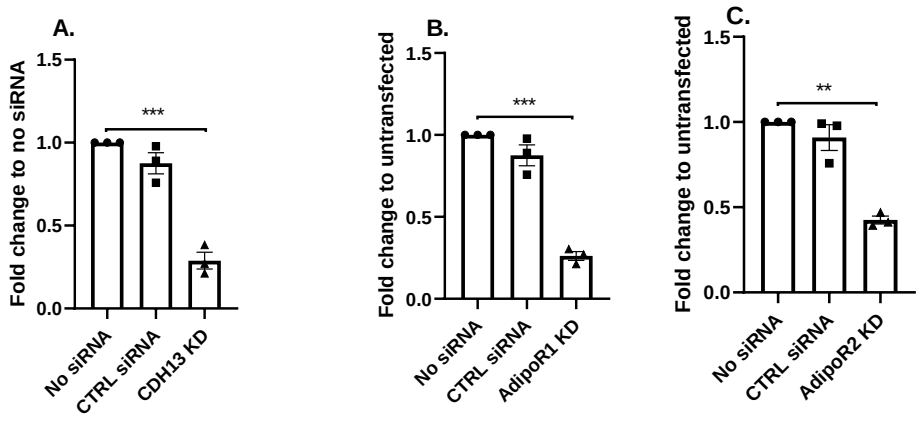


Figure 3.7 PCR confirmation of adiponectin receptors knockdown on BEC. BEC were cultured for 24 hours and transfected with siRNA against CDH13, AdipoR1 or AdipoR2 or scramble versions of the target siRNA (CTRL). As a control, cells were incubated in the transfection buffer alone (No siRNA). After 48 hours, BEC were stimulated with TNF-α/IFN-γ for 24 hours. **(A)** expression of CDH13, **(B)** AdipoR1 or **(C)** AdipoR2 were evaluated by qPCR and data are plotted as fold change to the no siRNA control. ANOVA showed a significant effect of transfection on gene expression of adiponectin receptors. Data are shown as mean ± SEM for n = 3 independent experiments using 1 BEC donor. **= $p<0.01$ and *** $p<0.005$ by Bonferroni's multiple comparison post-test.

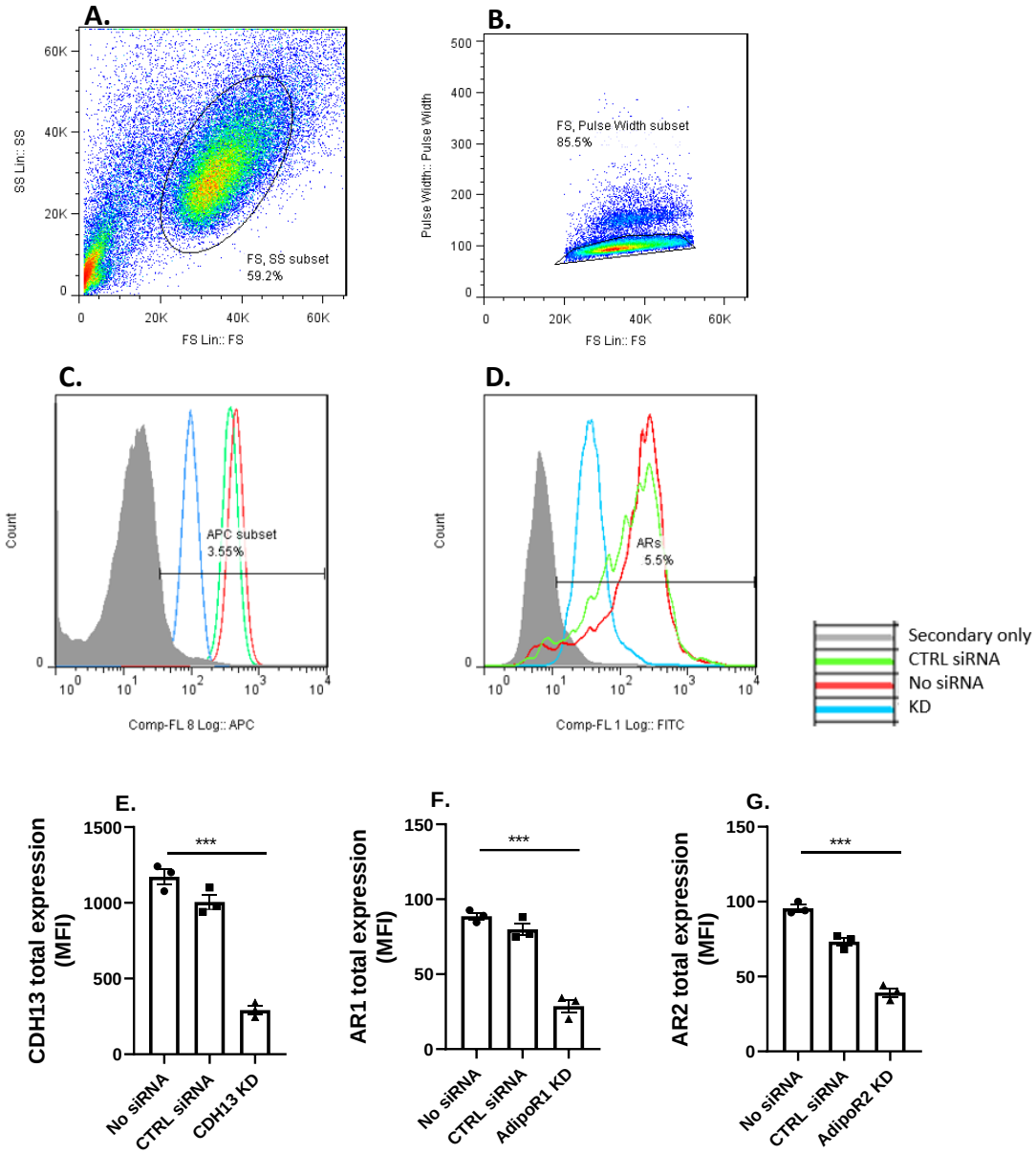


Figure 3.8 Flow cytometry confirmation of adiponectin receptors knock down on BEC. BEC were cultured for 24 hours and transfected with siRNA against CDH13, adipoR1 or adipoR2 or scramble versions of the target siRNA (CTRL siRNA). As a control, cells were incubated in the transfection buffer alone (No siRNA). After 48 hours, BEC were stimulated with TNF-α/IFN-γ for 24 hours. **(A)** BEC population was identified using forward vs. side scatter (FSC vs SSC). **(B)** Single cells were identified by using FSC vs Pulse Width. Expression of **(C)** CDH13 and **(D)** adipoR1/R2 were measured by low cytometry based on the secondary only and quantified as MFI of (E) CDH13, (F) adipoR1 and (G) adipoR2. ANOVA showed a significant effect of transfection on protein expression of adiponectin receptors. Data are shown as mean ± SEM for n = 3 independent experiments using 2 BEC donors. ***= P<0.001, by Bonferroni's multiple comparison posttest.

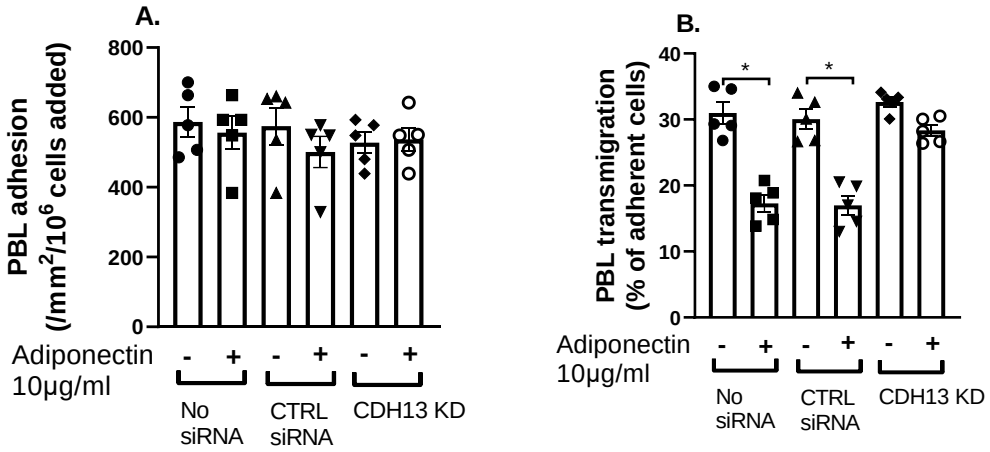


Figure 3.9 Effect of CDH13 knockdown on the ability of adiponectin to inhibit lymphocyte recruitment to inflamed endothelium. BEC were cultured for 24 hours and transfected with siRNA against CDH13 or scramble versions of the target siRNA (CTRL siRNA). As a control, cells were incubated in the transfection buffer alone (No siRNA). After 48 hours, BEC were stimulated with TNF-α/IFN-γ in presence or absence of adiponectin at 10µg/ml for 24 hours following the transfection. PBL were isolated and (1×10^6 cells/ml) added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. Data are shown as mean ± SEM for n =5 independent experiments for using 2 BEC donors and 5 different lymphocyte donors. * = $p<0.05$ by paired t-test compared to no adiponectin treated BEC.

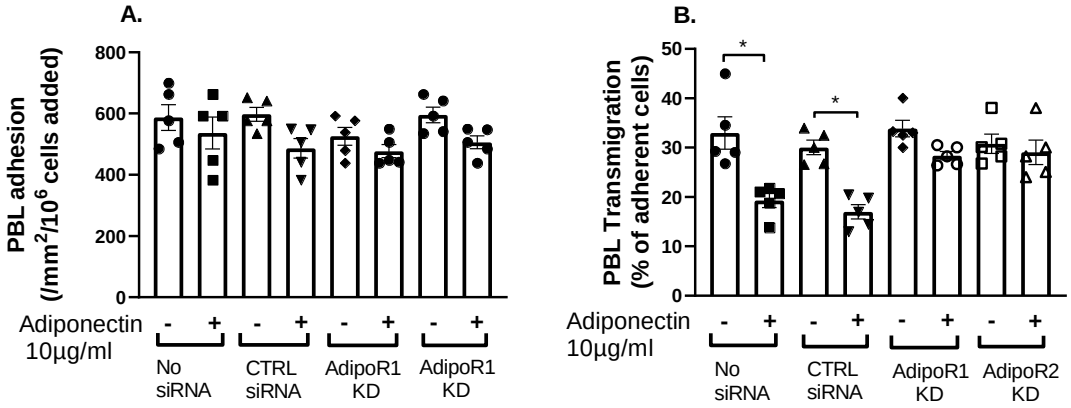


Figure 3.10 Effect of AdipoR1 or R2 knockdown on the ability of adiponectin to inhibit lymphocyte recruitment to inflamed endothelium. BEC were cultured for 24 hours and transfected with siRNA against adipoR1 or adipoR2 or scramble versions of the target siRNA. As a control, cells were incubated in the transfection buffer alone (No siRNA). After 48 hours, BEC were stimulated with TNF-α/IFN-γ for 24 hours in presence or absence of adiponectin at 10µg/ml for 24 hours following the transfection. PBL were isolated and (1×10^6 cells/ml) added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. Data are shown as mean ± SEM for n =5 independent experiments for transmigration assay using 2 BEC donors and 5 different lymphocyte donors. *= $p<0.05$, by paired t-test compared to no adiponectin treated BEC.

3.2.6 Evaluating Expression of CDH13 by BEC

Previous studies demonstrate that changes in tissue adiponectin and CDH13 protein levels are synchronized at tissue levels (Takeuchi et al., 2007), however the modulation of CDH13 in inflammation is not clear. Here, we measured the surface and total (surface and intracellular) expression of CDH13 on inflamed endothelial in presence or absence of adiponectin. As shown in figure 3.11-A, surface expression of CDH13 was increased in cytokine stimulated BEC compared to baseline levels and remained unchanged by the addition of adiponectin for either 24 hour or the final 1 hour. However, total (both surface and intracellular) expression of CDH13 was decreased following cytokine-stimulation of BEC when compared to baseline (Figure 3.11-B). In contrast, adiponectin treatment for either the duration of the 24 hours cytokine-stimulation or for just the final hour reversed the effects of the cytokines on CDH13 total levels, bringing them back to the levels observed at baseline (Figure 3.11-B). These data indicate that adiponectin is able to modulate the expression patterns of CDH13 during inflammation.

To clarify localization of CDH13 and adiponectin at the cellular level, BEC were stimulated with cytokines and expression of CDH13 was evaluated using confocal microscopy following adiponectin treatment. As shown in Figure 3.12-A, in n=1 the intracellular level of adiponectin was increased after adiponectin supplementation of BEC (Figure 3.12-A), suggesting uptake into the BEC. As anticipated the expression of CDH13 was decrease following cytokine-stimulation, although adiponectin treatment had no obvious effect in total amount of CDH13 (Figure 3.12-B). CDH13 seemed to localize more in nucleus (Figure 3.12 C-F). Additional experiments are required to make any definitive conclusion on this.

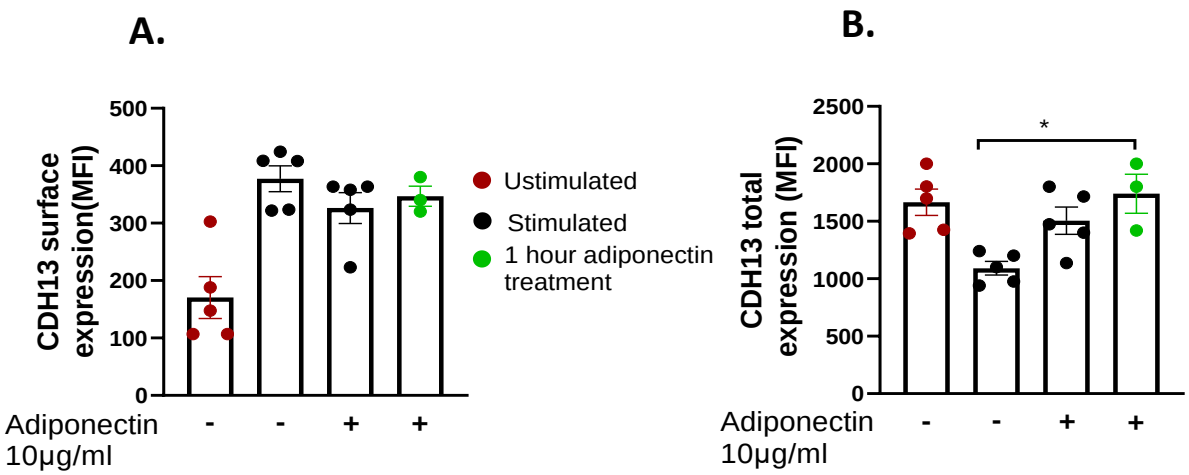


Figure 3.11 Effects of adiponectin treatment on CDH13 expression in stimulated endothelial cells. BEC were cultured for 24 hours, left untreated (red dots) or stimulated with TNF-α/IFN-γ in the absence or presence of adiponectin at 10 µg/ml, for 24 hours or for the final 1 hour (green dots) of the 24 hours cytokine treatment. CDH13 **(A)** surface and **(B)** intracellular expression were measured using flowcytometry and quantified as MFI. ANOVA showed a significant effect of adiponectin treatment on CDH13 expression. Data are shown as mean ± SEM for n = 3-5 independent experiments using 2 BEC donors. *= $p<0.05$, by Dunnett post-test.

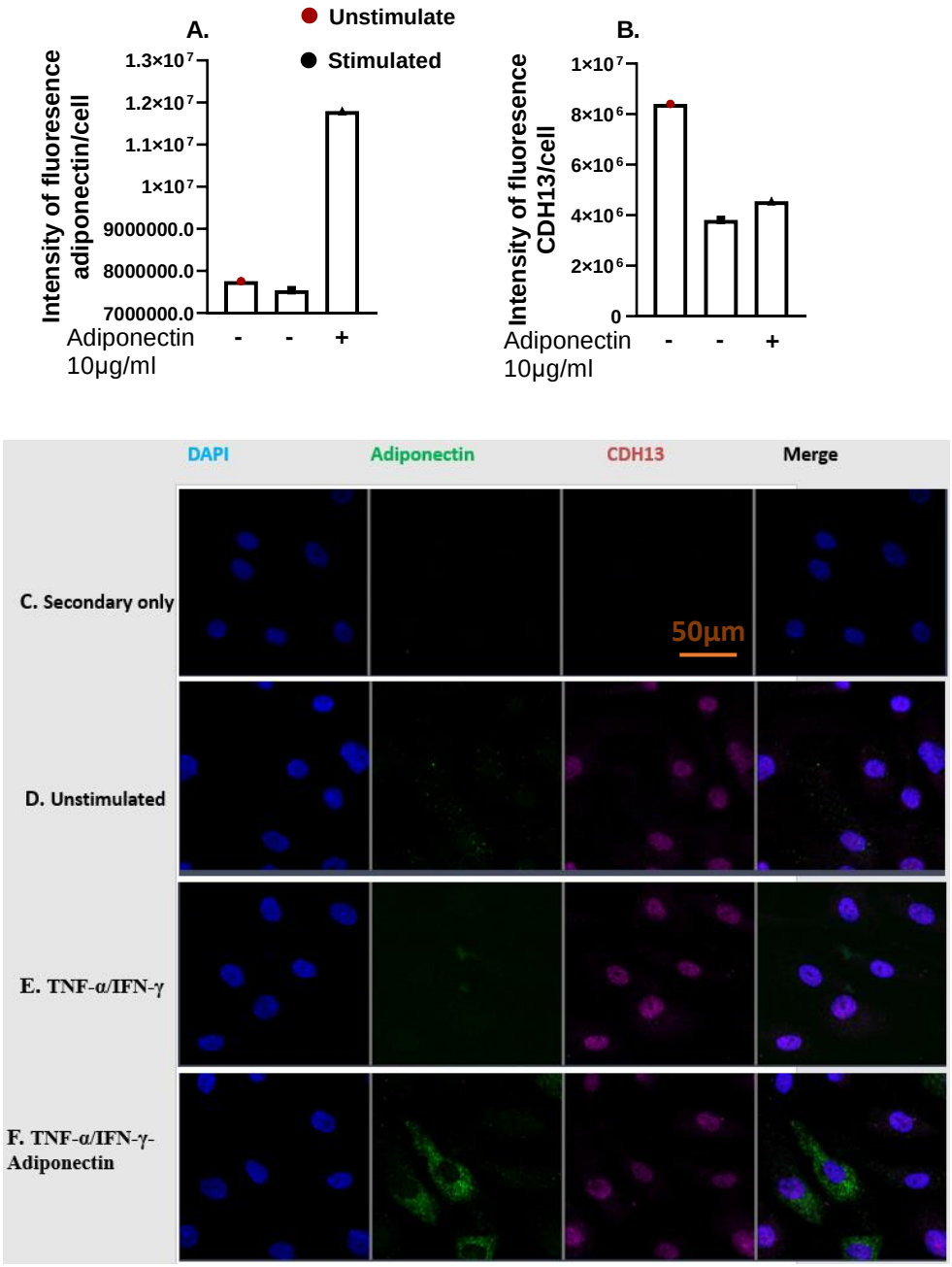


Figure 3.12 Confocal microscopy imaging of the localization of CDH13 and adiponectin in BEC. BEC were cultured for 24 hours, left untreated or stimulated with TNF-α/IFN-γ in the absence or presence of adiponectin at 10 µg/ml, for 24 hours. Adiponectin and CDH13 cellular expression were measured using confocal microscopy and quantified as the average fluorescence of **(A)** adiponectin and **(B)** CDH13. Representative images acquired using confocal microscopy showing separate and merged fluorescent channels from cells in **(C)** secondary only, **(D)** unstimulated, **(E)** stimulated with TNF-α and IFN-γ, **(F)** stimulated + Adiponectin 10µg/ml. Data are shown as mean ± SEM for n = 1 experiment using 1 BEC donors. Scale bar=50µm for all images.

3.3 Discussion

In this study to measure the functionality of PEPITEM pathway, we first quantified the AdipoR1R2 expression on PBMC subsets. We found higher expression of AdipoRs on B-cells, monocytes and CD3-CD56+ NK cells from healthy controls. Using an established model of lymphocyte migration, we showed that adiponectin can strongly inhibit PBL transmigration by acting on PBL (as previously published), (Chimen et al., 2015) and also directly on BEC (original finding). Investigating the role of adiponectin receptors in PEPITEM pathway, we observed expression of CDH13, AdipoR1 and R2 on BEC. By knocking down these individual receptors in the BEC, we found an abrogated PEPITEM pathway in absence of each of these receptors individually, suggesting that they were all able to contribute to the adiponectin binding and subsequent down-stream signaling. In addition, using flow cytometry we evaluated the interaction of CDH13 with adiponectin and we found that adiponectin is able to modulation the expression patterns of CDH13 during inflammation.

3.3.1 Expression of AdipoR1/R2 in immunity

We aimed to measure AdipoRs expression on PBMC subsets of healthy donors to understand the immunomodulatory role of adiponectin. We found that AdipoR1 and AdipoR2 are expressed on all PBMC subsets, but mainly on B-cells, NK-cells and monocytes. Our data are in agreement with previous studies that demonstrated AdipoR expression on PBMC subsets (Shen et al., 2007; Pang and Narendran, 2008; Chimen et al., 2015). Gene expression of AdipoR1 and AdipoR2 were previously demonstrated in human monocytes with AdipoR1 more abundant than AdipoR2 (Chinetti et al., 2004), which is consistent with our data showing the higher protein

expression of AdipoR1 not only on monocytes but also on B cells and NKs from healthy people. Later, Pang et al, extended the previous data, by indicating the protein and mRNA expression of AdipoR1/R2 on B cells and T cells (Pang and Narendran, 2008) and Chimen et al, also reported a high expression of AdipoRs on B cell Chimen et al., 2015). Although earlier studies focus on immunoregulatory role of adiponectin the myeloid-monocytic lineage, the presence of adiponectin receptors on lymphocyte subsets indicates a wider immunomodulatory capacity for adiponectin.

3.3.2 Measurement of lymphocyte transmigration *in vitro*

Here, we measured the lymphocyte recruitment using our *in vitro* static transmigration assay. Transmigration of PBLs has already been studied on activated HUVEC and HDMEC in both static and flow-based assays (Chimen et al., 2015). Static transmigration assay is easy to set up. However, it does not allow to analyse dynamic aspects of the leukocytes behavior such as capture from flow and rolling along endothelium (McGettrick et al., 2009). We stimulated the BEC with cytokines to mimic inflammatory environment to ensure efficient PBL adhesion and transmigration across the endothelium. Indeed, the activation of endothelial cells with TNF-α and IFN-γ has been found necessary for efficient recruitment of PBLs (McGettrick et al., 2009) as it induced proper CAMs upregulation and chemokines response in endothelium (CXCL9-11) to support PBL adhesion and transmigration (McGettrick et al., 2009). In fact, adhesion and transmigration of PBL in cytokine activated BEC were demonstrated in 6-8 minutes after their addition to BEC monolayer (McGettrick et al., 2009). In our study we focused on transmigration of lymphocytes as PEPITEM pathway is T cell specific (Chimen et al., 2015).

3.3.3 Evidence on immunomodulatory effects of adiponectin

Many of previous studies have shown the protective effects of adiponectin on vascular health as described in introduction (section 1.8.6). However, only few of them investigated the role of adiponectin in the regulation of leukocytes trafficking. Intravital microscopy data from adiponectin-knock out ($Ad^{-/-}$) mice showed a significant increase in leukocyte-endothelium interactions, as absence of adiponectin enhanced the monocyte rolling and increased the monocyte adhesion in the microcirculation (Ouedraogo et al., 2007). This was attributed to reduced levels of endothelial NO in absence of adiponectin (Ouedraogo et al., 2007). Moreover, their immunohistochemistry data showed that adiponectin deficiency was associated with increased expression of CAMs (E-selectin and VCAM-1) in the aortic vascular endothelium of mice (Ouedraogo et al., 2007). Interestingly, systemic administration of the globular adiponectin to these knock out mice, significantly decreased the CAMs expression and also monocyte-endothelium interactions, and restored the production of physiologic levels of endothelial NO in aortic endothelium (Ouedraogo et al., 2007). Collectively the authors suggested a potential pharmacologic role of adiponectin in regulating leukocyte-endothelium interactions by modulating the endothelial NO levels (Ouedraogo et al., 2007).

Relevant to our study, the role of adiponectin in the regulation of lymphocyte trafficking across HUVEC was previously investigated by Chimen et al, for the first time (Chimen et al., 2015). They have shown that AdipoR1/R2 expressed by B cells triggers an anti-inflammatory pathway inhibiting lymphocyte migration across endothelial cells, which is in agreement with our data showing 1 hour adiponectin treatment of PBLs inhibit their migration through inflamed BEC instead of HUVEC. Here we examined the performance of both juvenile and adult BEC in response to adiponectin in order to optimise our *in vitro* model for future experiments. Indeed, there is no

evidence supporting the advantage of using juvenile versus adult BEC for *in vitro* experiments. However, there are studies showing age related modulation of endothelial cells that would affect their behaviour in response to cytokines: for example, protein expression of P-selectin and VCAM-1 in aorta of 24-month-old rats were reported higher compared to 6-month-old rats, which was along with increased levels of myeloperoxidase used as a marker for leukocyte infiltration, suggested a higher cytokine response in older endothelium (Zou et al., 2006). Furthermore, there is evidence showing the decreased levels of NO in old cultured HUVEC compared to young HUVEC (Yoon et al., 2010). Since the endothelial NO levels have been shown to be associated with CAMs levels as discussed previously (Ouedraogo et al., 2007) In our study, we observed slightly higher levels of PBL adhesion in adult BEC compared to juvenile, which might be due to stronger reaction of adult BEC to cytokine treatment. However, the transmigration of PBL were not affected by the age of BEC, suggesting age related modulation of CAMs rather than chemokines support PBL recruitment in adult BEC.

In addition to age related discrepancies in endothelial cells, there are also evidence showing organ-specific heterogeneity in endothelial reactivity to inflammatory stimuli as well (Molema, 2010; Gunawardana et al., 2021). In a very recent study investigating tissue specific response of vascular endothelial cells to inflammation *in vitro* observed the highest adhesion of PBMC subsets (T cell, B cells and monocytes) to human aortic endothelial cells in response to 4 hours TNF-α stimulation compared to other vascular beds from liver, lunge, and renal, and this was associated with the highest VCAM-1 expression in human aortic endothelial cells (Gunawardana et al., 2021). This study further supports the idea that tissue-specific endothelial heterogeneity results in unequal inflammatory response in vasculature.

In addition to heterogeneity in endothelium function, there are some leukocyte-associated factors in different cohorts that may affect their interaction with endothelium. For example and relevant to our study, it has been shown that the AdipoR expression are decreased during ageing process (Chimen et al., 2015). As these receptors initiate the PEPITEM pathway, therefore including participants with different ages may affect the result of experiment. Similarly, other factors, such as age-related increase in expression of LFA-1 on lymphocytes (Powers et al., 1992), which plays an important role in T cells recruitment, might affect the study. Furthermore, sexual dimorphisms may cause some discrepancies in experimental results as reported in many studies (Chaves-Pozo et al., 2012; Kay et al., 2015). Females are reported to demonstrate relative protection from inflammatory conditions (Kay et al., 2015), as a murine zymosan-induced peritonitis model revealed that female C57BL/6 mice recruited lower levels of monocytes and neutrophils into peritoneum compared to male mice (Kay et al., 2015). Given that adiponectin levels were shown to change upon sexual maturation, sex related discrepancies might be taken into consideration in studies.

3.3.4 Anti-inflammatory effects of adiponectin acting on BEC

For the first time, we could show that the inhibitory effect of adiponectin in T cell migration is not only mediated through PBL, but also through acting on BEC, which led us to consider the involvement of adiponectin receptor expressed on BEC. There are previous evidence showing the expression of AdipoR1/R2 and CDH13 on endothelial cells and that they mediate protective effects of adiponectin (Takeuchi et al., 2007). For example Zhang et al, showed overexpression of AdipoR1/R2, induced via adenovirus, in TNF-α–stimulated HUVEC resulted in decreased

expression of ICAM-1 and NF-κB activation (Zhang et al., 2009). In addition, *in vivo* data from same study showed that overexpression of AdipoR1/R2 in carotid arteries of rat significantly reduced the expression of ICAM-1 *in vivo* (Zhang et al., 2009). Later study by Zhao et al, reported the presence of AdipoR1 on HUVEC rescues the endothelial cells from HG induced apoptosis via activating AMPK (Zhao et al., 2011). Overall, these studies provide evidence for the fact that adiponectin is able to exerts its protective and anti-inflammatory effects via acting on BEC through binding to AdipoR1/R2.

There are also evidence supporting the absence CDH13 independently results in vascular problems due to a lack of adiponectin signaling (Denzel et al., 2010). Using CDH13 knock out mice, Denzel et al, showed that in the absence of CDH13, adiponectin failed to accumulate in cardiac tissue and that this was associated with higher levels of adiponectin in circulation (Denzel et al., 2010). In addition, both CDH13 deficient and adiponectin deficient mice showed the same levels of infarct size in cardiac tissue (Denzel et al., 2010). This was further supported with another *in vivo* study where they investigated the role of CDH13 in a mice model of hind limb ischemia where blood flow was disrupted surgically in one limb (Parker-Duffen et al., 2013). They found that CDH13 deficiency in this model resulted in an impaired blood flow recovery compared with wild-type controls, similar to what they observed in adiponectin deficient model of these mice (Parker-Duffen et al., 2013). Furthermore, administration of exogenous adiponectin to adiponectin knock out mice was able to improve the revascularization in these mice (Parker-Duffen et al., 2013). However, this treatment strategy had no effect in CDH13 knock out mice. These data together demonstrates that adiponectin's protective functions is highly depend on CDH13 in different vascular beds.

In our study, to ensure the involvement of CDH13 and AdipoRs in T cell migration, we proceeded to measure the pathway in absence of AdipoR1/R2 and CDH13 in BEC as we have shown expression on these cells. Previously, preliminary data from our group (M. Chimen and M. Hussain) showed that the inhibitory effects of adiponectin on PBL migration was lost upon CDH13 and AdipoRs combined knock-down in BEC. Here, as no receptor antagonists for CDH13 or adiponectin receptors were available at the time of investigate, we opted to use siRNA knockdown the CDH13, AdipoR1 or R2 individually to investigate their roles in lymphocyte trafficking. Our data demonstrates the ability of these receptors to individually influence PBL migration and thus potentially impact on the PEPITEM pathway and for the first time our study highlight s an important capacity of CDH13 in regulating leukocyte trafficking.

3.3.5 Adiponectin-CDH13 interaction

Although many studies confirmed the importance of CDH13 in protective effects of adiponectin, alterations in CDH13 expression in inflammation is not clear. To address this, we tried to measure both surface and intracellular expression of CDH13 in base line and activated BEC with TNF-α and IFN-γ. Preliminary (unpublished) flow cytometry data from our lab (M. Chimen and M. Hussain), with n=1 experiment, has shown that CDH13 surface expression was decreased in inflamed HUVEC and adiponectin treatment of HUVEC increase the surface amount of CDH13, suggesting that CDH13 is shed upon inflammation and that supplementation of BEC with adiponectin was able the keep the CDH13 in surface of the endothelial cells and rescue it from being shed. This was further supported by a western blot data (unpublished) from our group (J. Reyat) where he showed increased protein amount of CDH13 in the supernatant of cytokine

treated endothelial cells compared to unstimulated, suggesting that CDH13 is shed during inflammation. These preliminary data are consistent with our hypothesis that, CDH13 binds to adiponectin on surface of endothelial cells and presented the adiponectin to AdipoRs on B cells to trigger the PEPITEM pathway. Here we tried to reproduce these experiments with more N numbers, however our flow cytometry data does not agree with these preliminary data, as we observed an increased surface expression of CDH13 in stimulated BEC, which does not agree with CDH13 being shed in inflammation. Indeed, in our study, first we failed to obtain a valid data from surface expression of CDH13 using flow cytometry, since we saw a dramatic reduction in BEC population. Later we optimised our protocol by staining BEC in plates and we achieved adequate population in flow cytometry. However, these discrepancies between our data, and previous preliminary data obtained in our lab, might be related to the source of adiponectin, or the type of endothelial cells were used in our study as previous experiments were done on HUVEC which might function differently compared to BEC.

In agreement with our flow cytometry data, overexpression of CDH13 on endothelial cells was shown in oxidative stressed HUVEC using immunohistochemistry, where they interpret this elevation as a protect against stress-induced apoptosis (Joshi et al., 2005). Later the same group provided evidence that oxidative stress induced-CDH13 on endothelial cells can be shed from endothelial cells in amounts indicating the extent of activation or damage (Philippova et al., 2011). Indeed they showed higher levels of CDH13 in culture medium from stressed/apoptotic HUVEC compared to normal proliferating HUVEC using immunoblotting (Philippova et al., 2011). Very recent research has described a different mechanism for CDH13 signalling, reporting internalization of adiponectin through endosomal system after binding to CDH13 on surface of F2 endothelial cells (CDH13-expressing cells), and accumulation in site of exosome generation inside

multivesicular bodies (Obata et al., 2018). This study interpreted adiponectin protective effects through its contribution to biogenesis and release of exosomes containing ceramides, which were dependent on CDH13, but not on AdipoR1 or AdipoR2 expression in F2 endothelial cells, as knockdown of AdipoR1 or AdipoR2 in these cells had no effect on adiponectin-stimulated exosome production (Obata et al., 2018). That said, this would appear to be an unlikely mechanism to explain our data, as we find higher surface expression upon inflammation.

There are studies showing colocalization of CDH13 and adiponectin at cellular levels; Adiponectin was not detected in myocytes of CDH13 deficient mice (Parker-Duffen et al., 2013). However, absence of AdipoR1/R2 had no effect on cellular levels of adiponectin (Parker-Duffen et al., 2013)., suggesting that CDH13 is essential for adiponectin localization. To examine this activity in endothelial cells in our study, we tried to evaluated interaction of adiponectin and CDH13 at cellular levels using confocal imaging, however we saw no effects of adiponectin in cellular levels of CDH13. Indeed, our antibodies were not optimal enough as we found localization of CDH13 in nucleus, which was not expected, and we were not able to make any definitive conclusions on this. Further *in vitro* experiments with different secondary antibodies are needed to optimise this experiment in future, as the staining should not be nuclear as demonstrated in other studies. Once we had validated antibodies, additional conditions with knock down of CDH13 and AdipoRs could be undertaken to help us to investigate the role of CDH13 in adiponectin signalling.

3.3.6 Limitation

This study has limitations as our static *in vitro* assay did not allow us to analyse dynamic behaviour of leukocytes. Therefore, reproducing the data using flow assay would reflect the data

in a more physiological context. Moreover, the transfection of BEC with siRNAs caused a reduction in BEC population, which might have affected the transmigration and flow cytometry data. Thus, using better blocking options like antagonists of receptors would improve the data in these experiments.

3.3.7 Conclusion

We demonstrated that adiponectin prevents lymphocyte trans-endothelial migration by acting on PBL and directly on BEC. Our study also demonstrated a crucial role of CDH13 in regulating the trans endothelial migration of lymphocytes via PEPITEM pathway. However, our study does not provide a mechanism of effect for CDH13, and more *in vitro* experiments are needed to clarify the signal transduction of the CDH13.

4 AN EXAMINATION OF IMMUNOMODULATORY EFFECT OF HIGH GLUCOSE, METFORMIN AND, INSULIN

4.1 Introduction

Hyperglycaemia has been identified as a main factor in the development of diabetic microvascular disease (Morigi et al., 1998), but the mechanisms responsible for the toxicity of hyperglycaemia on endothelial functions and their implication for cell–cell interaction in the microcirculation are not clear (Morigi et al., 1998). Previous studies have shown increased endothelium-leukocyte interaction in hyperglycaemic conditions *in vitro* (Kumar et al., 2014) and *in vivo* (Booth et al., 2001). However, most studies in this area have studied the whole leukocyte population, whilst the effect of HG on lymphocyte migration remains unclear. In this chapter we explored the effects of HG on PBL interactions with the endothelium and on the ability of endothelium to support these interactions. Since metformin and insulin are widely used in patients with T2DM for their glycaemic control and beneficial vascular effects, we also studied their effects on leukocyte-endothelium interactions. To investigate the mechanisms of action in hyperglycaemia we evaluated the expression of adhesion molecules on BEC. Finally, since AdipoRs expression on B cells from T1DM patients was decreased limiting their production of PEPITEM (Chimen et al., 2015), we hypothesised that hyperglycaemia decreases the expression of AdipoRs and therefore the PEPITEM pathway in T2DM. Using an *in vitro* experiment, we evaluated the expression of AdipoRs on B-cells and other subsets of PBMCs following treatment with HG, metformin and/or insulin.

4.2 Results

4.2.1 Effect of HG on PBL adhesion and migration

HG has been shown to increase expression of pro-inflammatory cytokines in T cells, which lead to leukocyte activation and adhesion (Kumar et al., 2014). To determine whether hyperglycemia affects PBL transmigration in healthy controls, we used an *in vitro* human vascular tissue model to study the sole effect of HG treatment on either lymphocyte or endothelial cell behavior. Pre-treating BEC with HG for 24 hours had no effect on PBL adhesion (Figure 4.1-A), but significantly reduced PBL transmigration when compared to BEC treated with normal glucose (Figure 4.1-B). In contrast, pre-treating PBL with HG for 1 hour had no effect on their adhesion or their migration (Figure 4.1-C and D). However, exposing the PBL to HG for 24 hours significantly increased both their adhesion (Figure 4.1-E) and migration when compared to the cells treated with normal glucose (Figure 4.1-F). Thus, suggesting that HG differently affects the endothelium and PBL behavior to influence lymphocyte trafficking.

4.2.2 Effect of Metformin on PBL adhesion and migration

Metformin is widely used for the treatment of T2DM and acts primarily by reducing the hepatic glucose output and improving insulin sensitivity (Cameron et al., 2016). However, in both experimental and clinical studies metformin is associated with anti-inflammatory response, which is exerted irrespective of its anti-hyperglycaemic properties (Hattori et al., 2015; Cameron et al., 2016). To determine whether metformin could influence lymphocyte recruitment in a normal glucose environment, we pre-treated either BEC or lymphocytes prior to assessing PBL adhesion and migration to TNF-α/IFN-γ-stimulated BEC. Metformin treatment of either cell type had no effect on PBL adhesion to or migration through TNF-α/IFN-γ-stimulated BEC (Figure 4.2), indicating metformin does not regulate endothelium-lymphocyte interaction under normal glucose conditions.

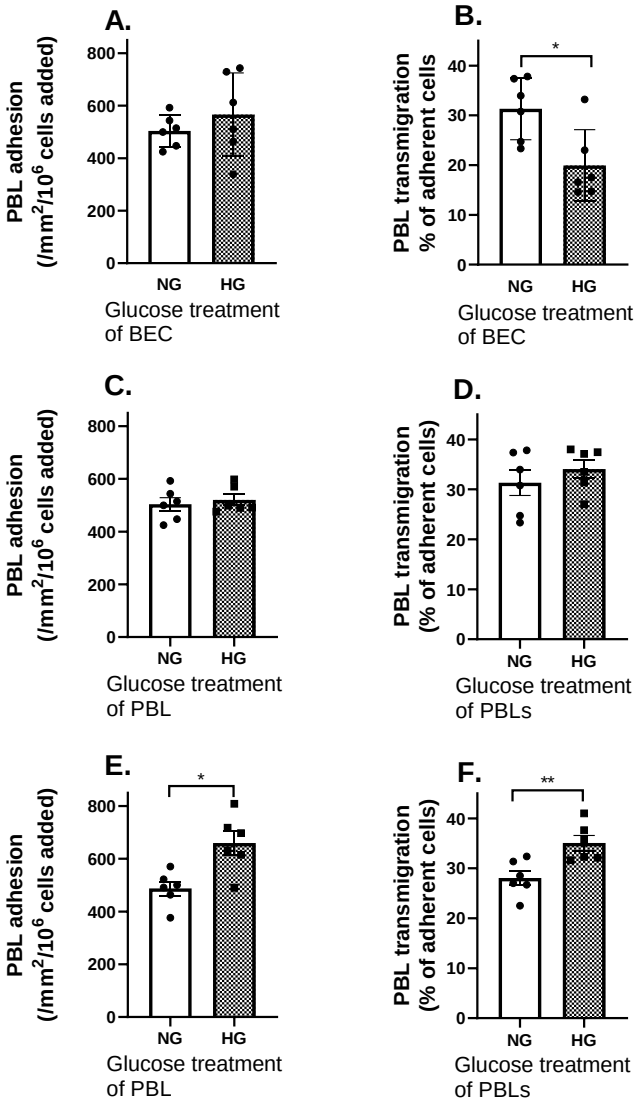


Figure 4. 1 The effect of high glucose treatment on lymphocyte recruitment to inflamed endothelium. BECs were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ and exposed to normal (5mM; NG) or high dose (30mM; HG) for 24 hours. PBLs were isolated, **(A, B)** left untreated or **(C-F)** treated with NG or HG for **(C, D**) 1 hour or **(E, F)** 24 hours. PBLs (1×10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A, C, E)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B, D, F)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. Data are shown as mean ± SEM for n = 6 independent experiments, using 2 different BEC donors and 6 different lymphocyte donors. *= $p<0.05$ and ** = $p<0.01$ by paired t-test compared to NG.

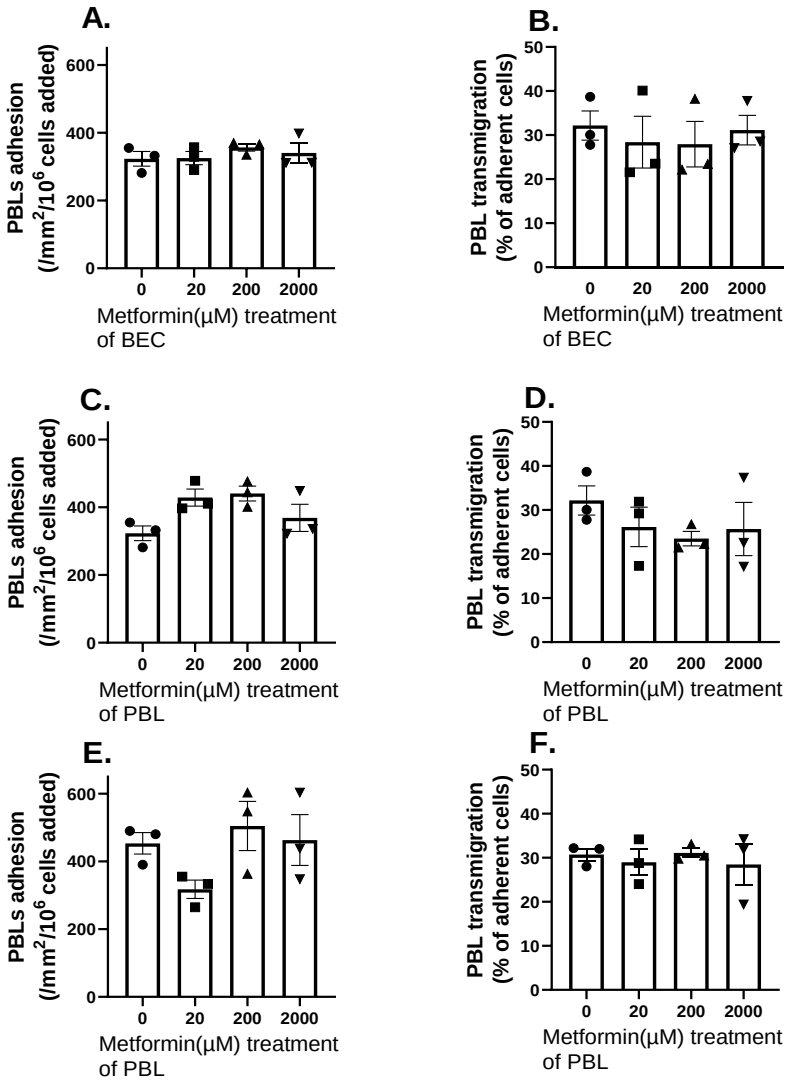


Figure 4.2 The effect of metformin treatment on lymphocyte recruitment to inflamed endothelium. BECs were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ in the **(C-F)** absence or **(A, B)** presence of metformin at 20 μM, 200 μM and 2000 μM (2mM) for 24 hours. PBLs were isolated, **(A, B)** left untreated or **(C-F)** treated with metformin at 20 μM, 200 μM and 2000 μM for **(C, D)** 1 hour or **(E, F)** for 24 hours. PBLs (1×10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A, C, E)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B, D, F)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. In **A-F**, ANOVA showed no effect of metformin concentration on PBL adhesion or transmigration. Data are shown as mean ± SEM for n = 3 independent experiments, using 1 BEC donor and 3 different lymphocyte donors.

1.1.1 The effect of metformin or high glucose treatment on monocyte or neutrophil recruitment to inflamed endothelium

Hyperglycemia-induced endothelial cell dysfunction in diabetes has been reported to enhance the leukocyte interaction with endothelium leading to vascular inflammation (Burlet and Jain, 2013; Sundararajan et al., 2019; Wang et al., 2019). One of the earliest events in this inflammatory process is increased binding of neutrophils (Berezin, 2019) and monocytes (Babu et al., 2012) to endothelial cells. Here, pre-treating BEC with HG or metformin had no effect on monocyte (Figure 4.3) or neutrophil (Figure 4.4) adhesion to or migration through TNF-α/IFN-γ-stimulated BEC. These data suggest the effects of HG are specific to lymphocyte recruitment, whilst once again metformin treatment is unable to not modulate leukocyte recruitment, specifically monocyte or neutrophil recruitment.

4.2.3 Combined effect of high glucose and metformin treatment on lymphocyte recruitment to inflamed endothelium

Although metformin treatment of either BEC or PBL had no effect on lymphocyte recruitment in a normal glucose environment (Figure 4.2**)**, we tested whether metformin could influence the PBL recruitment post HG treatment. Metformin treatment of BEC post HG treatment had no effect on PBL adhesion (Figure 4.5-A), but significantly increased lymphocyte transmigration back to the levels observed on BEC cultured in normal glucose (Figure 4.5-B). In contrast, metformin treatment of PBL post HG treatment had no effect on their adhesion to or migration through TNF-α/IFN-γ-stimulated BEC (Figure 4.5-C and D). These data indicate that metformin treatment of BEC restores the lymphocyte transmigration to control levels.

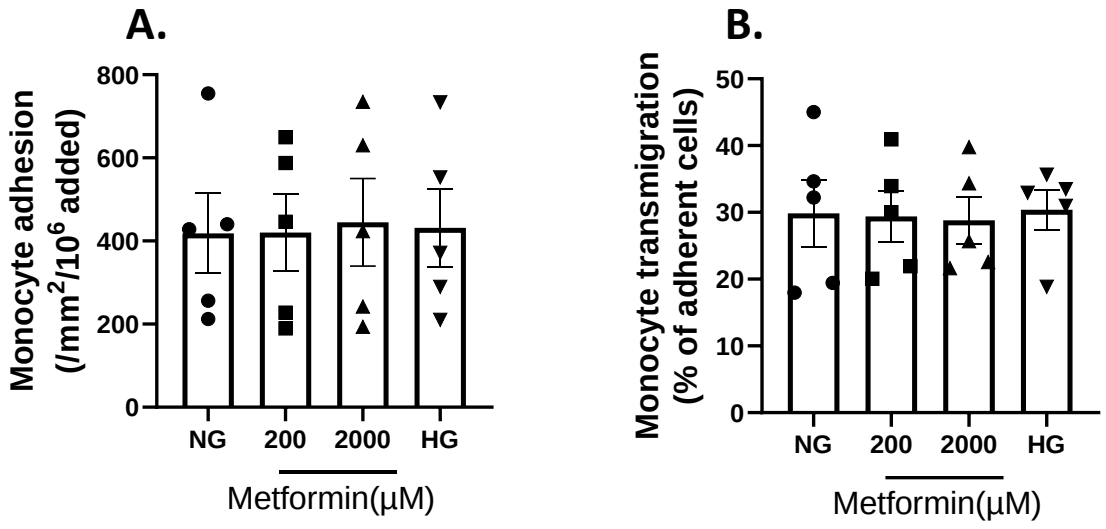


Figure 4.3 The effect of metformin or high glucose treatment on monocyte recruitment to inflamed endothelium. BECs were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ and exposed to normal glucose in absence (5mM; NG) or presence of metformin (200 μM, 2000 μM) or high glucose (30mM; HG) for 24 hours. Purified monocytes (1×10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A)** The number of adherent monocytes expressed as the number of cells/$mm^2/10^6$ cells added. **(B)** Monocyte transmigration expressed as the percentage of adherent cells that had transmigrated. In **A-B**, ANOVA showed no effect of treatment on monocyte adhesion or transmigration. Data are shown as mean ± SEM for n = 5 independent experiments, using 2 different BEC donors and 5 different blood donors.

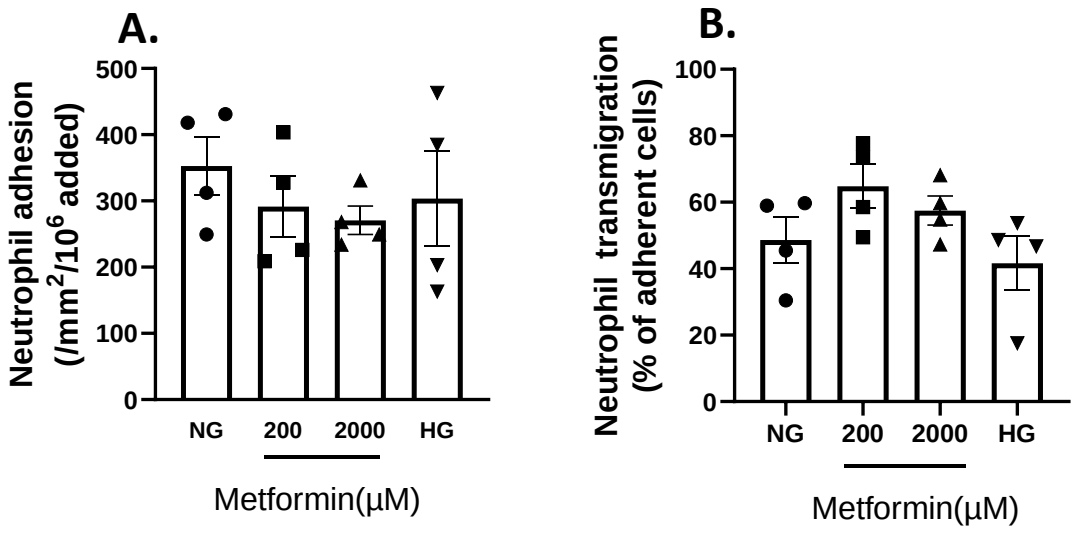


Figure 4.4 The effect of metformin or high glucose treatment on neutrophil recruitment to inflamed endothelium. BECs were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ and left at normal glucose (NG) or exposed to metformin (200µM**,** 2000µM) in normal glucose or high glucose only (30mM; HG) for 24 hours. Neutrophils (1×10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. (**A**) The number of adherent neutrophils expressed as the number of cells/$mm^2/10^6$ cells added. (**B**) Neutrophil transmigration expressed as the percentage of adherent cells that had transmigrated. In **A**-**B**, ANOVA showed no effect of treatment on neutrophil adhesion or transmigration. Data are shown as mean ± SEM for n = 5 independent experiments, using 2 different BEC donors and 5 different blood donors.

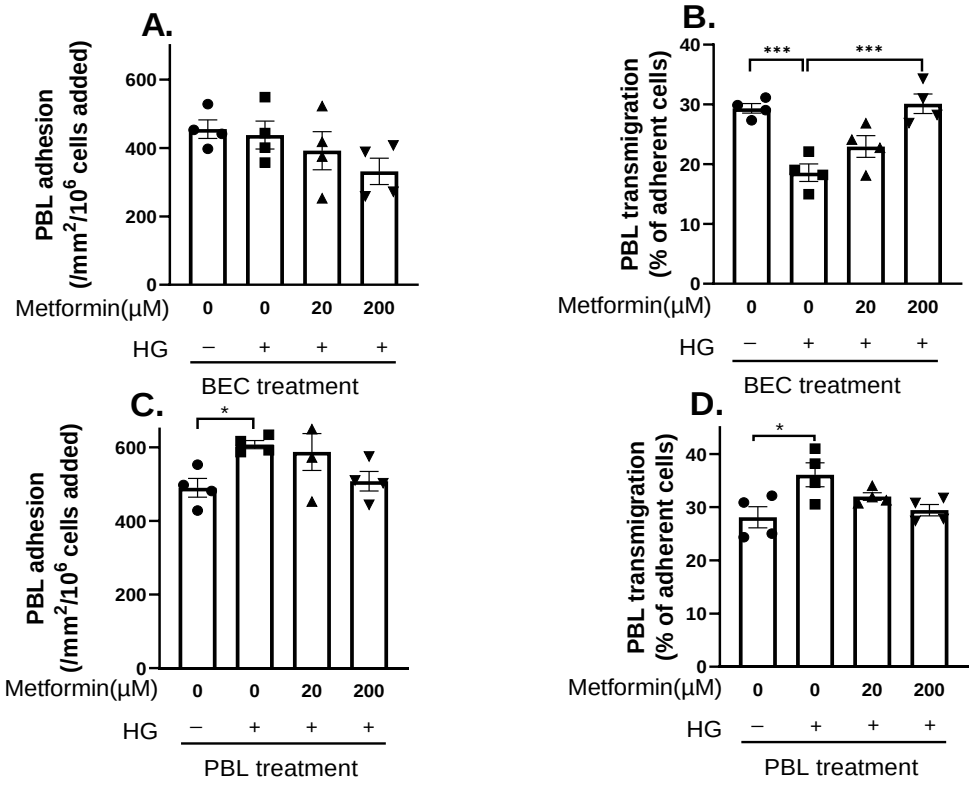


Figure 4.5 Combined effect of high glucose and metformin treatment on lymphocyte recruitment to inflamed endothelium. BEC were stimulated with TNF-α/IFN-γ in **(C, D)** normal or **(A, B)** high glucose (HG) for 24 hours, followed by metformin treatment (20μM and 200μM) for 4 hours. PBLs were isolated and **(A, B)** left untreated or **(C, D)** treated with HG for 24 hours, followed by metformin treatment (20 μM, 200 μM) for 4 hours. PBLs (1×10^6 cells/ml) were added to **(A, B)** treated or **(C, D)** untreated BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. **(A, C)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B, D)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. In **B**, ANOVA showed a significant effect of metformin on lymphocyte migration; $P<0.001$. Data are shown as mean ± SEM for n = 4 independent experiments, using 1 BEC donor and 4 different lymphocyte donors. ***= $P<0.001$ compared to HG, no metformin treatment by Dunnett post-test.

4.2.4 Effect of Insulin on PBL adhesion and migration

Insulin is one of common treatment options for many patients with T2DM (Cahn et al., 2015). Alterations in insulin regulation are shown to compromise the immune function in T2DM (Xiu et al., 2014). Insulin exerts its effects on immune cells by binding to the insulin receptor, which is extensively expressed on neutrophils and monocytes/macrophages (Gyurko et al., 2006). There are controversial findings regarding the effects of insulin on leukocyte trafficking: Insulin was reported to increased neutrophil-endothelial interaction; while it was shown to reduce monocyte adhesion to endothelium *in vivo* (Okouchi et al., 2002). To our knowledge, there are no studies showing the direct effects of insulin on lymphocyte recruitment. We found that insulin treatment of either BEC or PBL had no effect on PBL adhesion to or migration through TNF-α/IFN-γ-stimulated BEC (Figure 4-6), indicating insulin is unable to regulate endothelium-lymphocyte interaction under normal glucose conditions.

4.2.5 Combined effect of high glucose and insulin treatment on lymphocyte recruitment to inflamed endothelium

We showed no effects of insulin on PBL recruitment through the inflamed endothelium in NG environment. Next, we aimed to investigate whether insulin could influence the PBL recruitment post glucose treatment. Insulin treatment of BEC post HG treatment had no effect on PBL adhesion (Figure 4.7-A), but significantly increased lymphocyte transmigration back to the levels observed on BEC cultured in normal glucose (Figure 4.7-B). In contrast, insulin treatment of PBL post HG treatment had no effect on their adhesion to or migration through TNF-α/IFN-γ-stimulated BEC (Figure 4.7-C and D). These data indicate that insulin treatment of BEC restores the lymphocyte transmigration to control levels.

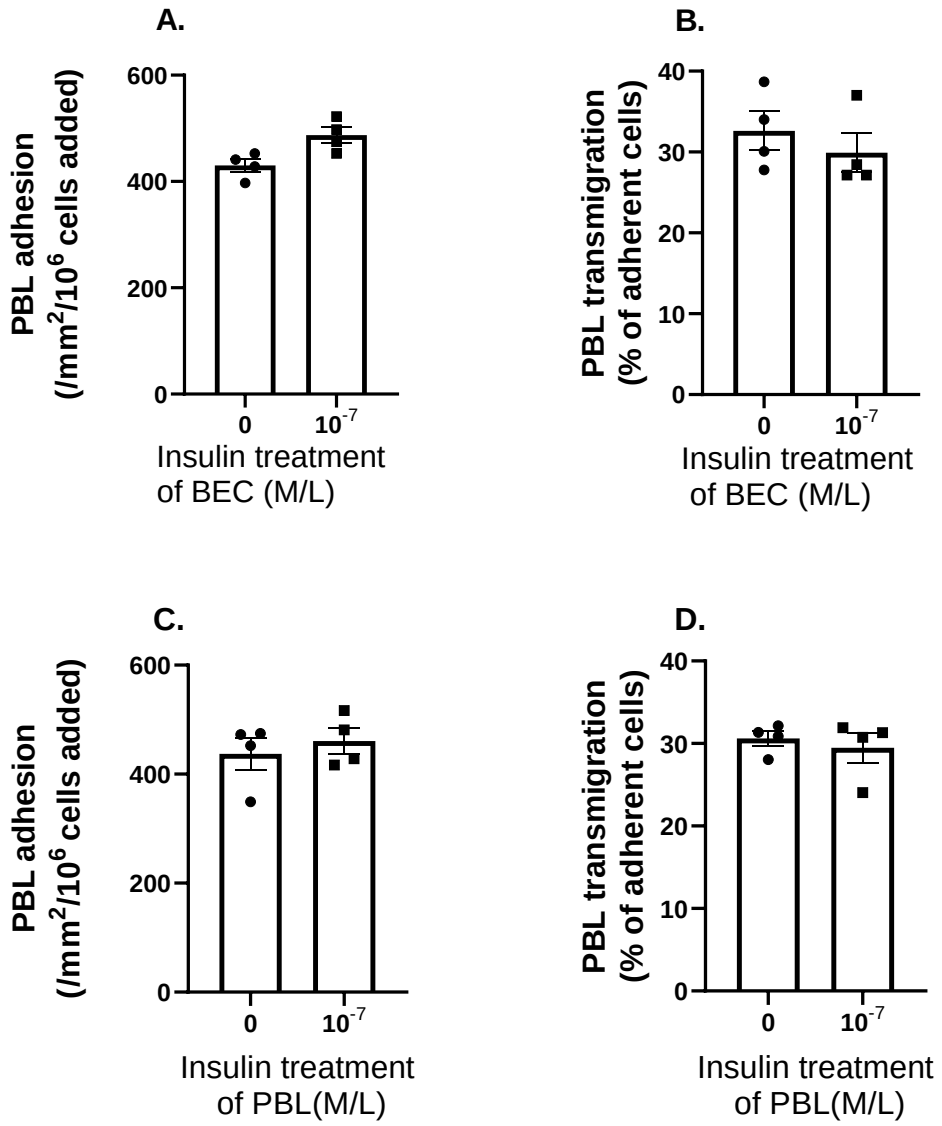


Figure 4.6 The effect of insulin treatment on lymphocyte recruitment to inflamed endothelium. BEC were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ in the (**C** and **D**) absence or (**A** and **B**) presence of insulin at 10^{-7}M/L for 24 hours. PBLs were isolated, (A, B) left untreated or (**C, D**) treated with insulin at 10^{-7}M/L for 24 hours. PBLs (1×10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. (**A, C**) The number of adherent PBLs expressed as the number of cells/mm^2/10^6 cells added. (**B, D**) Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. In A-F, ANOVA showed no effect of insulin concentration on PBL adhesion or transmigration. Data are shown as mean ± SEM for n = 4 independent experiments, using 1 BEC donor and 4 different lymphocyte donors.

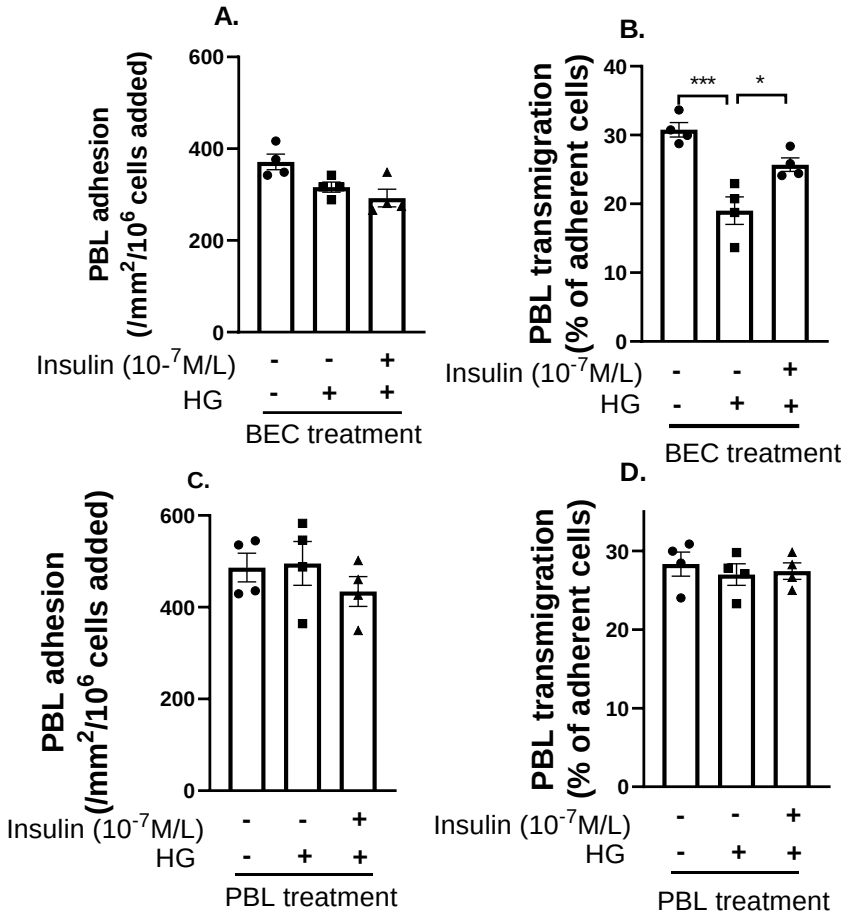


Figure 4.7 Combined effect of high glucose and insulin treatment on lymphocyte recruitment to inflamed endothelium. BEC were stimulated with TNF-α/IFN-γ in (**C, D**) normal or (**A, B**) high glucose (HG) for 24 hours, followed by insulin treatment (10^{-7}M/L) for 24 hours. PBLs were isolated and (**A, B**) left untreated or (**C, D**) treated with HG for 1 hours, followed by insulin treatment (10^{-7}M/L) for 1 hours. PBLs (1×10^6 cells/ml) were added to (**A, B**) treated or (**C, D**) untreated BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired and analysed offline. (**A, C**) The number of adherent PBLs expressed as the number of cells/mm^2/10^6 cells added. (**B, D**) Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. In A and B, ANOVA showed a significant effect of insulin on lymphocyte migration; Data are shown as mean ± SEM for n = 4 independent experiments, using 1 BEC donor and 4 different lymphocyte donors*= $p<0.05$, **= $P<0.01$ and ***= $P<0.001$, compared to HG treatment by Dunnett post-test.

4.2.6 Effects of high glucose or metformin on the expression of AdipoR1/2 on PBMC subsets

Reduced AdipoRs expression on B cells from patients with T1DM (Chimen et al., 2015) led us to hypothesise that the regulatory PEPITEM pathway is also modulated in T2DM individuals. Therefore, we tested if HG or metformin could modulate the pathway by influencing the AdipoRs expression on B cells of healthy donors. We previously showed that AdipoRs are mainly expressed on B cells, NK-cells, and monocytes. We found no difference in surface expression or frequency of AdipoR1/2 in leukocyte subsets following 24 hours treatment with HG or metformin under normal glucose conditions (Table 4.1). Suggesting HG and/or metformin does not influence lymphocyte recruitment through modulating AdipoRs.

4.2.7 Combined effect of high glucose and metformin on adiponectin receptors expression by PBMCs subsets

Since the metformin showed no effect on surface expression or frequency of AdipoR1/2 in leukocyte subsets in NG condition, we tested the effect of metformin on expression of these receptors post HG treatment of PBL. As shown in Table 4.2, metformin treatment of PBL had no significant effects on AdipoR1/R2 expression on B cells, NKs and monocyte populations.

4.2.8 Combined effect of high glucose and insulin on adiponectin receptors expression by PBMCs subsets

To investigate the combined effect of HG and insulin on adiponectin receptors expression, we did slightly different experiment. We exposed the PBL to HG only for 1 hour, followed by 1 hour insulin treatment. In B cells, glucose treatment for 2 hours (which was used as a HG control) significantly increased the surface expression of AdipoR1 (Figure 4.8-A), and AdipoR2 (Figure 4.8-A) compared to NG. We observed no difference in AdipoR1/R2 expression in insulin treated compared to HG group (Figure 4.8). We also found no significant difference in AdipoR1/R2 expression in NKs and monocyte population (Figure 4.8-E) Indicating insulin is not able to modulate AdipoRs in HG condition.

Table 4.1 Effect of high glucose and metformin treatment on Adiponectin receptors expression by PBMCs subsets.

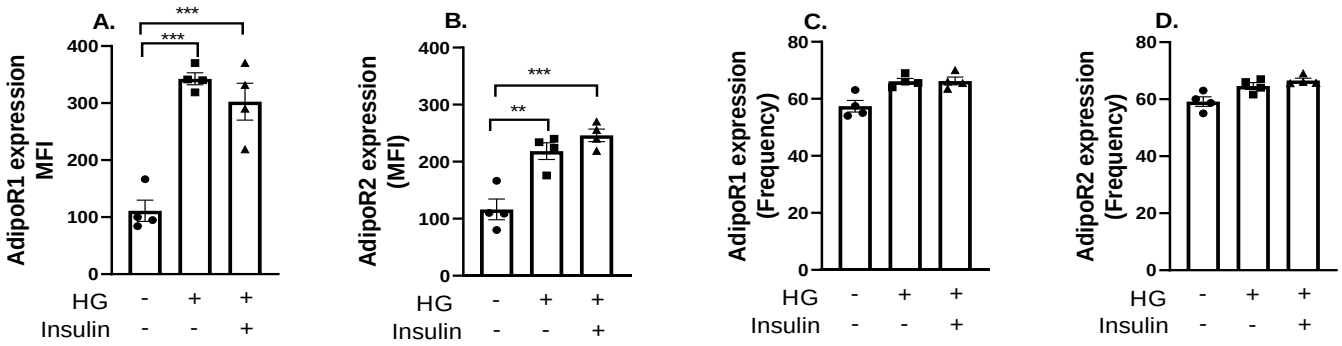
		AdipoR1				AdipoR2			
		MFI		Frequency		MFI		Frequency	
Leukocyte subset	Condition	Fresh blood	Leukocyte cone	Fresh blood	Leukocyte cone	Fresh blood	Leukocyte cone	Fresh blood	Leukocyte cone
B cell	Baseline	111.3 ± 13.64	222.6 ± 58.87	74.42 ± 2.761	83.67 ± 2.338	115.2 ± 37.06	99.08 ± 40.73	71.95 ± 4.499	74.50 ± 2.802
	NG	71.22 ± 20.04	100.6 ± 28.07	73.20 ± 3.439	72.50 ± 6.062	32.27 ± 9.476	65.14±9.663	61.78 ± 6.659	65.07 ± 2.130
	HG	93.64 ± 39.56	64.44 ± 18.51	71.43 ± 2.454	69.57 ± 4.921	31.42 ± 7.349	58.14 ± 21.58	58.10 ± 5.776	65.40 ± 11.51
	Metformin (200 µM)	105.2 ± 23.23	86.14 ± 9.975	73.98 ± 2.574	71.17 ± 3.835	41.74 ± 10.93	42.97±11.57	61.40 ± 4.793	63.90 ± 2.801
	Metformin (2000 µM)	56.94 ± 19.66	80.78 ± 33.38	66.63 ± 2.360	72.93 ± 3.205	40.97 ± 15.58	51.29 ± 20.32	58.06 ± 3.426	63.00 ± 6.409
Monocyte	Baseline	252.3 ± 72.26	324.5 ± 52.44	85.21 ± 2.667	86.78 ± 2.914	112.4 ± 60.71	167.0 ± 131.7	85.73 ± 2.586	85.43 ± 2.589
	NG	241.6 ± 58.45	166.0 ± 41.86	73.05 ± 1.552	86.80 ± 1.457	72.70 ± 31.11	82.57 ± 45.53	60.43 ± 8.173	79.85 ± 6.138
	HG	185.9 ± 42.05	136.6 ± 25.22	72.58 ± 2.071	84.40 ± 4.055	46.13 ± 14.91	58.18 ± 25.35	62.63 ± 2.855	76.72 ± 11.42
	Metformin (200 µM)	330.0 ± 37.63	302.8 ± 73.34	73.45 ± 1.291	89.18 ±0.5862	52.48 ± 7.105	122.3 ± 67.75	64.65 ± 3.452	81.18 ± 5.817
	Metformin (2000 µM)	168.3 ± 18.69	130.6 ± 21.73	71.23 ± 3.658	87.13 ± 1.571	73.03 ± 19.75	136.9 ± 81.37	63.35 ± 4.804	77.18 ± 10.19
NK	Baseline	131.5 ± 31.50	185.2 ± 62.41	78.98 ± 6'018	80.66 ± 3.567	62.67 ± 11.71	38.05 ± 1.567	76.08 ± 2.762	75.46 ± 3.666
	NG	77.95 ± 27.62	121.2 ± 24.49	67.25 ± 5.433	65.95 ± 13.69	27.35 ± 11.08	38.01 ± 7.557	64.23 ± 3.943	59.18 ± 9.122
	HG	74.35 ± 25.51	90.42 ± 12.75	66.14 ± 4.436	68.08 ± 14.47	21.63 ± 4.993	69.25 ± 26.28	64.77 ± 3.943	59.12 ±11.29
	Metformin 200 µM	111.8 ± 24.31	187.5 ± 43.22	67.73 ± 5.209	82.03 ± 4.975	36.07 ± 10.90	45.44 ± 18.44	63.43 ± 4.615	72.23 ± 2.225
	Metformin (2000 µM)	63.28 ± 21.05	41.85 ± 41.85	66.20 ± 4.452	66.82 ± 13.06	28.37 ± 13.43	50.11 ± 15.59	63.97 ± 3.89	58.68 ± 9.045

AdipoR1 - Adiponectin Receptor 1; AdipoR2 - Adiponectin Receptor 2; HG – 30mM glucose; MFI – mean fluorescence intensity; NG – 5mM glucose. ANOVA showed no significant effect of treatment on adiponectin receptor expression. Data are mean ± SEM for n = 7 independent experiments, using 4 different blood donors and 3 different leukocyte cones.

Table 4.2 Combined effect of high glucose and metformin on adiponectin receptors expression by PBMCs subsets.

		AdipoR1 MFI	AdipoR1 Frequency	AdipoR2 MFI	AdipoR2 Frequency
B-cells	Baseline	111.3±1.6	57.40±2.03	116.3±18.3	59.18±1.5
	HG	94.8±18.3	55.87±5.1	46.66±12.9	59.2±1.1
	HG+ metformin	98.1±13.2	57.1±65	58.77±65	58.86±1.4
Monocyte	Baseline	146.75±19.4	85.7±1.19	92.8±14.8	84.4±2.7
	HG	138.43±12.7	85.4±2.2	72.32±10.3	85.43±1.7
	HG+ metformin	144.30±13.4	84.5±1.5	81.11±11.2	84.1±1.2
NKs	Baseline	112.4±20.2	57.1±1.6	61.88±6.51	64±6.2
	HG	94.2±13.1	55.6±2.3	55.87.4±13.6	66.01±4.4
	HG+ metformin	110.02±20	57.8±1	64.76±16.6	64.22±8.6

AdipoR1 - Adiponectin Receptor 1; AdipoR2 - Adiponectin Receptor 2; HG – 30mM glucose; Metformin 200 µM; MFI – mean fluorescence intensity; ANOVA showed no significant effect of treatment on adiponectin receptor expression. Data are mean ± SEM for n = 4 independent experiments, using 4 different blood donors.



E.		AdipoR1 MFI	AdipoR1 Frequency	AdipoR2 MFI	AdipoR2 Frequency
Monocyte	Baseline	146.75±19.4	85.7±1.19	92.8±14.8	84.4±2.7
	HG	144.4±22.4	86.25±1.4	79.78±25.78	84.38±1.44
	HG+Insulin	123.65±20.29	87.4±0.4	67.73±30.9	84.39±1.8
NKs	Baseline	112.4±20.2	57.1±1.6	61.88±6.51	64±6.2
	HG	104.2±14.7	59.40±3.77	58.25 ±9.25	63±6
	HG+Insulin	104.7±10.73	59.70±3.19	52.22 ±9.22	60±6.3

Figure 4.8 Combined effect of high glucose and insulin on adiponectin receptors expression by PBMCs subsets. PBMCs were isolated from whole blood and treated with HG (30mM) for 1 hour, followed by insulin treatment (10^{-7}M/L) for 1 hours. AdipoRs expressions were measured using flow cytometry and quantified as frequency of cells expression AR1/2 or MFI on (**A-D**) B-cells, (**E**) monocytes and NKs. Data are mean ± SEM from n=4 independent experiments using 4 different blood donors. ** = $p<0.01$ and *** =$p<0.001$ by Bonferroni's multiple comparison posttest.

4.2.9 Effect of high glucose, metformin, or insulin treatment on expression of endothelial adhesion molecule

It is unclear by which mechanism hyperglycemia alters the function of vascular cells and leukocyte trafficking in T2DM. There are evidence showing the variations in the expression of adhesion molecules on the vasculature, enhancing leukocyte adhesion in patients with T2DM. Here, we analysed whether the treatment of BEC with HG, metformin or insulin could modulate the surface expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin. As expected, we saw up-regulation of ICAM-1 (Figure 4.9-A) and VCAM-1 (Figure 4.9-C and D) in response to cytokine treatment. Surface expression of E-selectin did not significantly change at this time point (24 hour) (Figure 4.9-E). HG treatment of BEC increased surface expression of ICAM-1(Figure 4.9-A) and frequency of both VCAM-1 (Figure 4.9-D) and E-selectin (Figure 4.9-F) compared to BEC treated in normal glucose. Treatment with metformin had no significant effect on surface expression or frequency of BEC expressing these adhesion molecules when compared to TNF-α/IFN-γ stimulated BEC control (Figure 4.9**)**, indicating metformin does not modulate adhesion molecules expression in NG condition. However, insulin treatment increased surface expression (Figure 4.9-C) and frequency (Figure 4.9-D) of VCAM-1 compared to TNF-α/IFN-γ stimulated control BEC. Metformin or Insulin treatment of BEC post HG treatment had no effect on surface expression or frequency of BEC expressing these adhesion molecules when compared to TNF-α/IFN-γ stimulated HG treated BEC (Figure 4.9).

We also analysed the gene expression of adhesion molecules in the same conditions we tested for protein expression. We saw up-regulation of ICAM-1 (Figure 4.10-A), VCAM-1 (Figure 4.10-B) and, E-selectin (Figure 4.10-C) in response to cytokine treatment. However, we saw no difference in gene expression of adhesion molecules in HG -cytokine stimulated BEC when

compared to HG -unstimulated BEC (Figure 4.10). Treatment with metformin or insulin had no significant effect on BEC expressing these adhesion molecules when compared to TNF-α/IFN-γ stimulated BEC control (Figure 4.10**)**. Metformin or insulin treatment of BEC post HG treatment had no effect on gene expression of adhesion molecules when compared to TNF-α/IFN-γ stimulated HG treated BEC (Figure 4-10). The data indicates that, HG suppresses gene expression of adhesion molecules in response to cytokine stimulation.

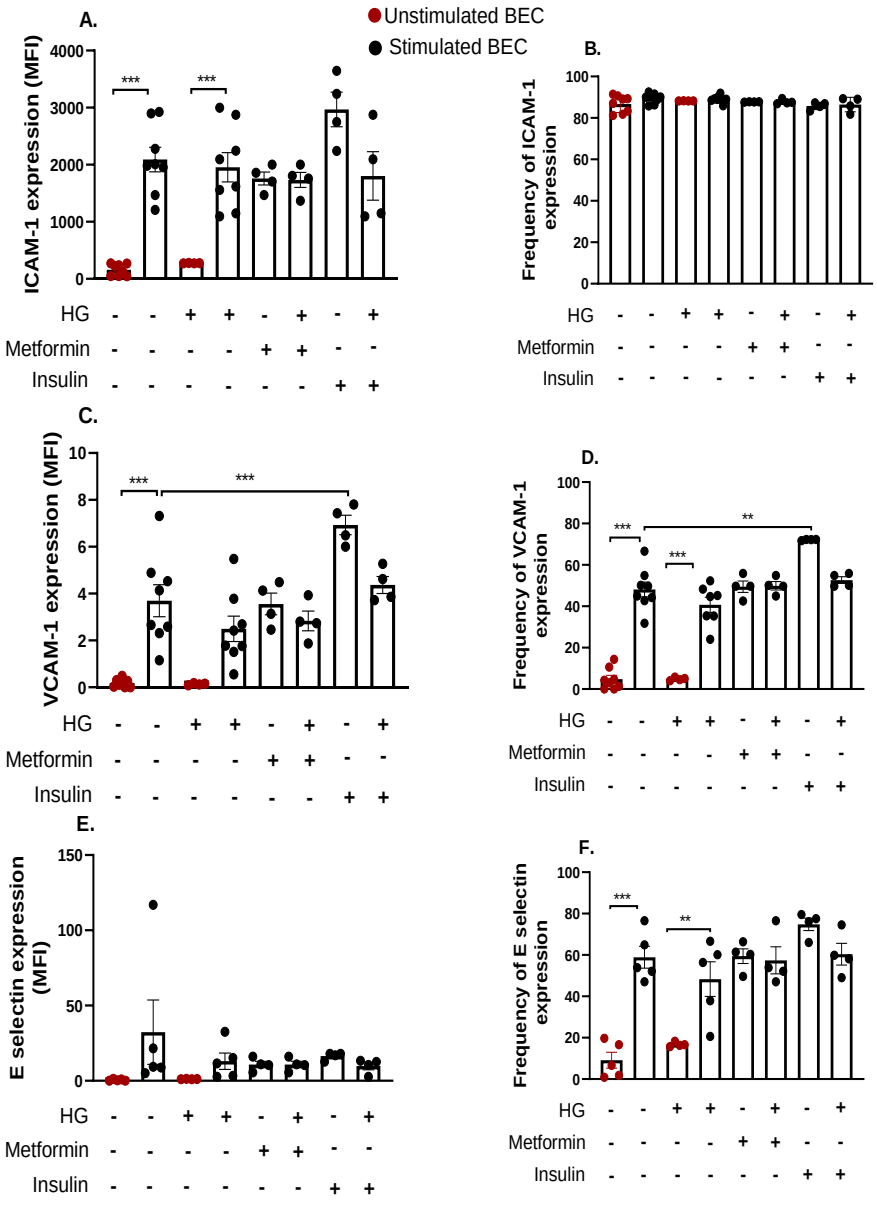


Figure 4-9. Effect of high glucose, metformin, or insulin treatment on surface expression of endothelial adhesion molecule. BECs were stimulated with TNF-α/IFN-γ in presence or absence of high glucose (HG), metformin (200μM) or insulin (10^{7}M) for 24 hours. For combined treatments, BEC were stimulated with TNF-α/IFN-γ in high glucose (HG) for 24 hours, followed by metformin (200μM) or insulin treatment (10^{-7}M/L) for 24 hours.The surface expression of (**A** and **B**) ICAM-1, (**C** and **D**) VCAM-1 and (**E** and **F**) E-selectin was measured by flow cytometry and quantified as median fluorescence intensity (MFI) or frequency of the cells expressing these molecules. In **A, C,D** and **F**, ANOVA showed a significant effect of treatment on adhesion molecules surface expression; P<0.001. Data are shown as mean ± SEM for at least n = 5 independent experiments, using 2 different BEC donors. ***= p<0.001 by Dunnett posttest compared to untreated BEC.

.

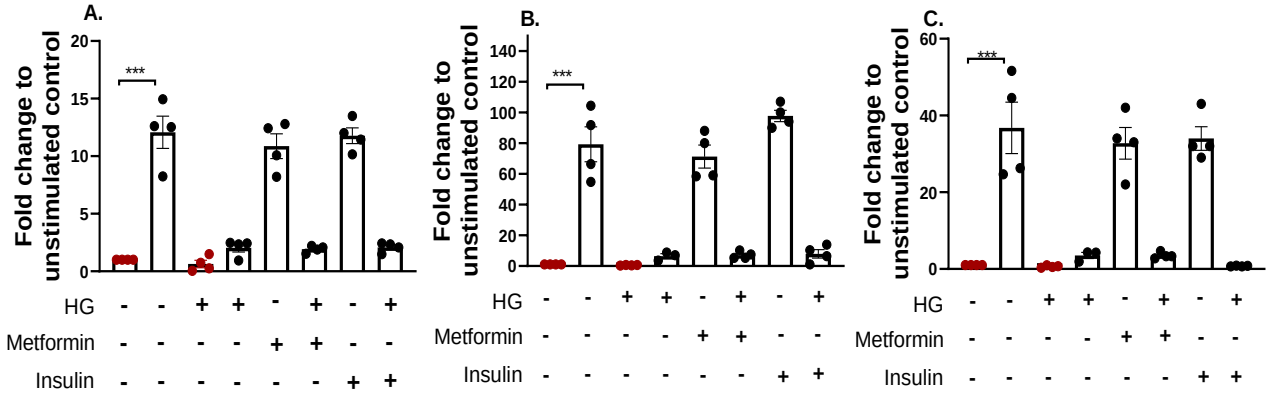


Figure 4.10 Effect of high glucose, metformin, or insulin treatment on gene expression of endothelial adhesion molecule. BEC were stimulated with TNF-α/IFN-γ in presence or absence of high glucose (HG), metformin (200µM/ml) or insulin (10^{7}M/L) for 24 hours. For combined treatments, BEC were stimulated with TNF-α/IFN-γ in high glucose (HG) for 24 hours, followed by metformin (200µM) or insulin treatment (10^{-7}M/L) for 24 hours. The gene expression of **(A)** ICAM-1, **(B)** VCAM-1 and (**C)** E-selectin was measured by qPCR and quantified as fold change to the unstimulated control. In **A, B** and **C** ANOVA showed a significant effect of treatment on adhesion molecules gene expression; Data are shown as mean ± SEM for n = 4 independent experiments, using 2 different BEC donors. ***= p<0.001 by Dunnett post-test compared to untreated BEC.

4.3 Discussion

In this chapter, we aimed to investigate how HG, metformin and insulin modulates the PEPITEM pathway. Using an *in vitro* adhesion model, we found that HG significantly influenced the ability of endothelial cells to support PBL transmigration, such that levels were much lower those seen in normal glucose conditions. Interestingly, we observed no changes in surface expression of adhesion molecule on BEC surface that would explain this reduction. Crucially this effect was reversed by insulin supplementation, as would be the physiological response post digestion, or supplementation with the T2DM treatment metformin. Thus, indicating immunomodulatory effects of insulin and metformin. By contrast, HG treatment for 24 hours, but not 1 hour, enhanced the adhesive and migratory properties of PBL on inflamed BEC, but this was not altered by metformin or insulin. We found no changed in surface expression or frequency of AdipoR1/2 on B cells in response to HG, metformin, or insulin to explain this increase.

We therefore propose that HG differently affects the endothelium and PBL behaviour to support the lymphocytes transmigration through inflamed BEC. We also suggest metformin or insulin treatment of BEC restores the lymphocyte transmigration to control levels, which should clinically translate to decreased vascular complications that are commonly associated with T2DM.

4.3.1 Evidence of High Glucose actions on endothelial cells and mechanisms involved

Since the effects of hyperglycaemia on leukocyte-endothelium interactions have mostly been investigated using *in vivo* animal models or in T2DM subjects, in which both leukocytes and endothelium are simultaneously exposed to HG, there is currently very little evidence defining the sole effect of HG on BEC or leukocytes behaviour to compare our results against. As such this

discussion will focus on what evidence exists for endothelium or leukocytes responses to HG and modulation by metformin and/or insulin. Indeed, the studies around the effect of HG on endothelial cells and leukocyte recruitment are conflicting: In a recent study, cultured endothelial cells isolated from mice injured tissue exposed to HG at 25 mM and TNF-α stimulation for 16 hours showed decreased gene expression of ICAM-1 in HG-cytokine stimulated cells compared to NG-cytokine stimulated (Qiu et al., 2020). No significant differences were detected among the non-activated counterparts. This agrees with our data showing suppressed gene expression of ICAM-1 in response to HG in activated endothelium. We similarly observed no change in gene expression of ICAM-1 in response to HG in non-activated endothelium, indicating upregulation of adhesion molecules gene expression in response to cytokine stimulation regardless of glucose concentration. In contrast to our result showing unaffected neutrophils recruitment in HG treated BEC, the same study also reported an attenuated neutrophil transmigration in HG treatment group using the transwell system and was postulated to be due to reduced expression of ICAM-1 (Qiu et al., 2020). Of note, ICAM-1 regulates BEC permeability in inflamed tissues (Han et al., 2016) and is known to support leukocyte rolling, adhesion and transmigration across the endothelium (Sumagin et al., 2008). Although the attenuation of CAMs was only observed at mRNA level in our study, there is also report of this decreased at protein levels in response to HG (Bačun et al., 2008). An *in vitro* study demonstrated, protein expression of ICAM-1 on human aortic endothelial cells after treatment with 16.5 mM glucose for 4 hours was lower than with 12.0 mM glucose in respect to 5.5 mM glucose, suggesting the cytotoxicity of glucose at high concentrations (Bačun et al., 2008). Indeed, it has been demonstrate that exposure of HUVEC to HG at 30 mM (HG concentration used in our study) for 48> hours can promote apoptosis compared to control cultures exposed to 5 mM glucose; as measured by [^{3}H] thymidine assays where increased DNA fragmentation was observed

in HG treated HUVEC, suggesting potential endothelial damage by hyperglycemia in diabetic patients (Baumgartner-Parzer et al., 1995). Our data adds further weight to these findings, where HG treatment of BEC not only decreases gene expression of ICAM-1, but also reduces VCAM-1 and E selectin gene expression. However, this attenuation was not translated to protein levels which is required for the function of these adhesion molecules. Therefore, reduced recruitment of PBL in HG treated BEC cannot be due to modulation of CAMs expression in our study. More in vitro experiments assessing other factors, such as chemokine response in HG condition, are needed clarify the mechanism involved in this process.

In contrast to our findings with HG, there are studies showing the upregulation of CAMs in response to transient HG conditions *in vitro*: Treatment of HUVEC for 48 hours with HG (25 mM) shown to increase ICAM-1, VCAM-1 and E-selectin protein expression compared to 5mM NG control (Huang et al., 2019). In addition, both 12 and 24 hour treatment of HUVEC with HG at 25 mM increased gene expression of these CAMs compared to NG control (Lo et al., 2018). The effects of chronic HG (which is realistically exist in T2DM condition) on inflammatory properties of endothelial cells were investigated by Ciortan et al. where using a 3D model of human aortic valvular endothelial cells they showed increase gene expression of VCAM-1 and E-selectin in endothelial cells after 14 days of HG (25 mM) treatment compared to normal glucose (5 mM), (Ciortan et al., 2020). As the cell surface expression of CAMs on endothelial cells can only be studied *in vitro*, discrepancies between these results may be related to differences in the concentration of glucose, as well as the exposure time of endothelial cells to glucose and the source of endothelial cells used in these experiments. Moreover, short-term

experiments *in vitro* have their limitations and the effects of HG when used long-term *in vivo* could differ from those observed in *in vitro*.

For the first time we shown that transient HG treatment of endothelium decreased lymphocyte recruitment, which was reversed by metformin and insulin treatment back to normal level. In fact, several studies have demonstrated that metformin and other hypoglycaemic drugs protect the endothelium from the development of vascular impairment (Mather et al., 2001; Arunachalam et al., 2014). However, the mechanisms for this effect remain unknown. Metformin-mediated decrease in CAM expression has been implicated as a possible mechanism for such effects (Abbasi et al., 2004). Metformin treatment of patients with T2DM for 12 weeks has been shown to result in a significant decrease in plasma sV-CAM levels with no changes in sICAM-1 and sE-selectin compared to baseline levels (Abbasi et al., 2004). As the soluble CAMs are representative of their endothelial expression this suggests metformin's capacity to modulate the leukocyte recruitment. The combined effects of HG and metformin *in vitro* was investigated by Bačun et al in human aortic endothelial cells; showing HG treatment of endothelial cells at 12.0 mM increased the expression of E-selectin, VCAM-1 and ICAM-1, and metformin (10 µM) administration in the medium with 12.0 mM glucose for 4 hours additionally enhanced E-selectin and VCAM-1 expression compared to effects of corresponding glucose concentration (Bačun et al., 2008). In contrast and in same study, it was shown that metformin treatment of human aortic endothelial cells (10 µM) in the medium with 16.5mM glucose for 4 hours had no significant effect on protein expression of ICAM-1, E-selectin and VCAM-1 compared to effects of corresponding glucose concentration (Bačun et al., 2008), which is in agreement with our findings demonstrating no change in expression of CAMs by endothelial cells in response to metformin in HG condition. However, in our study metformin administration was post glucose treatment of BEC and with

higher concentration at (200 µM) but still in therapeutic range. We also used higher concentration of glucose (30mM) with longer exposure time (24 hours) in our study. In fact, we showed that short-term effect of metformin on CAMs expression on BEC *in vitro* is unlikely to provide the mechanisms for metformin's beneficial effects on cardiovascular complications in diabetes. However, we cannot exclude a possibility that a long-term administration of metformin may result in significant changes in CAM expression of BEC. Furthermore, since it is reported that decrease in plasma soluble CAMs, such as E-selectin, is dependent on improvement in glycemia, this may indicate that metformin *in vivo* effects could be indirect via glucose control in patients with T2DM.

Although insulin and metformin exert their effects via different signalling pathways, our results demonstrated that insulin and metformin similarly influenced the endothelium supporting the PBL recruitment. In physiological conditions, insulin is released in response to high blood glucose post digestion and brings the blood sugar back to normal level by binding to insulin receptors. However, in conditions like T2DM where insulin resistance is developed due to insulin signalling inhibition, a series of immune responses occurs that exacerbate the inflammatory state, which leads to hyperglycaemia (Berbudi et al., 2020). Thus, understanding the mechanisms by which insulin acts would identify the cellular signalling network linking the immune system and metabolism in T2DM. Several reports have investigated the role of hyperinsulinemia on the endothelium (Federici et al., 2002; Taguchi et al., 2014), the condition which exists in T2DM. But there is not much known about how insulin modulated the endothelium behaviour at physiological concentrations. For the first time we showed that in HG environment, insulin at physiological concentrations restores the endothelium function to support the lymphocyte transmigration. Indeed, there are studies supporting the modulation of CAMs expression by insulin *in vitro:* Aljada et al. reported decreased gene and protein expression of ICAM-1 on human aortic endothelial cells

after exposing them to physiological concentrations of insulin (100 and 1000 μU/mL) for 2 days, which was postulated due to an increase in endothelial nitric oxide synthase (NOS) expression compared to non-insulin treated control (Aljada et al., 2000). As insulin increases NO bioavailability, reduced CAMs expression after insulin treatment was expected in our study. Contrary to expectations, we found that insulin-induced protein expression of one adhesion molecule, VCAM-1, which might partly explain the slight but not significant increased adhesion of PBL in insulin (10^{-7}M/L) treated BEC in NG condition. In agreement with our finding insulin was shown to increase protein expression of selectively VCAM-1 on HUVEC at 10^{-9} to 10^{-7} M/L concentrations in a dose dependent manner after 24 incubations (Madonna et al., 2004). Similar to our results they found no change in protein expression of ICAM-1 and E-selectin under similar conditions. To dissect potentially implicated signal transduction pathways, they individually treated HUVEC with known pharmacological inhibitors of two distinct insulin signalling pathways (PI3′-kinase and MAP kinase) which were not investigated in our study. They could show that 30 minutes of pre-treatment with PI3′-kinase inhibitor potentiated the effect of insulin on VCAM-1 surface expression compared to non-pre-treated cells (Madonna et al., 2004), which suggests activation of MAP-kinase pathway at 10^{-7} M/L concentration of insulin as used in our study. Therefore, indicating that insulin might be important mediator in activation of endothelium and progression of atherosclerosis in states of insulin resistance. In contrast to NG condition, BEC differently responded to same concentration of insulin in HG condition in our study. Unchanged expression of CAMs on BEC despite increased transmigration of PBLs in respond to insulin in HG condition suggests that immunomodulatory effects of insulin were not mediated through adhesion molecules or possibly activation of MAP kinase signalling pathway in our study. However, evaluating only the CAMs expression in our study was not optimal to investigate the

mechanism of action for insulin. Thus, more *in vitro* studies using inhibitors for CAMs, MAP-kinase and PI3′-kinase pathway are needed to clarify the possible mechanisms involved in HG condition.

4.3.2 Evidence of High Glucose actions on leukocytes and mechanisms involved

Our *in vitro* model allowed us to separately evaluate the behaviour of BEC and leukocytes in response to HG. Interestingly we observed an opposite effect of HG on lymphocyte - as both adhesion and transmigration of PBLs were increased in response to 24 HG treatment suggesting activation of lymphocytes in hyperglycaemic environment. In addition, this behaviour of PBLs was not affected by metformin or insulin supplementations of PBL post HG treatment. Indeed, activation of lymphocyte in response to HG was shown in a previous *in vitro* study, where they showed that HG treatment of Jurkat T-cells at 15mM increased their expression of IL-17, and downstream signalling cascades resulted in the expression of TNF-α and chemokines CXCL5, CXCL1, IL-8, CCL2 (Kumar et al., 2014), which functionally increased their adhesion to HUVEC. Here, our study lacks the information about whether HG modulates the expression of any activation marker on lymphocytes, however it reflects the functional activation of PBLs in response to HG. Increased expression of integrins LFA-1 and VLA-4, which are considered as leukocyte activation marker, were reported higher on leukocytes of patients with T2DM compared to healthy control (Stulc et al., 2012). Since firm adhesion of lymphocytes is only possible if the right signals activate these integrins on lymphocytes so that they bind their endothelial cell borne receptors ICAM-1 and VCAM-1 (Shamri et al., 2005), it would be worth investigating the expression of these integrins in HG condition in the future experiments. Therefore, in our study in the absence of any discernible changes in CAMs expression by BEC in response to HG, we

anticipate that HG might be acting on the signalling process that modulates integrin function in lymphocytes.

Although our results demonstrate no modulatory effects of metformin and insulin on PBL behaviour in HG condition, there are studies reporting lymphocyte-suppressing effects of metformin in T2DM patients where administration of metformin at higher dose of 3 gr/day and 1 gr/day for 3 month decreased lymphocyte (Krysiak and Okopien, 2012) and monocyte (Krysiak and Okopien, 2013) release of IFN-γ and TNF-α respectively compared to placebo. However, the behaviour of these leukocytes was not looked and compared in these studies.

Like metformin, insulin at physiological concentration did not affect the PBL recruitment in HG environment. Since in patient with T2DM glucose homeostasis is impaired and hyperinsulinemia has developed, leukocytes are exposed to pathophysiological concentrations of insulin in these subjects and to mimic their behaviour using *in vitro* experiments with higher concentrations of insulin are needed.

Investigating the possible mechanisms modulating leukocyte recruitment in HG condition, we also looked at the expression of AdipoR1/R2 on B cells of HG treated PBMCs as these receptors are important part of the PEPITEM pathway and binding to adiponectin they regulate the trafficking of lymphocytes (Chimen et al., 2015). Our findings from previous chapter showed that AdipoRs are highly expressed on B cells, monocytes, and NKs in contrast are very low expressed by lymphocytes. We know that AdipoRs expression is decreased on B cells from T1DM patients (Chimen et al., 2015). However, the pathophysiology of T1DM is different from T2DM and there are not many studies investigating the expression of these receptors by immune cells in T2DM to compare our results against. Among these few studies we can mention is the Alberti et al. study where they evaluated the gene expression of AdipoRs by lymphocytes in three groups of

obese subjects, one with normal glucose tolerance, obese with impaired glucose tolerance, and obese with T2DM (Alberti et al., 2007). Their findings followed a trend of decreased AdipoR1 expression when moving from obese subjects to obese with impaired glucose tolerance, which was associated with higher plasma insulin and lower adiponectin levels (Alberti et al., 2007). Indeed, insulin was already shown to reduce AdipoR1/R2 RNA expression in mice skeletal muscle, which supports the hypothesis that an alteration of AdipoRs expression together with lower serum adiponectin contributes to the development of insulin resistance in a sort of vicious cycle (Tsuchida et al., 2004). Surprisingly, AdipoR1/R2 RNA expression tended to increase in T2DM compared to obese with impaired glucose tolerant group. However, the T2DM subjects in this study were receiving glucose lowering drugs that might affect the AdipoRs expression. In our study, we saw no modulation of AdipoRs expression on any of PBMC subsets in response to 24-hour HG treatment, although 2-hour HG treatment of PBMCs increased AdipoRs on B cells in some experiments which can be explain as an immediate protective reaction to HG treatment. Furthermore, subsequent metformin and/or insulin treatments post HG had no modulatory effects on these receptors. Indicating the effects of HG, metformin and insulin on lymphocyte trafficking do not seem to be mediated by these receptors. However, in our *in vitro* system, PBMCs only experienced a transient HG which is not very similar to chronic hyperglycaemic condition in T2DM. Indeed, one of our research aims in this study was to evaluate the PEPITEM pathway through measuring the AdipoRs expression particularly on B cells of patients with T2DM compared to aged match healthy control and monitor lymphocyte trafficking which would allow us to measure the pathway in patients with poor glycaemic compared to those with well glycaemic control. However, this experiment was not plausible due to covid restrictions prohibiting our access to patients' blood samples.

4.3.3 Limitations

A limitation of this study is our static *in vitro* assay which does not allow us to analyse dynamic behaviour of leukocytes in response to HG. Therefore, a more realistic system like flow assay in which PBL are perfused on the HUVEC monolayer at a specific shear stress (Luscinskas *et al.*, 1995; Bahra *et al.*, 1998; McGettrick *et al.*, 2009) is required to quantify the total capture, rolling, firm adhesion and transmigration in a more physiological context. Furthermore, our experiments do not reflect the natural environment which exists in T2DM, because cells are only exposed to transient HG which is not optimal to reflect the chronic hyperglycaemia of T2DM. Important factors such as adiponectin and insulin resistance and inflammatory cytokines can influence the leukocyte-endothelium behaviour and are absent in our *in vitro* system. Lack of experiments with blood samples of subjects with T2DM is another limitation of our study which could have provided us more insight into leukocyte trafficking in T2DM.

4.3.4 Conclusion

In conclusion we show that HG differently affects the endothelium and PBL behaviour and subsequent impact on lymphocyte trafficking. Moreover, metformin and insulin supplementation of endothelium restores its ability to support the PBL recruitment. However, our study is not able to clarify the mechanisms involved in this process. Further studies such as evaluating the chemokine response, inhibiting the CAMs, and blocking insulin signalling pathways are required to fully understand the pathway.

5 Immunomodulatory effects of PEPITEM in HFD-induced glucose intolerance mice

5.1 Introduction

In the previous chapter, we clearly demonstrated that adiponectin inhibited lymphocyte recruitment through inflamed BEC. Similarly, we found that PEPITEM treatment of PBL inhibits their migration through the inflamed BEC *in vitro* (Figure 5.1). The efficacy of PEPITEM *in vivo* using models of inflammation has been previously shown, where PEPITEM reduced the number of T cells in site of inflammation in a model of endotoxin-induced ocular inflammation (Chimen et al., 2015). The PEPITEM pathway was shown to be compromised in subjects with the T cell–driven disease, such as type 1 diabetes (T1DM) (Chimen et al., 2015). T2DM is also considered an immunocompromised disease (Berbudi et al., 2020), however, the pathogenesis of T2DM is different to that of T1DM (Zaccardi et al., 2016). There is no study demonstrating the modulation of the PEPITEM pathway in T2DM. Obesity is a promoter of T2DM, and is accompanied by chronic inflammation at tissue level (Malone and Hansen, 2019). Suppressing inflammation has been shown to improve blood glucose levels and insulin sensitivity in patients with T2DM (Larsen et al., 2007).

Given that PEPITEM regulates T cell trafficking, we evaluated the immune modulatory effects of PEPITEM in mice on an obesogenic diet similar to that experienced by T2DM patients. We hypothesis that the PEPITEM pathway is dysregulated in T2DM mice, therefore resulting in suppression of the homeostatic mechanism (PEPITEM) controlling T-cell trafficking during inflammation. We also hypothesis that an impaired PEPITEM pathway worsens the obesity induced fat inflammation, insulin resistance and pancreatic beta cells function in T2DM. In this chapter we evaluated the PEPITEM effects in two sets of *in vivo* experiments including mice on 6 or 12 weeks on HFD. We investigated if PEPITEM administration is able to decrease disease onset

in a mouse model of T2DM and affect the blood glucose or insulin secretion. We also evaluated how PEPITEM administration in a model of T2DM modulates leukocyte trafficking in different tissues such as blood, adipose tissue, LNs, spleen and peritoneal lavage.

5.2 Results

5.2.1 The effect of PEPITEM on PBLs migration across the endothelium in healthy controls

There is previous evidence supporting the inhibitory effects of PEPITEM on PBLs transmigration through the inflamed endothelium (Chimen et al., 2015). Here, we also examined the inhibitory capacity of PEPITEM on PBL adhesion and migration *in vitro*. PEPITEM had no effect on PBL adhesion (Figure 5.1A), but significantly reduced the migration of PBLs across adult BEC (Figure 5.1B). This data indicates PEPITEM is functional *in vitro*.

5.2.2 Immunomodulatory effects of PEPITEM in 6 week-HFD mice

Efficacy in both *in vitro* (Figure 5.1) and *in vivo* models of inflammation led us to investigate whether the PEPITEM pathway was compromised in obesogenic diet induced inflammation. The mouse model of HFD-induced obesity has become a valuable tool to understand the development of T2DM (Tang et al., 2013). HFD feeding of mice for 16–20 weeks, increase the body weight up to 20–30% when compared to normal-fed mouse (Tang et al., 2013). Although 4 weeks of HFD usually induce the hyperglycaemia, typical manifestation of obesity occurs after 16 weeks of HFD (Tang et al., 2013).

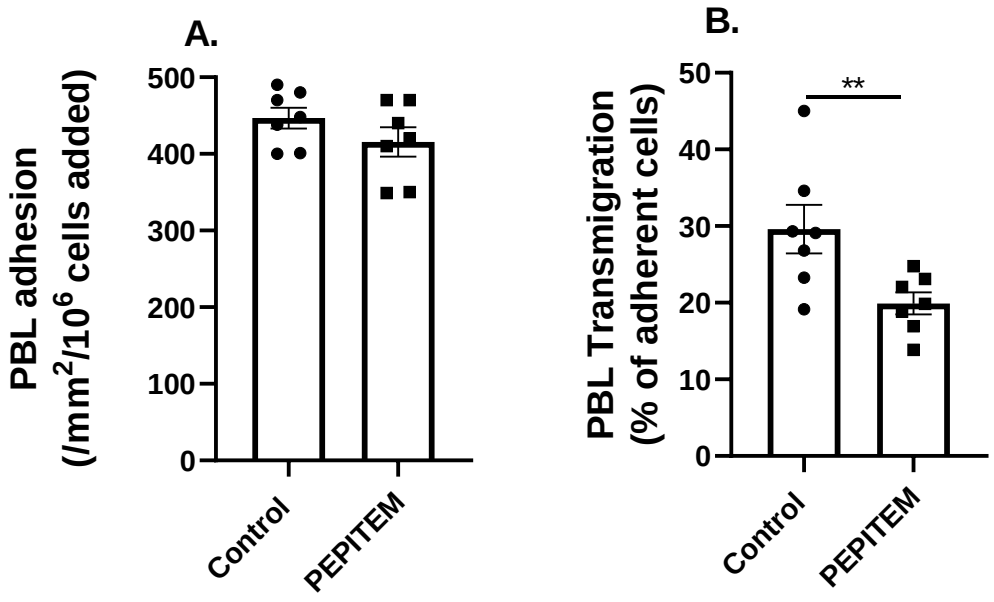


Figure 5.1 Effect of PEPITEM on lymphocyte migration through inflamed endothelium in static conditions. BEC were cultured for 24 hours prior to stimulation with TNF-α/IFN-γ for 24 hours. PBLs were isolated, left untreated (control) or treated with PEPITEM at 20ng/ml. PBLs (1×10^6 cells/ml) were added to BEC and allowed to adhere and migrate for 6 minutes 30 seconds at 37 °C. Digitised images of 5 random fields were acquired from BEC and analysed offline. **(A)** The number of adherent PBLs expressed as the number of cells/$mm^2/10^6$ cells added. **(B)** Lymphocyte transmigration expressed as the percentage of adherent cells that had transmigrated. Data are shown as mean ± SEM for n = 7 independent experiments using 2 different BEC donors and 7 different lymphocyte donors. **= $p<0.01$ compared to control, by paired t-test.

5.2.2.1 Effect of PEPITEM administration on body weight and glucose tolerance in 6 week-HFD mice

Here, the mice were placed on HFD for 6 weeks and treated with PEPITEM or PBS administrated via mini pumps. The osmotic minipumps are designed to deliver their contents for 6 weeks according to the manufacturer's guidelines. Body weight was assessed weekly and glucose tolerance was measured at week 3 and 6 following 5h fasting. Body weight steadily and similarly increased in both groups (Figure 5.2-A). There was no difference in AUC for body weight between two groups (Figure 5.2-B). Fasting blood glucose levels at baseline were not different between the two groups at week 3 (Figure 5.2-C) and week 6 (Figure 5.2-E) of HFD. Additionally, blood glucose concentrations returned close to baseline for both treatment groups following IPGTT at week 3 (Figure 5.2-C) and week 6 (Figure 5.2-E) of HFD. The AUC for IPGTT was not different between two groups at week 3 (Figure 5.2-D, P value=0.1373) and week 6 (Figure 5.2-F, P value=0.3092) of HFD. These data indicate PEPITEM had no effect on glucose tolerance and insulin resistance in mice on HFD for 6 weeks.

5.2.2.2 The effect of PEPITEM on pancreatic beta cell mass in 6-week HFD- mice

Although insulin resistance is described as a the main pathophysiological feature in T2DM, recent studies have shown that a functional deficiency of pancreatic islets is also important in the development of T2DM (Inaishi and Saisho, 2020). Previous reports have established that pancreatic beta cell mass in subjects with obesity increases, to compensate for insulin resistance (Polonsky et al., 1988). However, , the beta cell mass was reported to decrease by approximately 40% once the glucose intolerance develops in T2DM (Butler et al., 2003). Here, we aim to investigate whether the PEPITEM administration in a mice model of glucose intolerance can affect

the beta-cell mass and the number of pancreatic islets compared to PBS treated mice. Therefore, we isolated the mice pancreas at the end of 6 weeks on HFD and stained for insulin and glucagon. The islet images acquired using slide scanner as shown in Figure 5.3 and quantified as the number of the islets/10000µm^2 of whole area. PEPITEM had no effect on number of the pancreatic islets/10000µm^2 of whole sectioned area (Figure 5.4-A) However, it significantly reduced the area of islets/10000µm^2 of whole sectioned area compared to PBS treated (Figure 5.4-B). These data indicate, administration of PEPITEM in 6-week HFD- mice avoids the expansion of pancreatic beta cell mass.

5.2.2.3 Quantification of leukocytes in 6-week HFD-mice treated with PEPITEM vs PBS

Given that obesity and T2DM are accompanied by chronic inflammation at a tissue level, we evaluated the immunoregulatory effects of PEPITEM during inflammation caused by obesogenic diet and during T2DM. We investigated whether PEPITEM could affect T cells or other leukocyte subsets trafficking into different vascular beds. We quantified the number of leukocyte subsets in the blood, LN, spleen, adipose tissue, and peritoneal lavage in mice using flow cytometry. The number of CD45$^+$ cell, B cells, age associated B cells, CD8$^+$ T cells, T regs, eosinophils and neutrophils remained unchanged in all these tissues in PEPITEM treated mice compared to PBS treated mice (Table 5.1). However, PEPITEM treatment significantly decreased the number of CD4$^+$ T cells (Figure 5.5-A) and senescent T cells (Figure 5.5-B) in visceral adipose tissue. In addition, PEPITEM treatment significantly decreased the number of T cells (Figure 5.5-C), senescent T cells (Figure 5.5D) and macrophages (Figure 5.5E) in peritoneal lavage compared to PBS treated mice. These data indicates that the regulatory effects of PEPITEM in visceral fat and peritoneal lavage inflammation in in 6-week HFD-mice.

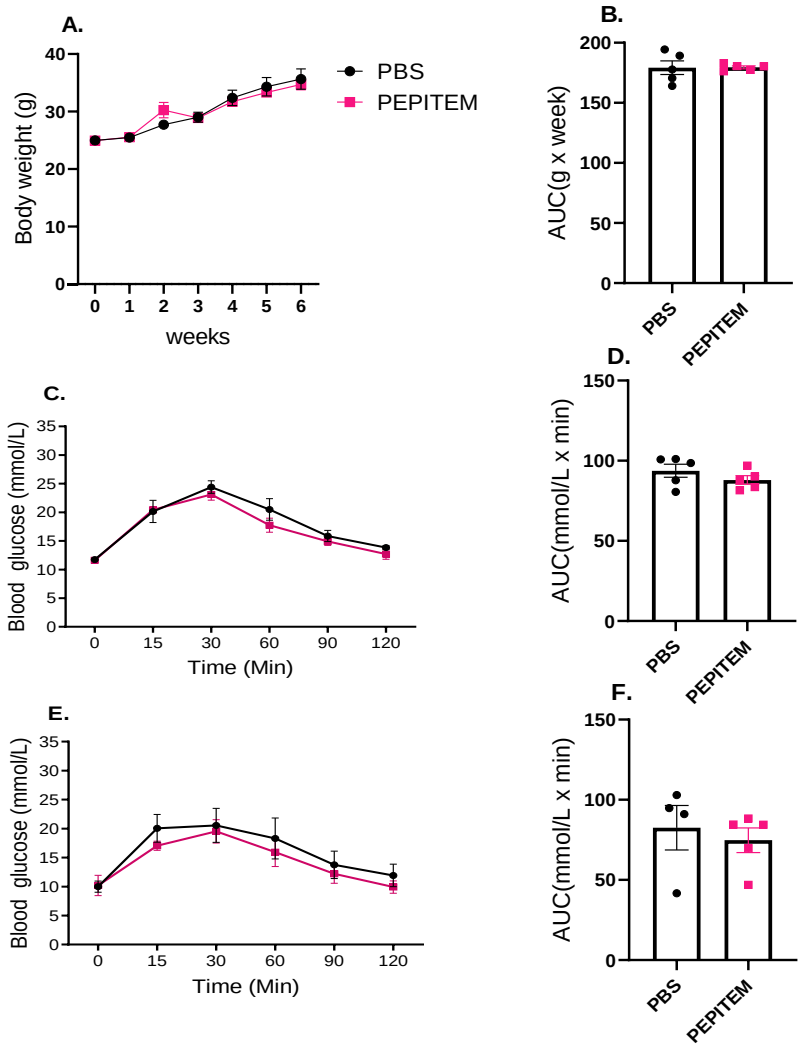


Figure 5.2 Effect of PEPITEM administration on body weight and glucose tolerance in HFD- mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 6 weeks and received 73µg/day PEPITEM (*red squares*) or PBS (*black circles*) administrated via mini pumps for the 6 weeks of HFD. **(A)** Body weight was assessed weekly and **(B)** quantified as the area under the curve. Blood glucose concentrations was measured at **(C)** week 3 and **(E)** week 6 of HFD: The mice were fasted for 5 hours, and baseline blood glucose was measured at 0 min, then 1g glucose/kg body weight was injected intraperitoneally. Glucose levels were measured regularly at 15, 30, 60, 90 and 120 minutes and quantified as AUC for **(D)** week 3 and **(F)** week 6 of HFD. Data are shown as mean ± SEM for n =1 independent experiment using n =5 mice per group.

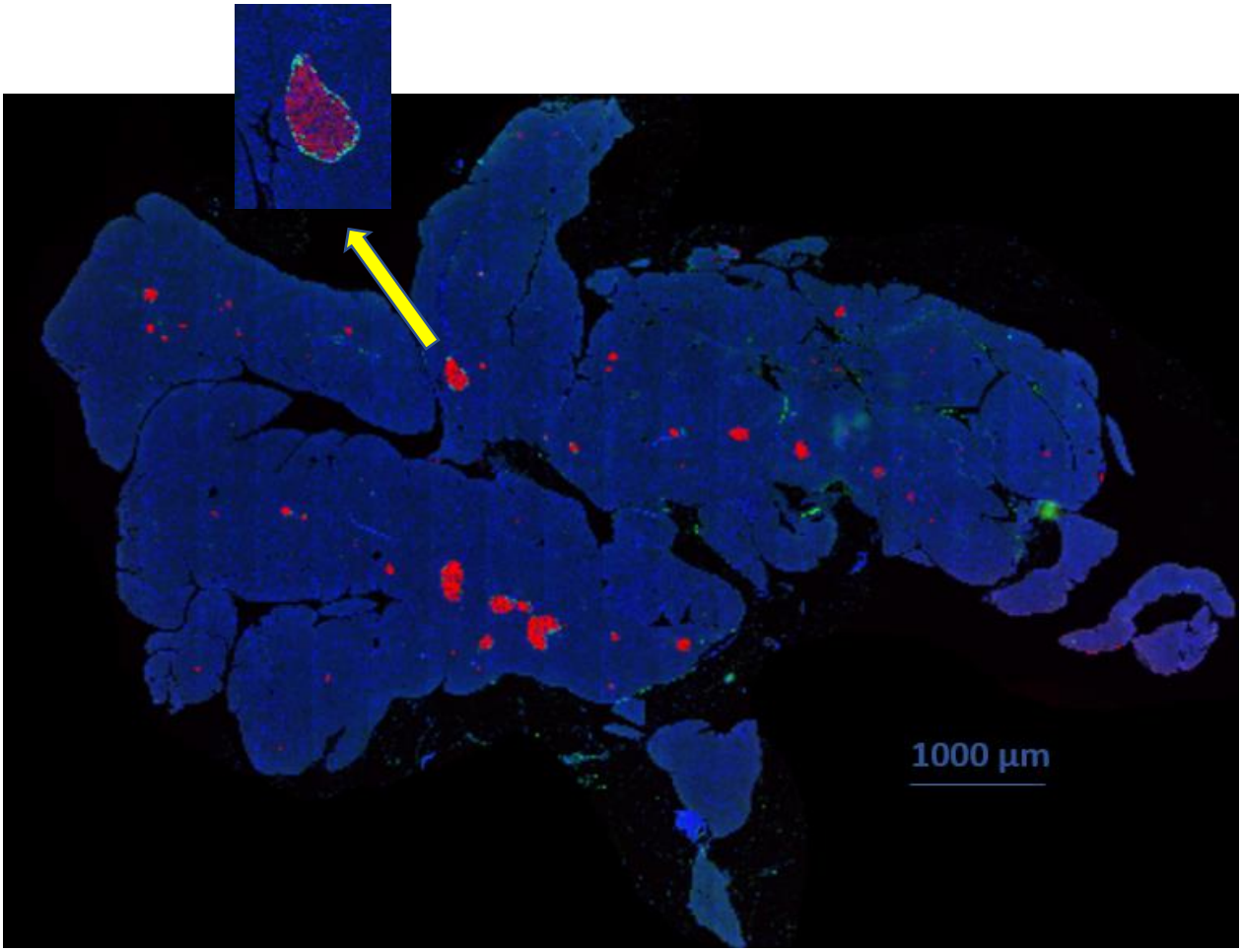


Figure 5.3 Representative image of pancreatic islets in mice pancreas. The isolated pancreas was fixed, paraffin embedded, sectioned, and stained for insulin and glucagon. Representative images acquired using slid scanner showing merged fluorescent channels in whole sectioned area using red for beta cells, green for alpha cells and DAPI for nucleus. The zoomed area represents one pancreatic islet. Scale bar =1000**µ**m

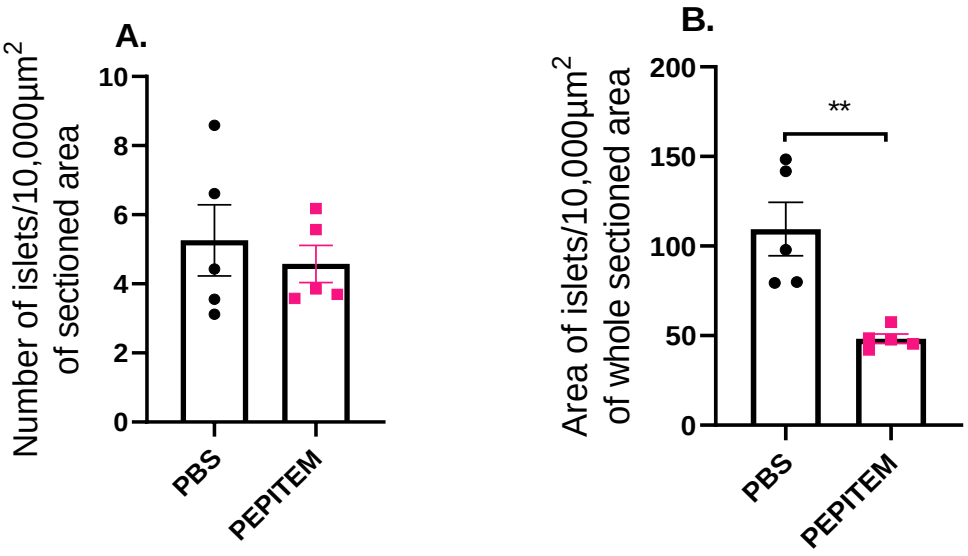


Figure 5.4 Quantification of pancreas islets number and area in HFD-mice treated with PEPITEM vs PBS. The C57Bl/6 Mice were placed on HFD for 6 weeks and received PEPITEM at 3.5mg/ml via mini pumps during 6 weeks of HFD. Pancreas was removed, and pancreatic islets were stained and imaged and quantified as **(A)** the average number of the islets/10000µm^2 of sectioned area and **(B)** the average area of islets/10000µm^2 of sectioned area. Data are shown as mean±SEM for n = 1 independent experiment using n=5 mice per group. **= p<0.01 by unpaired t-test compared to PBS treated.

Table 5.1 Quantification of leukocyte subsets in 6-week HFD-PEPITEM vs HFD-PBS mice

Leukocyte subset	Blood/ml		Visceral adipose Tissue/g		Subcutaneous adipose Tissue/g		Spleen/g		LNs		Peritoneal lavage/ml	
	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM
$CD45^+$ cell number	116307± 9038	108159± 3169	42655± 14492	48882± 12226	28655± 3311	30882± 2353	37777237± 3795124	40914684± 3937809	137761± 9322	168338± 17547	78712± 1249	82160± 3683
B cell number	37645± 2128	44837± 5613	2599± 357.6	2259± 257.8	1799± 152.3	1859± 100.9	18017788± 2422941	20549882± 3272735	26811± 5889	24771± 5804	32617± 4658	32838± 3015
Age associate B cell number	5091± 566.0	6895± 834.7	2067± 198.1	1627± 158.3	1027± 32.36	1007± 53.37	1605879± 280099	1723345± 164344	241.3± 49.36	294.2± 56.49	7052± 729.9	6190± 682.5
T cell number	35785± 4418	40481± 3827	30354± 3586	22952± 4381	14954± 1491	16152± 1673	5812659± 1284432	6841248± 1093347	99419± 1338	105056± 3970	**Figure 5.4C**	**Figure 5.4C**
$CD4^+$ T cell number	17686± 2435	19895± 3260	**Figure 5.4A**	**Figure 5.4A**	9454± 483.8	9483± 417.3	3898541± 262349	3727943± 393053	30624± 6550	34302± 2347	13560± 1231	14538± 1383
$CD8^+$ T cells number	6764± 1059	5868± 978.0	1884± 305.6	1956± 511.3	1484± 145.0	1356± 122.8	2658353± 341183	31795± 1446	32976± 760.9	34302± 2347	4557± 461.1	4464± 369.0
T reg number	140.0± 5.055	138.7± 13.06	181.0± 25.57	161.1± 17.58	153.0± 10.71	135.1± 12.90	3299± 452.8	3249± 495.7	265.3± 38.10	233.1± 37.80	61.45± 5.657	58.24± 9.663
Senescent T cell number	1762± 363.0	1704± 393.5	**Figure 5.4B**	**Figure 5.4B**	545.5± 72.02	455.8± 56.55	694010± 71650	689293± 71412	11427± 712.8	11735± 470.1	**Figure 5.4D**	**Figure 5.4D**
Monocyte/ Macrophage number	14171± 1812	15697± 1004	44660± 9735	52891± 9545	13260± 1007	13691± 1260	317833± 37650	307157± 37216	341.7± 137.8	332.4± 114.4	**Figure 5.4E**	**Figure 5.4E**
Eosinophil number	1147± 68.55	910.7± 193.1	3843± 439.5	3121± 531.1	1643± 99.73	1721± 37.83	19308± 3064	15359± 3000	21.86± 10.79	55.30± 26.30	110.8± 4.912	133.8 29.50
Neutrophil number	8067± 885.1	7605± 1488	959.8± 60.78	1183± 163.7	839.8± 44.11	682.9± 75.47	260316± 18831	223631± 12099	96.57± 33.38	94.97± 51.71	23.00± 8.973	37.36± 17.43

T reg -Regulatory T cells: LN – Lymph Node. Unpaired t-test showed no significant effect of PEPITEM treatment on leukocyte number for the data presented in this table. Statistically significant data are represented in figure format **(Figure 5.4A-E)**. Data are shown as mean ± SEM for n = 1 independent experiments, using n=5 mice per group.

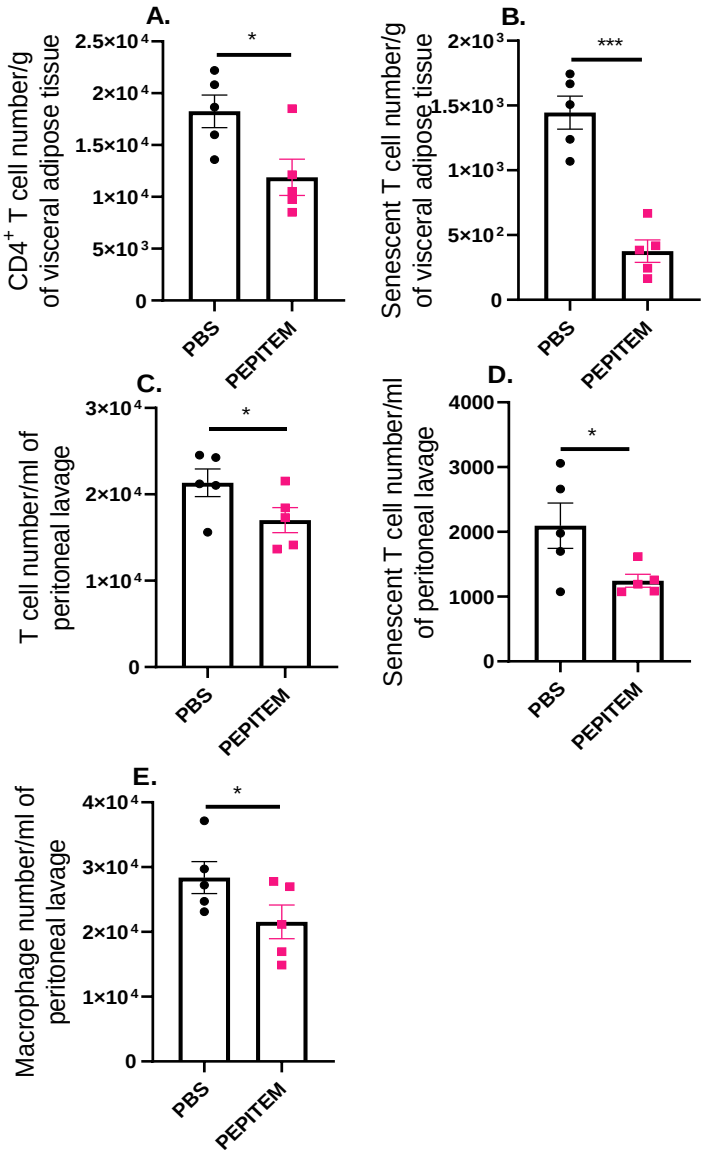


Figure 5.5 Quantification of leukocyte subsets in HFD-mice treated with PEPITEM vs PBS. The C57Bl/6 Mice were placed on HFD for 6 weeks and received PEPITEM at 3.5mg/ml via mini pumps during 6 weeks of HFD. **(A)** Visceral adipose tissue $CD4^+$ T cells, **(B)** visceral adipose tissue senescent T-cells, **(C)** peritoneal lavage T cells, **(D)** peritoneal lavage senescent T cells and **(E)** peritoneal lavage macrophages were quantified using flow cytometry analysis. Absolute number of immune cells were normalised to bead counts and plotted as number per g of adipose tissue and per ml of peritoneal lavage. Data are shown as mean±SEM for n = 1 independent experiment using n = 5 mice per group. *= $p<0.05$, and *** $p<0.005$ by unpaired t-test compared to PBS treated.

5.2.3 Immunomodulatory effects of PEPITEM in mice on HFD for 12 weeks

In previous experiment we used 6-week HFD-induced obesity to generate a glucose intolerance mice model to assess the immunomodulatory effects of PEPITEM in vivo. To achieve a model with worse glucose intolerance that more closely represented the T2DM physiopathology we extended the HFD duration to 12 weeks, to further explore the regulatory effects of PEPITEM. Due to the restriction of the Home Office licence, we were only allowed to implant one osmotic pump, as such we opted to administer PEPITEM for the last 6 weeks of HFD to assess its immunoregulatory potential as T2DM symptoms developed.

5.2.3.1 Effect of PEPITEM administration on body weight and glucose tolerance in 12-week HFD- mice

First, we explored whether the PEPITEM administration has any effect on weight gain and glucose tolerance during 12 weeks of HFD. Body weight was assessed weekly and glucose tolerance was measured at week 8 and 12 following 5 hours fasting. Body weight steadily and similarly increased in both groups (Figure 5.6-A). Once again, the AUC for body weight was not different between two groups (Figure 5.6-B). Fasting blood glucose levels at baseline were not different between the two groups at week 8 (Figure 5.6-C) and week 12 (Figure 5.6-E) of HFD. Additionally, blood glucose concentrations returned close to baseline for PBS treated group following IPGTT at week 8 and week 12 of HFD. We observed a slight increase in glucose intolerance in the PEPITEM-treated mice at week 8 (Figure 5.6-C) and 12 (Figure 5.6-E) as glucose levels did not return to baseline levels. However, the AUC for IPGTT data was not significantly different between two groups at week 8 (Figure 5.6-D) and week 12 (Figure 5.6-F) of HFD, indicating that PEPITEM had no effect on glucose tolerance and insulin resistance in mice on HFD for 12 weeks.

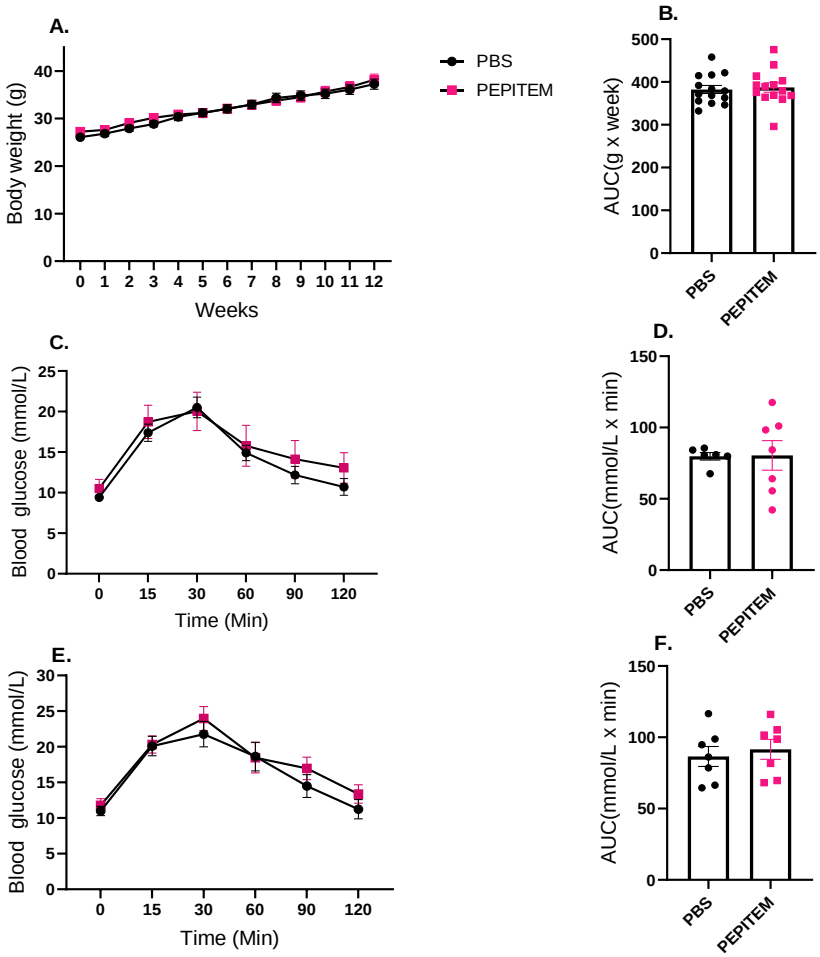


Figure 5.6 Effect of PEPITEM administration on body weight and glucose tolerance in HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM (*red squares*) or PBS (*black circles*) administrated via mini pumps for the last 6 weeks of HFD. **(A)** Body weight was assessed weekly and **(B)** quantified as the area under the curve. Blood glucose concentrations was measured at **(C)** week 8 and **(E)** week 12 of HFD: The mice were fasted for 5 hours, and baseline blood glucose was measured at 0 min, then 1g glucose/kg body weight was injected intraperitoneally. Glucose levels were measured regularly at 15, 30, 60, 90 and 120 minutes and quantified as AUC for **(D)** week 8 and **(F)** week 12 of HFD. Data are shown as mean ± SEM for n =1 independent experiment using n =14 mice per group.

5.2.3.2 The effect of PEPITEM on pancreatic beta cell mass in 12-week HFD- mice

Next, we explored whether administration of PEPITEM for 6 weeks can affect the islet number or their area in 12-week HFD-mice. The islet images acquired using slide scanner as shown in Figure 5.7. We observed no significant effect of PEPITEM on number of the pancreatic islets/10000µm^2 of whole sectioned area (Figure 5.8A). However, PEPITEM treatment significantly reduced the area of islets/10000µm^2 of whole sectioned area compared to PBS (Figure 5.8-B). These data indicate, administration of PEPITEM in 12-week HFD-mice avoids the expansion of pancreatic beta cell mass.

5.2.3.3 Quantification of leukocytes in 12-week HFD-mice treated with PEPITEM vs PBS

Finally, we explored the effect of PEPITEM on leukocyte trafficking in in the blood, LN, spleen, adipose tissue, and peritoneal lavage in mice using flow cytometry. We found no significant changes in number of CD8$^+$ T cells and T regs in any of the tissues (Table 5.2) in PEPITEM treated mice vs PBS. The number of age associated B cells (Figure 5.9-A), senescent T cells (Figure 5.9-B), and neutrophils (Figure 5.9-C) were reduced in blood of PEPITEM treated mice. We also observed reduced number of T cells (Figure 5.10-A), CD4$^+$ T (Figure 5.10-B), cells and senescent T cells (Figure 5.10-C) in visceral adipose tissue of PEPITEM treated mice. We found no significant change in number of any leukocyte subsets in subcutaneous adipose tissue in PEPITEM treated group (Table 5.2). In spleen, the number of CD45$^+$ cells (Figure 5.11-A), B cells (Figure 5.11-B), age associated B cells (Figure 5.11-C), and T cells (Figure 5.11-D) were increased in PEPITEM treated mice. However, neutrophil number (Figure 5.11-E) were reduced in this group. In LN, higher numbers of CD45+ cells (Figure 5.12-A), T cells (Figure 5.12-B) and eosinophils (Figure 5.12-C) were observed in PEPITEM treated group compared to control treated

animals. PEPITEM treatment only reduced the macrophage number in peritoneal lavage (Figure 5.13). These data indicates that administration PEPITEM in last 6 weeks of HFD-induced obesity modulates leukocyte trafficking in visceral fat, peritoneal lavage also in secondary lymphatic organs such as spleen and LNs.

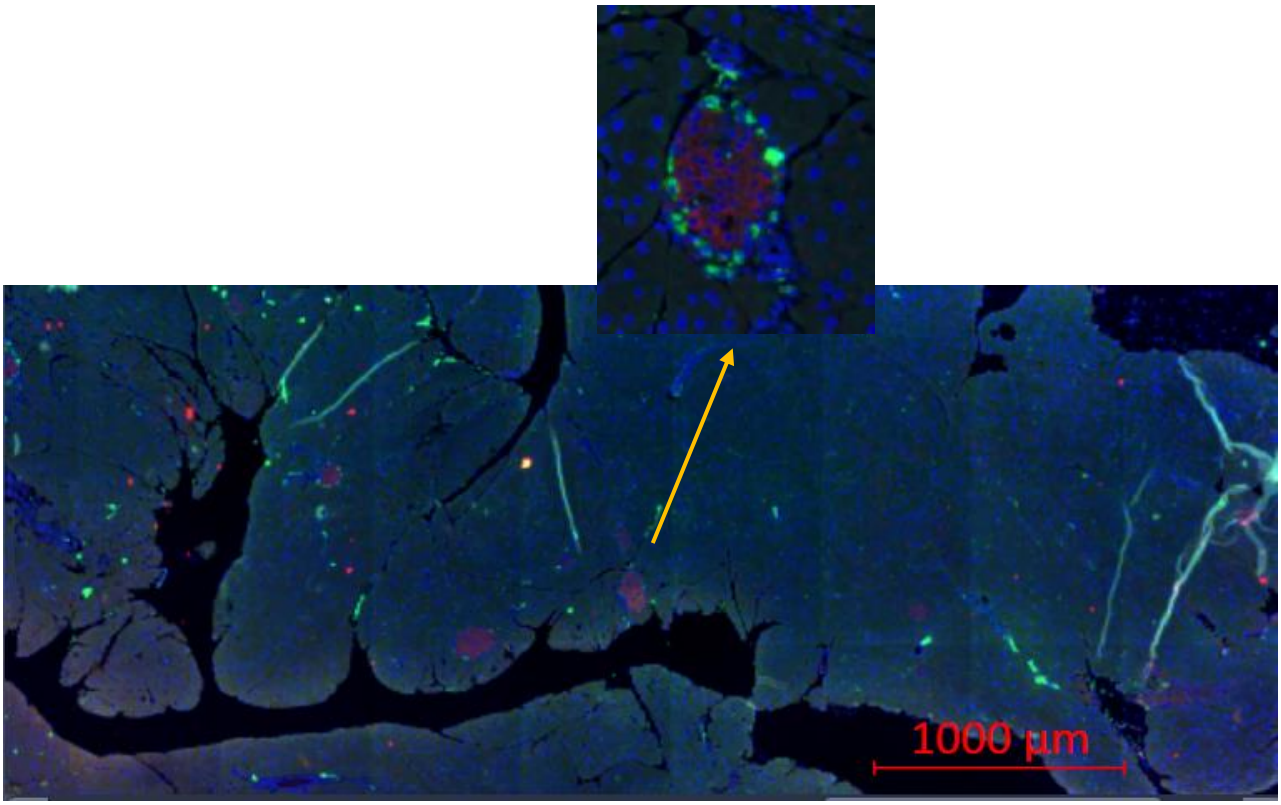


Figure 5.7 Representative image of pancreatic islets in mice pancreas. The isolated pancreas was fixed, paraffin embedded, sectioned, and stained for insulin and glucagon. Representative images acquired using slid scanner showing merged fluorescent channels in whole sectioned area using red for beta cells, green for alpha cells and DAPI for nucleus. The zoomed area represents one pancreatic islet. Scale bar =1000µm

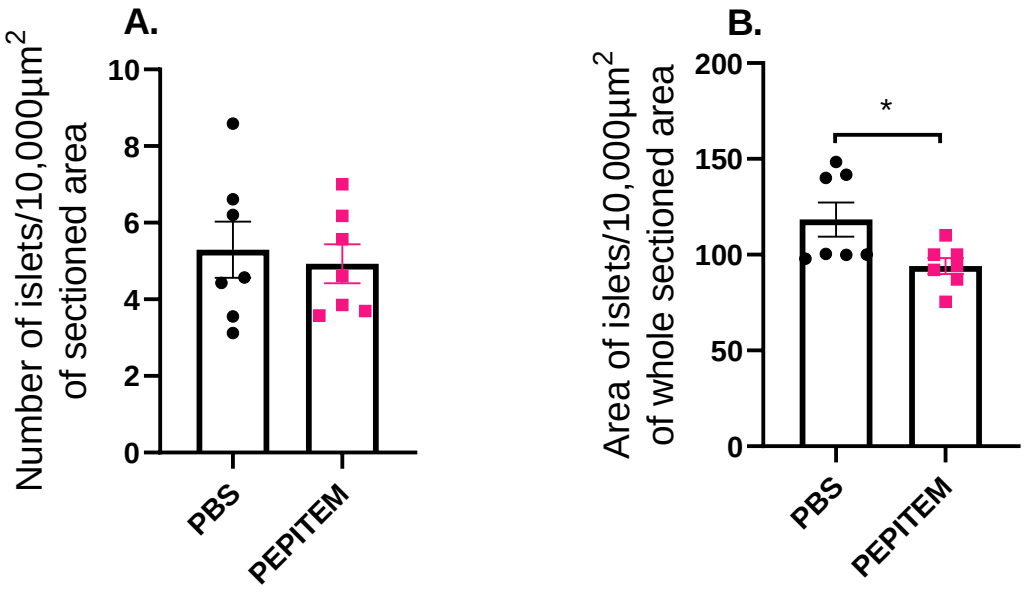


Figure 5.8 Quantification of pancreas islets number and area in HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM or PBS administrated via mini pumps for the last 6 weeks of HFD. Pancreas was removed, and pancreatic islets were stained and imaged and quantified as **(A)** the average number of the islets/10000**µ**m^2 of sectioned area and **(B)** the average area of islets/10000µm^2 of sectioned area. Data are shown as mean±SEM for n = 1 independent experiment using n=7 mice per group. *= $p<0.05$ by unpaired t-test compared to PBS treated.

Table 5.2 Quantification of leukocyte subsets in 12- week HFD-mice treated with PBS vs PEPITEM

Leukocyte subset	Blood/ml		Visceral adipose Tissue/g		Subcutaneous adipose Tissue/g		Spleen/g		LNs		Peritoneal lavage/ml	
	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM	PBS	PEPITEM
$CD45^+$ cell number	125413± 7680	126756± 12148	42328±5 478	50925± 5764	27399± 1277	27353± 1505	**Figure 5.8A**	**Figure 5.8A**	**Figure 5.9A**	**Figure 5.9A**	86512± 10012	84768±1 4061
B cell number	90359± 12190	116113± 11644	2615± 206.9	2540± 214.0	2257± 92.45	2195± 101.2	**Figure 5.8B**	**Figure 5.8B**	2723± 5750	31610± 5063	35959± 5160	25257±3 596
Age associate B cell number	**Figure 5. 6A**	**Figure 5. 6A**	1929± 126.1	2123± 118.9	1350± 49.26	1440± 51.59	**Figure 5.8C**	**Figure 5.8C**	399.0± 72.70	401.9± 76.86	8570± 1247	6372± 994.4
T cell number	20930± 3587	28771± 4010	**Figure 5. 7A**	**Figure 5. 7A**	24114± 810.0	22855± 798.0	**Figure 5.8D**	**Figure 5.8D**	**Figure 5.9B**	**Figure 5.9B**	23228± 3704	21997±3 547
$CD4^+$ T cell number	10255± 1821	14783± 2145	**Figure 5. 7B**	**Figure 5. 7B**	14857± 845.4	13654± 911.8	3506279±5 10247	5028258±8 12723	3840± 6313	53669± 7784	14177± 2182	12751±1 973
$CD8^+$ T cells number	7894± 1422	9929± 1302	1741± 202.9	1864± 217.5	904.8± 44.33	876.6± 59.01	2536683±3 66794	3311768±5 17992	3039± 1129	33409± 855.9	5730± 1089	5269±10 83
T reg number	59.05± 12.01	96.43± 23.67	384.5± 81.54	340.8± 77.51	74.88± 6.300	69.18± 7.382	4141± 718.1	4517± 857.0	311.1± 42.85	266.3± 31.22	46.07± 7.619	58.75± 7.399
Senescent T cell number	**Figure 5. 6B**	**Figure 5. 6B**	**Figure 5. 7C**	**Figure 5. 7C**	978.6± 49.70	920.1± 68.95	1325711±2 07466	1479041±2 35741	1033± 568.1	11741± 392.0	2245± 254.1	2421±44 1.5
Monocyte/ Macrophage number	15022± 1991	11864± 1070	42582±5 265	49601± 6083	24024± 1005	24866± 1017	704531± 99344	522682± 83250	357.2± 94.92	447.8± 95.50	**Figure 5.10**	**Figure 5.10**
Eosinophil number	784.2± 88.40	791.5± 112.0	4287± 593.7	4285± 678.1	3082± 151.7	2682± 209.3	27292± 4242	23901± 6099	**Figure 5.9C**	**Figure 5.9C**	4.406± 0.8045	4.273± 0.5858
Neutrophil number	**Figure 5. 6C**	**Figure 5. 6C**	1320± 128.3	1480± 1480	1046± 67.31	997.7± 50.01	**Figure 5.8E**	**Figure 5.8E**	64.90± 17.87	66.24± 20.77	169.8± 44.00	216.7± 50.73

T reg -Regulatory T cells: LN – Lymph Node. Unpaired t-test showed no significant effect of PEPITEM treatment on leukocyte number for the data presented in this table. Statistically significant data are represented in figure format **(Figure 5.6-10)**. Data are shown as mean ± SEM for n = 1 independent experiments, using n=14 mice per group.

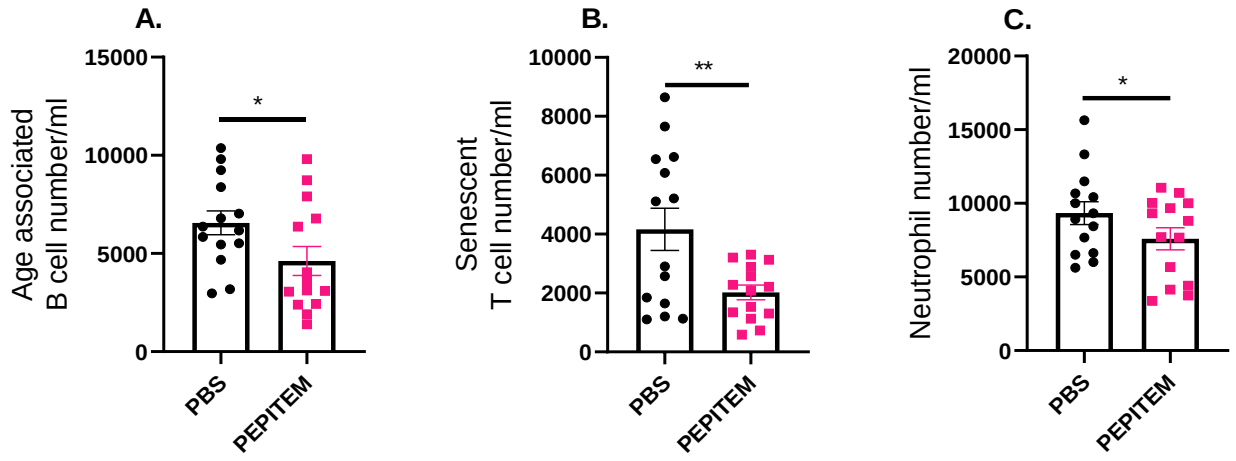


Figure 5. 9 Quantification of leukocyte subsets in blood of HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM or PBS administrated via mini pumps for the last 6 weeks of HFD. Blood **(A)** age associated B cells, **(B)** senescent T-cells and **(C)** neutrophils were quantified using flow cytometry analysis. Absolute number of immune cells were normalised to bead counts and plotted as number per ml of blood. Data are shown as mean±SEM for n = 1 independent experiment using n = 14 mice per group. *= $p<0.05$, ** = $p<0.01$ by unpaired t-test compared to PBS treated.

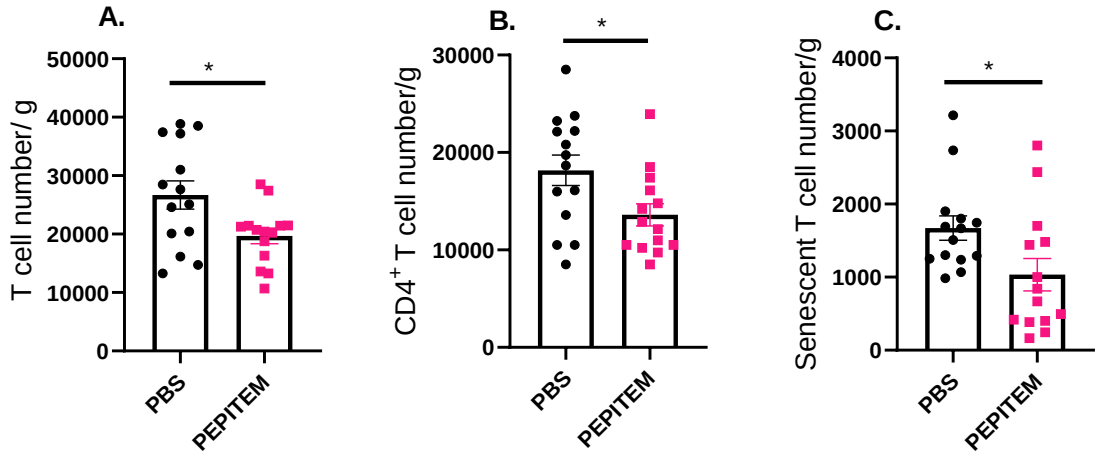


Figure 5.10 Quantification of leukocyte subsets in visceral adipose tissue of HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM or PBS administrated via mini pumps for the last 6 weeks of HFD. Visceral adipose tissue **(A)** T cells, **(B)** $CD4^+$ T cells and **(C)** senescent T-cells were quantified using flow cytometry analysis. Absolute number of immune cells were normalised to bead counts and plotted as number per g of tissue. Data are shown as mean±SEM for n = 1 independent experiment using n = 14 mice per group. *= $p<0.05$, by unpaired t-test compared to PBS treated.

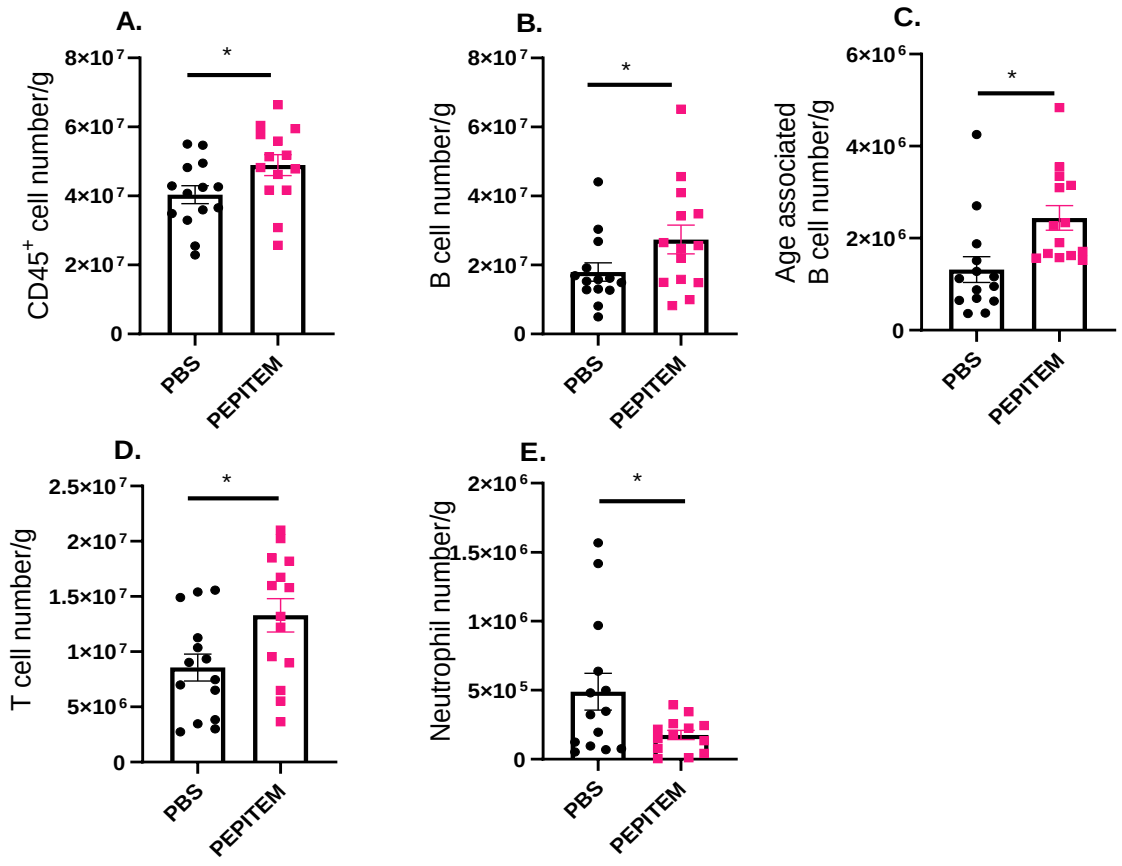


Figure 5.11 Quantification of leukocyte subsets in spleen of HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM or PBS administrated via mini pumps for the last 6 weeks of HFD. Spleen **(A)** CD45+ cells, **(B)** B cells **(C)** age associated B cells, **(D)** T cells, and **(E)** neutrophils were quantified using flow cytometry analysis. Absolute number of immune cells normalised to bead counts and plotted as number per g of tissue. Data are shown as mean±SEM for n = 1 independent experiment using n = 14 mice per group. *= $p<0.05$, by unpaired t-test compared to PBS treated.

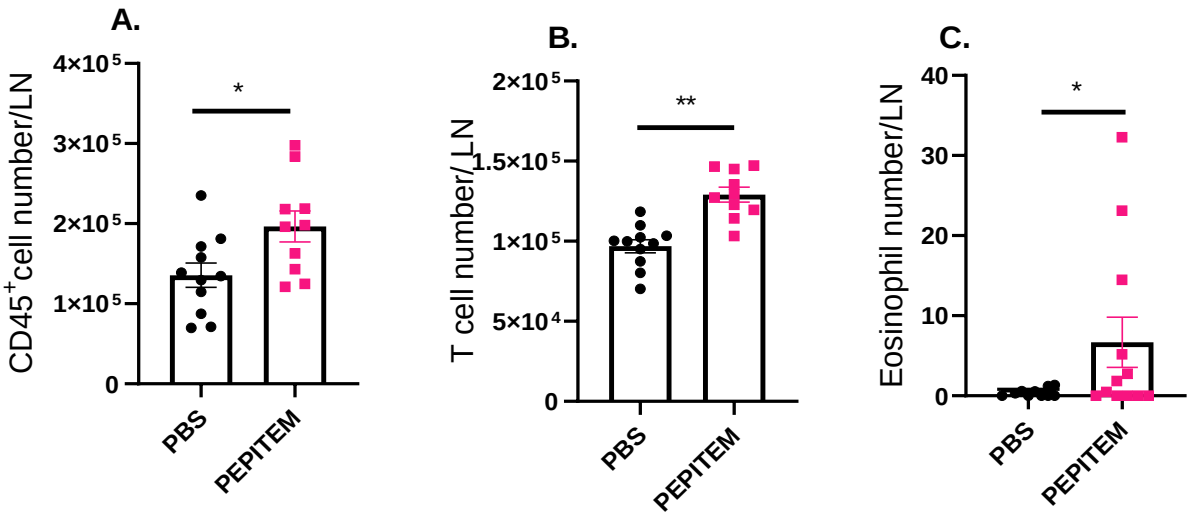


Figure 5.12 Quantification of leukocyte subsets in LN of HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM or PBS administrated via mini pumps for the last 6 weeks of HFD. Lymph node (LN) **(A)** CD45+ cells, **(B)** T cells and **(C)** eosinophils using flow cytometry analysis. Absolute number of immune cells normalised to bead counts and plotted as number per LN. Data are shown as mean±SEM for n = 1 independent experiment using n = 11 mice per group. *= $p<0.05$, ** = $p<0.01$ by unpaired t-test compared to PBS treated.

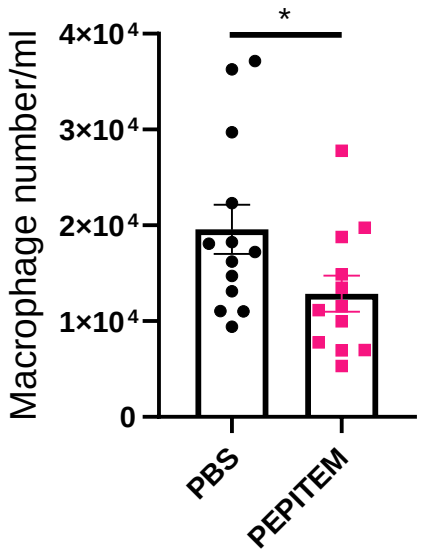


Figure 5.13 Quantification of macrophages in peritoneal lavage of HFD-mice treated with PEPITEM vs PBS. Male wt (C57Bl/6) mice were fed a high fat diet (HFD) for 12 weeks and received 3.5mg/ml PEPITEM or PBS administrated via mini pumps for the last 6 weeks of HFD. Peritoneal lavage macrophages were quantified using flow cytometry analysis. Absolute number of macrophages normalised to bead counts and plotted as number per ml. Data are shown as mean±SEM for n = 1 independent experiment using n = 13 mice per group. *= $p<0.05$, by unpaired t-test compared to PBS treated.

5.3 Discussion

Here using two sets of experiments, we explored the prophylactic as well as therapeutic capacity of a novel endogenous peptide, PEPITEM, on chronic inflammation caused by obesogenic diet in mice. We observed no direct metabolic action of PEPITEM in either sets of our *in vivo* experiments, despite a significant reduction in islets individual size of area compared to PBS treated group. Prophylactic PEPITEM treatment was only able to block the obesogenic diet-induced T cell and macrophage trafficking into adipose tissue and peritoneum. However, therapeutic administration of PEPITEM following feeding mice HFD dramatically altered the composition of leukocyte subsets in peripheral tissues, as well as secondary lymphoid organs.

To the best of our knowledge, this is the first study to investigate the metabolic aspects of PEPITEM and there is currently no evidence reporting the immune modulatory influence of PEPITEM in obesity/T2DM to compare our results against. Therefore, this discussion will focus on the evidence exists for obesity-related leukocyte trafficking focusing on adipose tissue.

5.3.1 Evidence on obesity related metabolic changes

Diet-induced obesity, widely used in rodents, has become one of the most beneficial tools to explore the impact of obesity on glucose metabolism and development of T2DM (Tang et al., 2013). Some studies suggest that high fat/high fructose diet is better to mimic the T2DM in animal models, as high-fructose diet can cause hyperinsulinaemia, while a high-fat diet can result in impaired pancreatic function of insulin secretion and glucose intolerance (Huang et al., 2004). Following 16–20 weeks of HFD, mouse will typically show 20–30% increase in body weight when compared to chow diet mouse (Tang et al., 2013). Here we observed approximately 30% increase

in body weight in both groups after feeding them HFD either for 6 or 12 weeks, which is similar to the clinical manifestation of diabetic patients in early stage as they are gaining body weight. We used male mice in our experiments as it is already stablished that C57BL/6 male mice have a greater propensity of gaining body weight than female mice to develop glucose intolerance (Hong et al., 2009). In our model although we have not include any chow diet control to compare whether obesity could cause necessary changes to impair the glucose tolerance, our results are comparable with previous studies showing higher fasting glucose (9.1 ± 0.3 vs. 7.9 ± 0.4 mmol/l) following 8 weeks of HFD compared to chow diet mice (Andrikopoulos et al., 2008). In both sets of experiments, we observed an impaired fasting glucose in week 8 (PBS:9.41±0.3, PEPITEM:10.5±1.11) and week 12 (PBS:10.9±0.6, PEPITEM: 11.8±0.9) of HFD, indicating that our HFD was only able to induce the initial metabolic changes take place in obese subjects as the fast glucose levels in both sets of experiments are higher compare to those shown in chow fat diet control mice in other studies (Andrikopoulos et al., 2008)

Here we used IPGTT as a simple approach to analyse the effect of diet/PEPITEM on metabolism (Dinger et al., 2018). Indeed we examined how well the body's cells are able to uptake glucose after injecting a given amount of sugar, which in turn is indicative of insulin secretion and insulin effect (Dinger et al., 2018). Here, our findings showed that blood glucose levels reached the fasting glucose levels in all IPGTT after 120-minute test, suggesting that despite impaired fasting glucose, insulin is still able to keep the blood glucose at fasting levels, showing no development of insulin resistance after feeding mice HFD for 6 or 12 weeks in our model.

Previous reports have clearly established the association between obesity and beta cell mass in both obese and T2DM subject. Several human studies have reported beta cell mass expansion by 20% to 90% in obese non diabetic subjects (Hanley et al., 2010; Saisho et al., 2013).

Immunohistochemistry data from Tschen et al. demonstrated a threefold expansion in beta cells mass in 6-week-old male C57BL/6 mice following 8 weeks HFD compared to control chow diet mice, which was due to increased proliferation of the pancreatic beta cells in response to decreased insulin sensitivity shown as increased fasting insulin levels in HFD mice (Tschen et al., 2009).In fact our study lacks the control mice on chow diet to evaluate the obesity related expansion of islets. However, we observed 2.2- and 1.2-fold reduction in islet size by prophylactic and therapeutic administration of PEPITEM respectively, suggesting prophylactic administration of PEPITEM was more efficiently reduced the obesity induced expansion of the islets.

Given that we observed no change in glucose homeostasis between two groups, it is unlikely that PEPITEM has modulate the blood insulin levels in treated group and more tests such as insulin tolerance test is needed to address whether PEPITEM affects insulin sensitivity. Our data suggests that PEPITEM limits HFD-induced pancreatic islet expansion independent of its effects on insulin sensitivity. However, the mechanism by which PEPITEM suppress the islets expansion is unknown and the sole effect of PEPITEM on beta cells mass in non-obese WT mice must be investigated to conclude whether PEPITEM is reversing the beta cell mass back to baseline or avoids the expansion of islets in general. Interestingly, like what we observed with PEPITEM, metformin, was shown to suppress the compensatory beta cell expansion induced by HFD in mice (Tajima et al., 2017). Indeed, they showed that 8 weeks of HFD was sufficient to induce adaptive beta cell mass expansion and proliferation in HFD mice compared to chow diet mice (Tajima et al., 2017). An 8-week metformin treatment suppressed the compensatory beta cell hyperplasia induced by HFD which was demonstrated by lower ratio of BrdU- or Ki67-positive proliferating beta cells in metformin treated HFD mice (Tajima et al., 2017). In search of mechanism by which metformin affected this, they roll out the possibility of beta cell apoptosis by showing unchanged

ratio of cleaved caspase-3-positive beta cells between the HFD group and HFD + metformin group using immunostaining (Tajima et al., 2017). Using 1 week HFD mice, while no insulin resistance was developed, they could show that 1 week metformin treatmentis able to suppress the 1-week HFD induced beta cell proliferation compared to only HFD mice, indicating metformin suppressed HFD-induced beta cell proliferation before the development of insulin resistance (Tajima et al., 2017). This was further supported by *in vitro* data where metformin directly blocked the glucose-induced replication of beta cells through suppressing the glucose-induced phosphorylation of mammalian target of rapamycin (which is essential for cell growth and proliferation). Since we also have seen a similar suppressing effect of PEPITEM on HFD induced beta cell expansion in a condition which the insulin resistance was not induced yet by HFD, this suggests a possible mechanism of action for PEPITEM to reduce the islets expansion by blocking the proliferation of beta cells before the development of insulin resistance. However, the involvement of extrinsic signals from other organs such as adipose tissue is also important for the regulation of adaptive beta cell expansion. These signals might influence the PEPITEM effects on the compensatory beta cell expansion, since PEPITEM has shown immunomodulatory effects in multiple tissues in our study. Based on the results of our study, mainly the prophylactic administration of PEPITEM has the potential to impose a preventive effect on compensatory beta cell expansion during an early disease stage independent of improvement in metabolic parameters.

5.3.2 Evidence on PEPITEMs actions in inflammation

Despite the lack of evidence about metabolic capacity of PEPITEM, there are few studies supporting the in vivo efficacy of PEPITEM in inflammation. For the first time Chimen et al.

showed the *in vivo* functionality of PEPITEM in various inflammatory induced conditions (Chimen et al., 2015); They generated a B cell–knock out mouse model of zymosan-induced peritonitis, with no capacity to produce PEPITEM. Zymosan induced more T cells infiltration into the peritoneum of B cell– knock out mice compared to WT controls, which was reversed with injection of PEPITEM to these mice with (Chimen et al., 2015). This was further supported with another model of inflammation where PEPITEM administration could suppress the lymphocyte infiltration into inflamed liver of *Salmonella*-infected C57BL/6 mice compared to PBS treated mice (Chimen et al., 2015). Most recently, Mastubara et al. reported reduced infiltration of double-negative T cells into the kidney of MRL/lpr mice treated with PEPITEM, which was concomitant with increased protein expression of CDH15 (receptor for PEPITEM) on endothelial cells of glomeruli and kidney arterioles of lupus mice (Matsubara et al., 2020), suggesting the modulation of PEPITEM/CDH15 axis in lupus nephritis. This improvement in inflammation translated into milder proteinuria in PEPITEM treated mice compared to non-treated MRL/lpr mice (Matsubara et al., 2020). Our study adds more evidence to this regulatory aspect of PEPITEM in governing lymphocytes trafficking in obesity induced chronic inflammatory condition. Indeed, we could show prophylactic administration of PEPITEM suppressed CD4+ T cells and senescent T cells infiltration into visceral adipose tissue and peritoneum in a 6-week HFD-mice. In addition, our data showed a reduction in number of macrophages in peritoneum of PEPITEM treated when both administrated prophylactic and therapeutic, suggesting the capacity of PEPITEM in regulating the innate immune system, either by limiting the lymphocyte recruitment or unknown direct influence over macrophages. This limited recruitment of macrophages to site of inflammation was also shown in Mastubara et al. study, where their immunohistochemistry analysis showed a decrease in the number of F4/80 + cells in the renal interstitium along with reduced deposition in C3 and

IgG in the glomeruli upon PEPITEM treatment, indicating macrophage and B cell activation was suppressed along with the decrease infiltration of T cells into the renal interstitium (Matsubara et al., 2020).

5.3.3 Obesity related changes in leukocyte trafficking in adipose tissue

In this study we observed a regulatory effect of PEPITEM on adipose tissue mostly by suppressing the $CD4^+$ T cells and senescent T cells recruitment. The mechanisms that regulate T cell trafficking in adipose tissue are largely unknown. Increased number of T cells in visceral adipose tissue has been previously shown in obese mice and human and are linked either to their recruitment or proliferation. For example, Duffat et al, showed more infiltration of $CD3^+$ T cells composed of $CD4^+$ and $CD8^+$ cytotoxic T cells into adipose tissue of obese non diabetic subjects, (Duffaut et al., 2009) which was explained by increased gene expression of chemokine CCL20 on mature adipocytes from obese people compare to lean (Duffaut et al., 2009). Moreover, flow cytometric analysis of an *in vivo* study showed higher numbers of T cells and macrophages in adipose tissue of male mice placed on 24-week HFD compared to normal diet lean mice (Wu et al., 2007). In this study the recruitment of T cells into adipose tissue of obese mice was postulated to higher protein and gene expression of RANTES and its receptor CCR5 in fat tissue of obese mice (Wu et al., 2007). HFD for 14 weeks, was reported to activate the proliferation of $CD4^+$ T cells in adipose tissue (Morris et al., 2013). In fact, they showed that adipose tissue macrophages are able to develop the proliferation $CD4^+$ T cells in adipose tissue (Morris et al., 2013). These data support the concept that adipose tissue macrophages govern an adaptive response by modulating T cell activation in obese fat (Morris et al., 2013). However, in another *in vivo* study early infiltration of adipose tissue B cells followed by increased T cell proceeded the accumulation

of macrophages in adipose tissue after 12 weeks of HFD (Duffaut et al., 2009). Using lymphocyte deficient mice, this study demonstrated absence of lymphocytes did not change the onset of obesity and insulin resistance status (Duffaut et al., 2009). Therefore suggested that the lymphocytes alone are not enough for the initiation of obesity-associated insulin resistance (Duffaut et al., 2009). Moreover, an exaggerated infiltration of macrophages in adipose tissue in absence of lymphocytes suggested a protective role for lymphocytes to control inflammation in adipose tissue (Duffaut et al., 2009). In our study however, since the number of visceral adipose tissue macrophages are unchanged in both prophylactic and therapeutic treatment with PEPITEM, it seems that PEPITEM exerts its immunomodulatory effect on adaptive cells in adipose tissue. Indeed, PEPITEM is known to regulate T cells trafficking *in vitro* and *in vivo* in previous studies. Here for the first time, we show its functionality in obesity induced inflammation. Although we have not measured the adiponectin levels here, it is already stablished that both circulating and adipose tissue expressed adiponectin levels are decreased in obese subjects (Lu et al., 2008) and our data suggests that administration of PEPITEM is able to restore the homeostatic pathway regulating T cell recruitment in adipose tissue of obese mice. However more experiments such as evaluating the expression of CDH15 by adipose tissue is needed to confirm the modulation of PEPITEM/CDH15 axis in obesity. Furthermore, the effect of PEPITEM on chemokine response in adipose might be necessary to better clarify its mechanism of action.

There are evidence showing the involvement of $CD8^+$ T cells in development of insulin resistance in obesity. It has been proposed that $CD8^+$ T cells improve insulin resistance through recruiting macrophages (Eller et al., 2011; Liu et al., 2021) As shown by Nishimura et al., the increased recruited $CD8^+$ T cells and macrophages in visceral adipose tissue of WT mice placed on HFD for 16 weeks was associated to insulin resistance in HFD mice compared to chow diet and

genetic depletion of $CD8^+$ T cells decreased macrophage infiltration and dampened the systemic insulin resistance (Nishimura et al., 2009) This suggests adipose tissue $CD8^+$ T cells and macrophages together are necessary for metabolic changes occurs in obesity. Thus, here in our study, unchanged number of $CD8^+$ T cells and macrophages in adipose tissue in PEPITEM treated mice, might be associated with unchanged systemic metabolic status between two groups.

In this study for the first time, we demonstrated the capacity of PEPITEM in regulating senescent lymphocytes in chronic inflammation. KLRG1 has been widely used in animal studies to identify senescent $CD4^+$ (Beyersdorf et al., 2007) and CD8 $^+$T cells (Voehringer et al., 2001). These studies provide evidence for strikingly lower cell divisions of $KLRG1^+$ T cells when compared with their $KLRG1^-$ counterpart. However, a recent *in vivo* mice study has revealed that, it is the loss of KLRG1 that marks out a population of senescence like cells (Herndler-Brandstetter et al., 2018). In this study they showed that $KLRG1^+$ effector $CD8^+$ T cells lose KLRG1 expression and differentiate into long-lived memory cells called ‘‘ExKLRG1’’memory cells which retained high cytotoxic and proliferative capacity distinct from other populations and contributed to effective anti-influenza and anti-tumor immunity (Herndler-Brandstetter et al., 2018). In our study the senescent T cells have been defined by phenotype only and the data showing their proliferative capacity is missing. Therefore, more functional experiments confirming their senescence is needed in future expeiments. Previous studies has shown increased number of senescent T cells in adipose tissue of HFD mice (Shirakawa et al., 2016; Kiran et al., 2021). Shirakawa *et al*. showed that CD4 $^+$ T cells expressing PD-1 and CD153 were increased in number in adipose tissue from mice fed a HFD for 3 months (Shirakawa et al., 2016). These T cells were similar to senescence $CD4^+$ T cells that normally increase with age. Also, their adoptive transfer from a HFD mice to a lean WT mice caused insulin resistance (Shirakawa et al., 2016). Kiran et al. found that 16 weeks of HFD

increases infiltration of activated T cells expressing KLRG1, a senescent marker, and induce polarization of macrophages towards an inflammatory M1 macrophages in epididymal adipose tissue of HFD mice which was associated with increased fasting blood glucose in HFD mice compared to lean mice (Kiran et al., 2021). In our study, although we have not included a normal chow diet mice to compare the HFD effects on senescent T cells, reduced number of T cells expressing KLRG1 were significantly decreased in visceral adipose tissue of PEPITEM treated group, suggesting PEPITEM might be able to restore the normal profile. However, reduced number of senescent T cells with PEPITEM was not associated with any change in metabolic parameters in our study, indicating PEPITEM may influence the immune system independent to metabolism.

Although, PEPITEM pathway emphasis on protective role of B cells in regulating lymphocyte recruitment in inflammatory condition, there are studies demonstrating the pathogenic role for B cells in the development of metabolic disorders in obesity induced inflammation. Indeed, Winer et al, showed 8 week-HFD-induced obese mice lacking B cells are protected from impaired fasting glucose and impaired glucose tolerance and insulin resistance compared to HFD-wt mice (Winer et al., 2011). B cell deficiency did not influence the glucose intolerance and insulin resistance in normal chow diet mice, indicating that metabolic influences of B cells require a HFD (Winer et al., 2011) Therefore it might be assumed that depletion of B cell might be a beneficial strategy in obesity induced T2DM. However, our results that PEPITEM can inhibit the trafficking of T cells in fat tissue, raises the possibility that the PEPITEM pathway can be a potential target to treat chronic inflammatory diseases.

5.3.4 Obesity related changes in leukocyte trafficking in secondary lymphatic organs

There are evidence showing obesity modulates leukocyte trafficking in secondary lymphoid organs. For example, frequency of $CD4^+$ and $CD8^+$ T cells reported higher in spleen and mesenteric LNs of obese compared to WT lean mice following 16 weeks of HFD (Kiran et al., 2021). However, in another *in vivo* study total number of lymphocytes determined using a phase-contrast microscope, reported lower in visceral LNs of mice on HFD for 10 weeks compared to those on chow diet (Kim et al., 2008). Their FACS data showed no changes in the percentages of $CD4^+$ T cells and $CD8^+$ T cells in the visceral lymph nodes of the obese compared to those in the nonobese mice (Kim et al., 2008). Moreover, they observed higher expression levels of Annexin V, (apoptosis marker), on lymphocytes of visceral lymph node in obese mice, indicating HFD-induced lymphocyte death happens in visceral lymph node cells (Kim et al., 2008). Overall, our data from spleen and inguinal LNs indicates that therapeutic but not prophylactic treatment of PEPITEM in HFD mice alters leukocyte trafficking to these organs mostly through affecting the adaptive immune cells. Reduced number of T lymphocytes in adipose tissue as a main source of inflammation in obesity along with lymphocyte increase in spleen and lymph nodes with no change in number of antigens presenting cells like macrophages in these organs, suggest that PEPITEM possibly retains the lymphocytes in secondary lymphoid organs. Thus, less lymphocytes are migrated to peripheral tissues. The mechanism by which PEPITEM retains the lymphocyte in SLO is not clear. More evaluations are needed to see if PEPITEM rescue the lymphocyte from HFD induced apoptosis in SLO.

It is widely accepted that S1P is one of the most important factors that regulates lymphocyte egress from the SLO to periphery via binding to receptor 1 ($S1P_1$) (Chiba et al., 2011). It has been stablished that in mice whose lymphocytes lack a single S1P receptor, ($S1P1^{+/-}$),

mature T cells are unable to exit SLO (Lo et al., 2005). Moreover, S1P1 overexpression in activated B cells reduces their accumulation in the spleen and increase the ability of activated T cells to exit the lymph nodes (Lo et al., 2005). It is also proposed that cyclical modulation of S1P1 on circulating lymphocytes is necessary to direct them to/from SLO, as expression of S1P1 receptor are reduced on lymphocytes during trafficking in blood and lymph and increase within the lymphoid organs (Lo et al., 2005; Tsai and Han, 2016). Thus, considering the importance of S1P1 in homing and egression of lymphocytes, modulation of S1P1 in SLO might be a possible mechanism by which PEPITEM influence the lymphocyte. Furthermore, homing of lymphocyte from blood into SLO highly depends on lymphocyte specific chemokines (CCL19, CCL21, CXCL12, and CXCL13), as their expression in SLO is necessary to attract the lymphocytes from circulation to SLO (Tsai and Han, 2016). Therefore, the effects of PEPITEM on the expression of these chemokines and their receptors might give us more insight on immunomodulatory effects of PEPITEM in SLO.

5.3.5 Limitations

Our study lacks a control group for mice on chow diet. Therefore, including a control group of lean mice would be beneficial to clarify whether either of the 6- or 12-week HFD made the sufficient metabolic as well as immune alterations required to achieve obesity related changes. Similarly including a group of control mice on normal chow diet with PEPITEM treatment would allow us to investigate the metabolic and immunomodulatory effects of PEPITEM itself at baseline levels in WT mice, which to our knowledge it is not investigated so far.

5.3.6 Conclusion

In conclusion we have observed PEPITEM therapy can limit the obesity induced islet expansion independent of improvement in metabolic status and is able to restore the homeostatic regulatory pathway in adipose tissue of obese mice. Thus, PEPITEM administration may be considered as a potential therapeutic tool to overcome the obesity relatted changes occures in early disease stage. However, more control groups including chow diet mice with or without PEPITEM administration is required to fully interpret this result.

6 GENERAL DISCUSSION

6.1 Summary of findings

In this thesis we first sought to understand and characterise the role of CDH13 in anti-inflammatory effects of adiponectin focusing on PEPITEM pathway. As previously published (Chimen et al., 2015) and this study also showed, adiponectin can strongly inhibit PBL transmigration via PEPITEM pathway by acting on PBL. In addition, we showed the inhibitory effect of adiponectin in T cell migration when acting directly on BEC, suggesting that endothelial response to adiponectin was sufficient to dampen PBL transmigration. Furthermore, we demonstrated that CDH13 and AdipoR1/R2 are expressed by BEC. There are previous evidence supporting that, in absence of adiponectin receptors, adiponectin loses its protective effects because of the lack of adiponectin signaling in different tissues (Denzel et al., 2010; Iwabu et al., 2010). Here, we showed that individual knock down of CDH13, AdipoR1 or AdipoR2 abrogates the capacity of adiponectin to inhibit PBL transmigration through the inflamed endothelium, indicating the ability of these receptors to individually influence PBL migration and thus potentially impact on the PEPITEM pathway.

Evaluating expression of CDH13 by BEC, we found increased surface expression and decreased total (both surface and intracellular) expression of CDH13 following cytokine-stimulation of BEC, which is in agreement with the previous evidence showed increased expression of CDH13 in oxidative stressed-experienced BEC (Joshi et al., 2005) and subsequent increased shedding from endothelial cells (Philippova et al., 2011). Here we showed that adiponectin treatment reversed the effects of the cytokines on CDH13 total levels bringing them back to the levels observed at baseline, suggesting the ability of adiponectin to modulation the expression patterns of CDH13 during inflammation. Indeed, colocalization of adiponectin together with CDH13 in vasculature was shown in previous studies suggesting their overlapping biological

effects (Ivanov et al., 2001; Takeuchi et al., 2007). However, the mechanisms of action of CDH13 are not clear and further studies are required to get a better understanding of adiponectin-CDH13 interactions.

Mimicking a HG condition similar to T2DM *in vitro*, we determined how hyperglycaemia effects PBL transmigration. Since the effects of hyperglycaemia on leukocyte-endothelium interactions have mostly been investigated using *in vivo* animal models or in T2DM subjects, in which both leukocytes and endothelium are simultaneously exposed to HG, using our *in vitro* transmigration assay we were able to define the sole effect of HG on BEC or leukocytes behaviour separately. For the first time we showed that HG differently effects the endothelium and PBL behaviour and subsequent impact on lymphocyte trafficking. We found that HG significantly reduced the ability of endothelial cells to support PBL transmigration, which was restored by insulin supplementation, as would be the physiological response post digestion, or supplementation with the T2DM treatment metformin. These findings emphasise the immunomodulatory capacity of metformin and insulin in hyperglycaemic conditions like T2DM regardless of their glucose lowering effects.

Furthermore, unchanged protein levels of CAMs in these experiments suggests that HG, metformin, and insulin are not influencing the PBL recruitment through modulation of CAMs in this study despite their previously shown capacity to modulate the CAMs (Aljada et al., 2000; Abbasi et al., 2004; Bačun et al., 2008; Huang et al., 2019). Therefore, more *in vitro* experiments assessing other factors, such as chemokine response in HG and supplemented conditions, are needed to clarify the mechanism involved in this process.

In contrast to what we saw in BEC, we found increased adhesion and transmigration of PBLs in response to HG treatment of PBLs, suggesting activation of lymphocytes in hyperglycemic

environment. Indeed, increased expression of leukocyte activation marker, such as integrins LFA-1 (αLβ2-integrin) and VLA-4 (α4β1-integrin), were reported higher on leukocytes of patients with T2DM (Stulc et al., 2012). Although our study reflects the functional activation of PBLs in response to HG, it lacks the data about whether HG modulates the expression of these markers on lymphocytes, which can be a suggested mechanism by which HG acts on lymphocytes. Moreover, since the AdipoRs expression, particularly on B cells, remained unchanged following HG and subsequent metformin and insulin treatment of PBLs, the modulation of these receptors is not suggested to be a possible mechanism for these treatments here. However, here PBLs only experienced a transient HG, which is not like chronic hyperglycemic condition in T2DM and evaluating the AdipoRs expression on B cells from subjects with T2DM would be beneficial to investigate the possible mechanisms of lymphocyte trafficking in this condition.

We clearly showed the efficacy of PEPITEM through reducing PBL migration across BEC *in vitro.* The *in vivo* functionality of PEPITEM was previously shown using different models of inflammation, where PEPITEM reduced the number of T cells in site of inflammation (Chimen et al., 2015; Matsubara et al., 2020). Here for the first time, we investigated the metabolic and immune modulatory aspects of PEPITEM in a HFD-induced chronic inflammation, while no insulin resistance was developed in mice. We found no improvement in metabolic parameters by either prophylactic or therapeutic administration of PEPITEM despite a significant limit in pancreatic islet expansion in both PEPITEM treatment conditions. This decrease was greater when PEPITEM was administrated prophylactic or in other word before the HFD-induced alterations was stablished. Our data suggests that PEPITEM limits HFD-induced pancreatic islet expansion independent of its effects on glucose homeostasis. Indeed, PEPITEM action here was similar to metformin mechanism of action, which has been shown to suppress the HFD-induced islet

expansion before the development of insulin resistance via blocking the glucose-induced replication of beta cells (Tajima et al., 2017). However, the mechanism by which PEPITEM suppress the HFD-induced islets expansion is unknown and the sole effect of PEPITEM on beta cells mass in non-obese wt mice has not been investigated yet.

For the first time, in this study we showed a regulatory effect of PEPITEM on visceral adipose tissue mostly by limiting T cells recruitment. Indeed, increased infiltration of T cells into adipose tissue was shown in interaction with innate immune cells (macrophages) in obese subjects (Duffaut et al., 2009; Morris et al., 2013). Here, decreased number of T cells along with unaffected number of macrophages in visceral adipose tissue in both prophylactic and therapeutic treatment with PEPITEM, suggest that PEPITEM exerts its immunomodulatory effect on adaptive cells in adipose tissue. Furthermore, decreased adiponectin levels (both in circulation and adipose tissue) has been previously reported in obesity (Lu et al., 2008) and suggests the possibility of impaired PEPITEM pathway in our HFD mice is more like, and which seems to be compensated by PEPITEM treatment through its ability to restore the homeostatic pathway regulating T cell recruitment in visceral adipose tissue.

$CD8^+$ T cells and macrophages are two major population of leukocytes that are known to induce insulin resistance and metabolic changes in fat inflammation (Eller et al., 2011; Liu et al., 2021), suggesting that unchanged systemic metabolic status between treated and untreated groups in our study might be associated with unchanged number of $CD8^+$ T cells and macrophages in adipose tissue in PEPITEM treated mice. Furthermore, the accumulation of senescent T cells in adipose tissue of HFD mice was shown in association with insulin resistance (Shirakawa et al., 2016; Kiran et al., 2021). In our study, reduced number of T cells expressing KLRG1 were significantly decreased in visceral adipose tissue of PEPITEM treated group, suggesting a

therapeutic potential of PEPITEM to restore the normal profile in adipose tissue. However, reduced number of senescent T cells with PEPITEM was not associated with any change in metabolic parameters in our study, indicating PEPITEM may influence the immune system independent to metabolism.

Our data from SLO (spleen and inguinal LNs) indicates that therapeutic, but not prophylactic, treatment of PEPITEM (when the 6-week HFD-induced damage is stablished) in HFD mice alters leukocyte trafficking to these organs mostly through affecting the adaptive immune cells. Both increase (Kiran et al., 2021) and decreased (Kim et al., 2008) number of lymphocyte were reported in LNs of HFD mice. Indeed, increased number of T lymphocytes in SLO along with no change in number of antigens presenting cells like macrophages in these organs suggest that PEPITEM possibly retains the lymphocytes in secondary lymphoid organs. However, the mechanism by which PEPITEM does this is not clear and more studies are required to see if PEPITEM effects the lymphocyte specific chemokines (Tsai and Han, 2016) in SLO or acts through modulating $S1P_1$ a (potent regulator of lymphocyte egress from SLO) (Chiba et al., 2011).

1.2 Future directions

CDH13

- Further evaluating the localization of CDH13, AdipoR1/R2 and adiponectin at cellular levels using confocal imaging by including more conditions, such as individual knock down of these receptors, would help to investigate the role of these receptors in adiponectin signaling.

- Further investigating the protein expression of CDH13 in inflammation vs baseline by including other techniques of protein measurement, such as western blot, to support the flow cytometry data and clarify CDH13 modulation in inflammation.

HG, metformin, and insulin

- Analysing dynamic behavior of leukocytes in response to HG, metformin, and insulin treatments to quantify their behavior in a more physiological context.

- Evaluating expression of leukocyte activation markers, such as integrins LFA-1 (αLβ2-integrin) and VLA-4 (α4β1-integrin), following HG treatment to confirm their activation in response to HG.

- Evaluating the endothelium-leukocyte interaction in response to pathological/prolonged exposure to insulin concentration similar to those observed in T2DM.

- Investigating the AdipoRs expression in PBMCs from patients with T2DM and measuring the capacity of adiponectin and PEPITEM in regulating PBL recruitment *in vitro*. *[This aspect of the project was delayed due to COVID]*.

PEPITEM

- Adding more control groups, including chow diet mice with or without PEPITEM administration to compare the PEPITEM effects with baseline levels, as well as investigate the metabolic and immunomodulatory effects of PEPITEM in non-obese WT mice.

- Evaluating the effects of PEPITEM on insulin secretion by beta cells either via performing insulin tolerance test or Glucose-Stimulated insulin Secretion (GSIS).

- Placing mice on HFD for longer timeframes, such as 16 weeks, to create a glucose intolerance mice model closer to T2DM to allow understanding of PEPITEM effects on metabolic and immune changes occur in T2DM.

- Measuring the adiponectin and PEPITEM levels in circulation in HFD mice to see how PEPITEM pathway is modulated in obesity, and check if PEPITEM administration via mini pumps effectively increased the PEPITEM serum levels

- Evaluating the expression of CDH15 by visceral adipose tissue to confirm the involvement of PEPITEM/CDH15 axis in adipose tissue in obese mice.

- Evaluating the chemokine response in visceral adipose tissue of obese mice to allow us to better clarify PEPITEM mechanism of action in regulating T cells.

- Evaluating the functional and proliferative capacity of KLRG1^{+} T cells to confirm their senescence.
- Evaluating the modulation of S1P1, as well as lymphocyte specific chemokines, in SLO to understand the mechanism by which PEPITEM retains the lymphocyte in SLO

In conclusion, we have shown a crucial role of CDH13 in functionality of PEPITEM pathway. We also showed that HG differently affects the endothelium and PBL behaviour. Moreover, metformin and insulin supplementation of endothelium restores its ability to support the PBL recruitment. Finally, we showed that PEPITEM therapy can limit the obesity induced islet expansion independent of improvement in metabolic status and is able to restore the homeostatic regulatory pathway in adipose tissue of obese mice. Thus, PEPITEM administration may be considered as a potential therapeutic tool to control the magnitude of obesity related inflammation and overcome the changes occurs in early disease stage.

Graphical abstract of our findings is shown Figure 6.1.

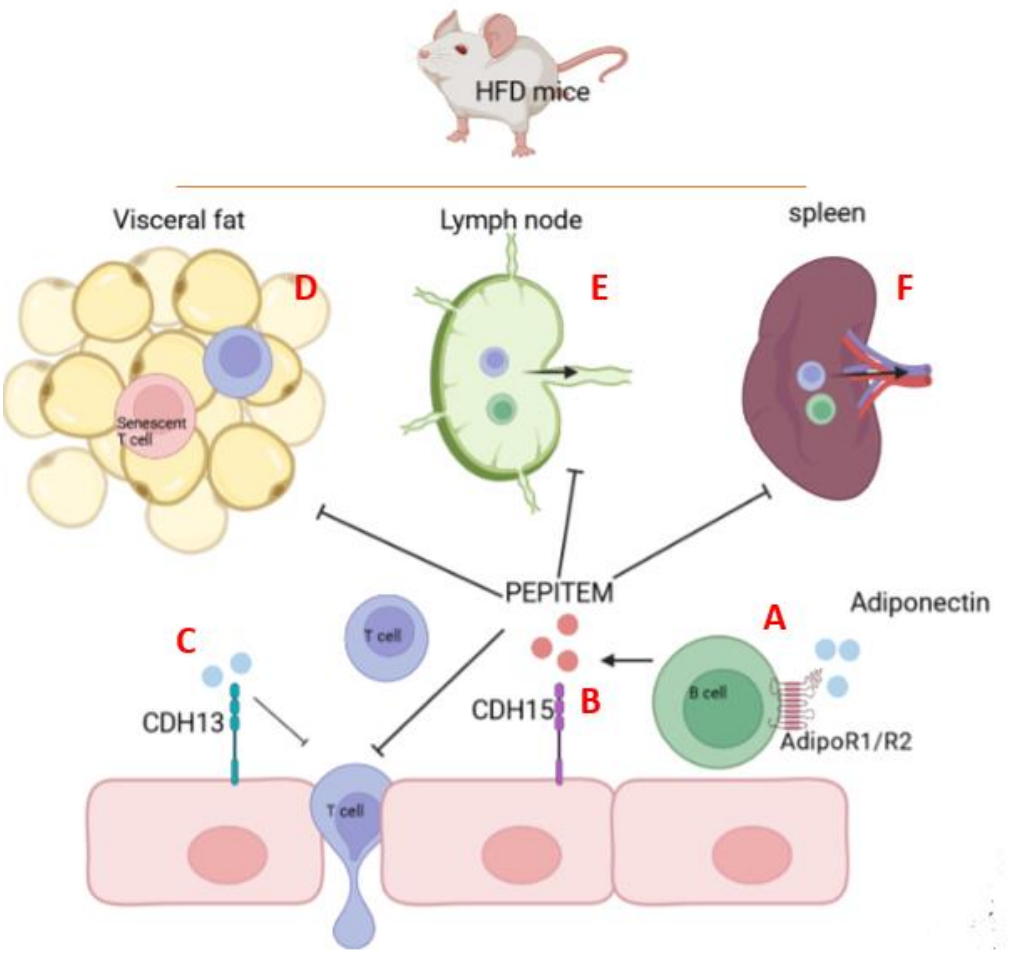


Figure 1.6 PEPITEM regulation of lymphocytes during inflammation. (A) Binding to AdipoR1/R2 on B cells, adiponectin induces PEPITEM production by B cells. (B) Binding to its endothelial receptor (CDH15), PEPITEM results in inhibition of T cell transmigration through inflamed endothelium. (C) Adiponectin signaling through CDH13 also inhibits T cell transmigration. (D) PEPITEM is able to inhibit T cell infiltration into the visceral adipose tissue and (E) suppress lymphocyte mobilization from secondary lymphatic organs such as lymph nodes and (F) spleen to peripheral tissues during HFD induced inflammation.

7 References

Abbasi, F., J. W. Chu, T. McLaughlin, C. Lamendola, E. T. Leary and G. M. Reaven (2004). "Effect of metformin treatment on multiple cardiovascular disease risk factors in patients with type 2 diabetes mellitus." Metabolism **53**(2): 159-164.

Abe, Y., B. El-Masri, K. T. Kimball, H. Pownall, C. F. Reilly, K. Osmundsen, C. W. Smith and C. M. Ballantyne (1998). "Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion." Arteriosclerosis, thrombosis, and vascular biology **18**(5): 723-731.

Adekola, K., S. T. Rosen and M. Shanmugam (2012). "Glucose transporters in cancer metabolism." Current opinion in oncology **24**(6): 650.

Ahmed, S. R., H. M. McGettrick, C. M. Yates, C. D. Buckley, M. J. Ratcliffe, G. B. Nash and G. E. Rainger (2011). "Prostaglandin D2 regulates CD4+ memory T cell trafficking across blood vascular endothelium and primes these cells for clearance across lymphatic endothelium." The Journal of Immunology **187**(3): 1432-1439.

Aiello, A., F. Farzaneh, G. Candore, C. Caruso, S. Davinelli, C. M. Gambino, M. E. Ligotti, N. Zareian and G. Accardi (2019). "Immunosenescence and its hallmarks: how to oppose aging strategically? A review of potential options for therapeutic intervention." Frontiers in immunology **10**: 2247.

Akbar, A. N. and J. M. Fletcher (2005). "Memory T cell homeostasis and senescence during aging." Current opinion in immunology **17**(5): 480-485.

Alberti, L., L. Gilardini, A. Girola, M. Moro, F. Cavagnini and C. Invitti (2007). "Adiponectin receptors gene expression in lymphocytes of obese and anorexic patients." Diabetes, Obesity and Metabolism **9**(3): 344-349.

Aljada, A., H. Ghanim, R. Saadeh and P. Dandona (2001). "Insulin inhibits NFκB and MCP-1 expression in human aortic endothelial cells." The Journal of Clinical Endocrinology & Metabolism **86**(1): 450-453.

Aljada, A., R. Saadeh, E. Assian, H. Ghanim and P. Dandona (2000). "Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothelial cells through stimulation of nitric oxide." The Journal of Clinical Endocrinology & Metabolism **85**(7): 2572-2575.

Andrikopoulos, S., A. R. Blair, N. Deluca, B. C. Fam and J. Proietto (2008). "Evaluating the glucose tolerance test in mice." American Journal of Physiology-Endocrinology and Metabolism **295**(6): E1323-E1332.

Arora, A., T. Behl, A. Sehgal, S. Singh, N. Sharma, S. Chigurupati, R. Kaur, S. Bhatia, A. Al-Harrasi and C. Vargas-De-La-Cruz (2021). "Free fatty acid receptor 1: a ray of hope in the therapy of type 2 diabetes mellitus." Inflammopharmacology **29**(6): 1625-1639.

Arunachalam, G., S. M. Samuel, I. Marei, H. Ding and C. R. Triggle (2014). "Metformin modulates hyperglycaemia-induced endothelial senescence and apoptosis through SIRT1." British journal of pharmacology **171**(2): 523-535.

Babu, P. V. A., H. Si, Z. Fu, W. Zhen and D. Liu (2012). "Genistein prevents hyperglycemia-induced monocyte adhesion to human aortic endothelial cells through preservation of the cAMP signaling pathway and ameliorates vascular inflammation in obese diabetic mice." The Journal of nutrition **142**(4): 724-730.

Bačun, T., T. Belovari, M. Suver, B. Dobrošević and L. Glavaš-Obrovac (2008). "Effects of hyperglycemia and metformin on expression of adhesion molecules on human aortic endothelial cells." Croatica Chemica Acta **81**(1): 157-162.

Barouch, F. C., K. Miyamoto, J. R. Allport, K. Fujita, S. E. Bursell, L. P. Aiello, F. W. Luscinskas and A. P. Adamis (2000). "Integrin-mediated neutrophil adhesion and retinal leukostasis in diabetes." Investigative ophthalmology & visual science **41**(5): 1153-1158.
Basta, G., S. T. Del and R. C. De (2004). "Advanced glycation endproducts: implications for accelerated atherosclerosis in diabetes." Recenti progressi in medicina **95**(2): 67-80.
Baumgartner-Parzer, S. M., L. Wagner, M. Pettermann, J. Grillari, A. Gessl and W. Waldhäusl (1995). "High-glucose–triggered apoptosis in cultured endothelial cells." Diabetes **44**(11): 1323-1327.
Berbudi, A., N. Rahmadika, A. I. Tjahjadi and R. Ruslami (2020). "Type 2 diabetes and its impact on the immune system." Current diabetes reviews **16**(5): 442.
Berezin, A. (2019). "Neutrophil extracellular traps: The core player in vascular complications of diabetes mellitus." Diabetes & Metabolic Syndrome: Clinical Research & Reviews **13**(5): 3017-3023.
Beyersdorf, N., X. Ding, J. K. Tietze and T. Hanke (2007). "Characterization of mouse CD4 T cell subsets defined by expression of KLRG1." European journal of immunology **37**(12): 3445-3454.
Bluestone, J. A., K. Herold and G. Eisenbarth (2010). "Genetics, pathogenesis and clinical interventions in type 1 diabetes." Nature **464**(7293): 1293.
Booth, G., T. J. Stalker, A. M. Lefer and R. Scalia (2001). "Elevated ambient glucose induces acute inflammatory events in the microvasculature: effects of insulin." American Journal of Physiology-Endocrinology And Metabolism **280**(6): E848-E856.
Boucher, J., A. Kleinridders and C. R. Kahn (2014). "Insulin receptor signaling in normal and insulin-resistant states." Cold Spring Harbor perspectives in biology **6**(1): a009191.
Boura-Halfon, S. and Y. Zick (2009). "Phosphorylation of IRS proteins, insulin action, and insulin resistance." American Journal of Physiology-Endocrinology and Metabolism **296**(4): E581-E591.
Brake, D. K., E. O. B. Smith, H. Mersmann, C. W. Smith and R. L. Robker (2006). "ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice." American Journal of Physiology-Cell Physiology **291**(6): C1232-C1239.
Brown, A. M. and B. R. Ransom (2007). "Astrocyte glycogen and brain energy metabolism." Glia **55**(12): 1263-1271.
Burlet, E. and S. K. Jain (2013). "Manganese supplementation reduces high glucose-induced monocyte adhesion to endothelial cells and endothelial dysfunction in Zucker diabetic fatty rats." Journal of Biological Chemistry **288**(9): 6409-6416.
Butler, A. E., J. Janson, S. Bonner-Weir, R. Ritzel, R. A. Rizza and P. C. Butler (2003). "β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes." Diabetes **52**(1): 102-110.
Cahn, A., R. Miccoli, A. Dardano and S. Del Prato (2015). "New forms of insulin and insulin therapies for the treatment of type 2 diabetes." The Lancet Diabetes & Endocrinology **3**(8): 638-652.
Cameron, A. R., V. L. Morrison, D. Levin, M. Mohan, C. Forteath, C. Beall, A. D. McNeilly, D. J. Balfour, T. Savinko and A. K. Wong (2016). "Anti-inflammatory effects of metformin irrespective of diabetes status." Circulation research **119**(5): 652-665.
Campa, C. C., E. Ciraolo, A. Ghigo, G. Germena and E. Hirsch (2015). "Crossroads of PI3K and Rac pathways." Small GTPases **6**(2): 71-80.
Campbell, J. E. and C. B. Newgard (2021). "Mechanisms controlling pancreatic islet cell function in insulin secretion." Nature reviews Molecular cell biology **22**(2): 142-158.
Carman, C. V. and T. A. Springer (2004). "A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them." The Journal of cell biology **167**(2): 377-388.
Chaudhuri, A. and P. O. Behan (2003). "Natalizumab for relapsing multiple sclerosis." The New England journal of medicine **348**(16): 1598.
Chaves-Pozo, E., I. Cabas and A. García-Ayala (2012). "Sex steroids modulate fish immune response." Sex steroids: 199-220.

Chiba, K., H. Kataoka, N. Seki, Y. Maeda and K. Sugahara (2011). "Fingolimod (FTY720), the sphingosine 1-phosphate receptor modulator, as a new therapeutic drug in multiple sclerosis." Inflammation and Regeneration **31**(2): 167-174.
Chimen, M., H. M. McGettrick, B. Apta, S. J. Kuravi, C. M. Yates, A. Kennedy, A. Odedra, M. Alassiri, M. Harrison and A. Martin (2015). "Homeostatic regulation of T cell trafficking by a B cell–derived peptide is impaired in autoimmune and chronic inflammatory disease." Nature medicine **21**(5): 467-475.
Chimen, M., H. M. McGettrick, B. Apta, S. J. Kuravi, C. M. Yates, A. Kennedy, A. Odedra, M. Alassiri, M. Harrison and A. Martin (2015). "Homeostatic regulation of T cell trafficking by a B cell–derived peptide is impaired in autoimmune and chronic inflammatory disease." Nature medicine **21**(5): 467.
Chinetti, G., C. Zawadski, J.-C. Fruchart and B. Staels (2004). "Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPARα, PPARγ, and LXR." Biochemical and biophysical research communications **314**(1): 151-158.
Cho, E., E. B. Rimm, M. J. Stampfer, W. C. Willett and F. B. Hu (2002). "The impact of diabetes mellitus and prior myocardial infarction on mortality from all causes and from coronary heart disease in men." Journal of the American College of Cardiology **40**(5): 954-960.
Ciortan, L., R. D. Macarie, S. Cecoltan, M. Vadana, M. M. Tucureanu, A. C. Mihaila, I. Droc, E. Butoi and I. Manduteanu (2020). "Chronic high glucose concentration induces inflammatory and remodeling changes in valvular endothelial cells and valvular interstitial cells in a gelatin methacrylate 3D model of the human aortic valve." Polymers **12**(12): 2786.
Civitarese, A., C. Jenkinson, D. Richardson, M. Bajaj, K. Cusi, S. Kashyap, R. Berria, R. Belfort, R. DeFronzo and L. Mandarino (2004). "Adiponectin receptors gene expression and insulin sensitivity in non-diabetic Mexican Americans with or without a family history of Type 2 diabetes." Diabetologia **47**(5): 816-820.
Cook-Mills, J. M., M. E. Marchese and H. Abdala-Valencia (2011). "Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants." Antioxidants & redox signaling **15**(6): 1607-1638.
Cooper, M. D. and M. N. Alder (2006). "The evolution of adaptive immune systems." Cell **124**(4): 815-822.
Crabtree, M. J., C. L. Smith, G. Lam, M. S. Goligorsky and S. S. Gross (2008). "Ratio of 5, 6, 7, 8-tetrahydrobiopterin to 7, 8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS." American Journal of Physiology-Heart and Circulatory Physiology **294**(4): H1530-H1540.
Crotty, S. (2015). "A brief history of T cell help to B cells." Nature Reviews Immunology **15**(3): 185-189.
Dandona, P., A. Aljada, P. Mohanty, H. Ghanim, W. Hamouda, E. Assian and S. Ahmad (2001). "Insulin inhibits intranuclear nuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect?" The Journal of Clinical Endocrinology & Metabolism **86**(7): 3257-3265.
De Nigris, V., G. Pujadas, L. La Sala, R. Testa, S. Genovese and A. Ceriello (2015). "Short-term high glucose exposure impairs insulin signaling in endothelial cells." Cardiovascular diabetology **14**(1): 1-7.
DeFuria, J., A. C. Belkina, M. Jagannathan-Bogdan, J. Snyder-Cappione, J. D. Carr, Y. R. Nersesova, D. Markham, K. J. Strissel, A. A. Watkins and M. Zhu (2013). "B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile." Proceedings of the National Academy of Sciences **110**(13): 5133-5138.
Denzel, M. S., M.-C. Scimia, P. M. Zumstein, K. Walsh, P. Ruiz-Lozano and B. Ranscht (2010). "T-cadherin is critical for adiponectin-mediated cardioprotection in mice." The Journal of clinical investigation **120**(12): 4342-4352.
Dimayuga, F. O., C. Wang, J. M. Clark, E. R. Dimayuga, V. M. Dimayuga and A. J. Bruce-Keller (2007). "SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells." Journal of neuroimmunology **182**(1-2): 89-99.

Ding, Y., N. D. Vaziri, R. Coulson, V. S. Kamanna and D. D. Roh (2000). "Effects of simulated hyperglycemia, insulin, and glucagon on endothelial nitric oxide synthase expression." American Journal of Physiology-Endocrinology and Metabolism **279**(1): E11-E17.
Dinger, K., J. Mohr, C. Vohlen, D. Hirani, E. Hucklenbruch-Rother, R. Ensenauer, J. Dötsch and M. A. A. Alcazar (2018). "Intraperitoneal glucose tolerance test, measurement of lung function, and fixation of the lung to study the impact of obesity and impaired metabolism on pulmonary outcomes." JoVE (Journal of Visualized Experiments)(133): e56685.
Donath, M. Y. and S. E. Shoelson (2011). "Type 2 diabetes as an inflammatory disease." Nature Reviews Immunology **11**(2): 98-107.
Drexler, H., S. Kurz, M. Jeserich, T. Münzel and B. Hornig (1995). "Effect of chronic angiotensin-converting enzyme inhibition on endothelial function in patients with chronic heart failure." The American journal of cardiology **76**(15): 13E-18E.
Duffaut, C., J. Galitzky, M. Lafontan and A. Bouloumié (2009). "Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity." Biochemical and biophysical research communications **384**(4): 482-485.
Duffaut, C., A. Zakaroff-Girard, V. Bourlier, P. Decaunes, M. Maumus, P. Chiotasso, C. Sengenès, M. Lafontan, J. Galitzky and A. Bouloumié (2009). "Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators." Arteriosclerosis, thrombosis, and vascular biology **29**(10): 1608-1614.
Dvorak, A. M. and D. Feng (2001). "The vesiculo–vacuolar organelle (VVO): a new endothelial cell permeability organelle." Journal of Histochemistry & Cytochemistry **49**(4): 419-431.
Ehses, J., G. Lacraz, M.-H. Giroix, F. Schmidlin, J. Coulaud, N. Kassis, J.-C. Irminger, M. Kergoat, B. Portha and F. Homo-Delarche (2009). "IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat." Proceedings of the National Academy of Sciences **106**(33): 13998-14003.
Eller, K., A. Kirsch, A. M. Wolf, S. Sopper, A. Tagwerker, U. Stanzl, D. Wolf, W. Patsch, A. R. Rosenkranz and P. Eller (2011). "Potential role of regulatory T cells in reversing obesity-linked insulin resistance and diabetic nephropathy." Diabetes **60**(11): 2954-2962.
Engeli, S., M. Feldpausch, K. Gorzelniak, F. Hartwig, U. Heintze, J. Janke, M. Möhlig, A. F. Pfeiffer, F. C. Luft and A. M. Sharma (2003). "Association between adiponectin and mediators of inflammation in obese women." Diabetes **52**(4): 942-947.
Esser, N., S. Legrand-Poels, J. Piette, A. J. Scheen and N. Paquot (2014). "Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes." Diabetes research and clinical practice **105**(2): 141-150.
Evia-Viscarra, M. L., E. R. Rodea-Montero, E. Apolinar-Jiménez, N. Munoz-Noriega, L. M. García-Morales, C. Leaños-Pérez, M. Figueroa-Barrón, D. Sánchez-Fierros and J. G. Reyes-García (2012). "The effects of metformin on inflammatory mediators in obese adolescents with insulin resistance: controlled randomized clinical trial." Journal of Pediatric Endocrinology and Metabolism **25**(1-2): 41-49.
Fan, Z. and K. Ley (2015). "Leukocyte arrest: Biomechanics and molecular mechanisms of β2 integrin activation." Biorheology **52**(5-6): 353-377.
Farhangkhoee, H., Z. A. Khan, H. Kaur, X. Xin, S. Chen and S. Chakrabarti (2006). "Vascular endothelial dysfunction in diabetic cardiomyopathy: pathogenesis and potential treatment targets." Pharmacology & therapeutics **111**(2): 384-399.
Federici, M., R. Menghini, A. Mauriello, M. L. Hribal, F. Ferrelli, D. Lauro, P. Sbraccia, L. G. Spagnoli, G. Sesti and R. Lauro (2002). "Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells." Circulation **106**(4): 466-472.

Feuerer, M., L. Herrero, D. Cipolletta, A. Naaz, J. Wong, A. Nayer, J. Lee, A. B. Goldfine, C. Benoist and S. Shoelson (2009). "Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters." Nature medicine **15**(8): 930-939.
Fidan, E., H. Onder Ersoz, M. Yilmaz, H. Yilmaz, M. Kocak, C. Karahan and C. Erem (2011). "The effects of rosiglitazone and metformin on inflammation and endothelial dysfunction in patients with type 2 diabetes mellitus." Acta diabetologica **48**(4): 297-302.
Foretz, M., B. Guigas and B. Viollet (2019). "Understanding the glucoregulatory mechanisms of metformin in type 2 diabetes mellitus." Nature Reviews Endocrinology **15**(10): 569-589.
Foretz, M., S. Hébrard, J. Leclerc, E. Zarrinpashneh, M. Soty, G. Mithieux, K. Sakamoto, F. Andreelli and B. Viollet (2010). "Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state." The Journal of clinical investigation **120**(7): 2355-2369.
Fowler, M. J. (2008). "Microvascular and macrovascular complications of diabetes." Clinical diabetes **26**(2): 77-82.
Frasca, D., A. Diaz, M. Romero, T. Vazquez and B. B. Blomberg (2017). "Obesity induces pro-inflammatory B cells and impairs B cell function in old mice." Mechanisms of ageing and development **162**: 91-99.
Fulop, T., A. Larbi, G. Dupuis, A. Le Page, E. H. Frost, A. A. Cohen, J. M. Witkowski and C. Franceschi (2018). "Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes?" Frontiers in immunology **8**: 1960.
Galkina, E. and K. Ley (2007). "Vascular adhesion molecules in atherosclerosis." Arteriosclerosis, thrombosis, and vascular biology **27**(11): 2292-2301.
Gao, D., M. Madi, C. Ding, M. Fok, T. Steele, C. Ford, L. Hunter and C. Bing (2014). "Interleukin-1β mediates macrophage-induced impairment of insulin signaling in human primary adipocytes." American Journal of Physiology-Endocrinology and Metabolism **307**(3): E289-E304.
Garde, A., I. W. Kenny, L. C. Kelley, Q. Chi, A. S. Mutlu, M. C. Wang and D. R. Sherwood (2022). "Localized glucose import, glycolytic processing, and mitochondria generate a focused ATP burst to power basement-membrane invasion." Developmental Cell **57**(6): 732-749. e737.
Giagulli, C., L. Ottoboni, E. Caveggion, B. Rossi, C. Lowell, G. Constantin, C. Laudanna and G. Berton (2006). "The Src family kinases Hck and Fgr are dispensable for inside-out, chemoattractant-induced signaling regulating β2 integrin affinity and valency in neutrophils, but are required for β2 integrin-mediated outside-in signaling involved in sustained adhesion." The Journal of Immunology **177**(1): 604-611.
Giagulli, C., E. Scarpini, L. Ottoboni, S. Narumiya, E. C. Butcher, G. Constantin and C. Laudanna (2004). "RhoA and ζ PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte in vivo homing." Immunity **20**(1): 25-35.
Goldberg, R. B. (2009). "Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications." The Journal of Clinical Endocrinology & Metabolism **94**(9): 3171-3182.
Goodyear, L. J., F. Giorgino, L. A. Sherman, J. Carey, R. J. Smith and G. L. Dohm (1995). "Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects." The Journal of clinical investigation **95**(5): 2195-2204.
Gordon, S. and F. O. Martinez (2010). "Alternative activation of macrophages: mechanism and functions." Immunity **32**(5): 593-604.
Grasso, C., C. Pierie, R. Mebius and L. van Baarsen (2021). "Lymph node stromal cells: subsets and functions in health and disease." Trends in Immunology **42**(10): 920-936.
Gu, H. F., J. Ma, K. T. Gu and K. Brismar (2013). "Association of intercellular adhesion molecule 1 (ICAM1) with diabetes and diabetic nephropathy." Frontiers in endocrinology **3**: 179.

Gunawardana, H., T. Romero, N. Yao, S. Heidt, A. Mulder, D. A. Elashoff and N. M. Valenzuela (2021). "Tissue-specific endothelial cell heterogeneity contributes to unequal inflammatory responses." Scientific reports **11**(1): 1-20.
Gustavsson, C., C.-D. Agardh, A. V. Zetterqvist, J. Nilsson, E. Agardh and M. F. Gomez (2010). "Vascular cellular adhesion molecule-1 (VCAM-1) expression in mice retinal vessels is affected by both hyperglycemia and hyperlipidemia." PloS one **5**(9): e12699.
Gyurko, R., C. C. Siqueira, N. Caldon, L. Gao, A. Kantarci and T. E. Van Dyke (2006). "Chronic hyperglycemia predisposes to exaggerated inflammatory response and leukocyte dysfunction in Akita mice." The Journal of Immunology **177**(10): 7250-7256.
Hada, Y., T. Yamauchi, H. Waki, A. Tsuchida, K. Hara, H. Yago, O. Miyazaki, H. Ebinuma and T. Kadowaki (2007). "Selective purification and characterization of adiponectin multimer species from human plasma." Biochemical and biophysical research communications **356**(2): 487-493.
Hadi, H. A. and J. Al Suwaidi (2007). "Endothelial dysfunction in diabetes mellitus." Vascular health and risk management **3**(6): 853.
Hall, J. E. and M. E. Hall (2020). Guyton and Hall textbook of medical physiology e-Book, Elsevier Health Sciences.
Han, X., Y. Wang, H. Chen, J. Zhang, C. Xu, J. Li and M. Li (2016). "Enhancement of ICAM-1 via the JAK2/STAT3 signaling pathway in a rat model of severe acute pancreatitis-associated lung injury." Experimental and therapeutic medicine **11**(3): 788-796.
Hanley, S. C., E. Austin, B. Assouline-Thomas, J. Kapeluto, J. Blaichman, M. Moosavi, M. Petropavlovskaia and L. Rosenberg (2010). "β-Cell mass dynamics and islet cell plasticity in human type 2 diabetes." Endocrinology **151**(4): 1462-1472.
Harjunpää, H., M. Llort Asens, C. Guenther and S. C. Fagerholm (2019). "Cell adhesion molecules and their roles and regulation in the immune and tumor microenvironment." Frontiers in immunology **10**: 1078.
Hattori, Y., K. Hattori and T. Hayashi (2015). "Pleiotropic benefits of metformin: macrophage targeting its anti-inflammatory mechanisms." Diabetes **64**(6): 1907-1909.
Hawley, S. A., F. A. Ross, C. Chevtzoff, K. A. Green, A. Evans, S. Fogarty, M. C. Towler, L. J. Brown, O. A. Ogunbayo and A. M. Evans (2010). "Use of cells expressing γ subunit variants to identify diverse mechanisms of AMPK activation." Cell metabolism **11**(6): 554-565.
Herder, C. and M. Roden (2011). "Genetics of type 2 diabetes: pathophysiologic and clinical relevance." European journal of clinical investigation **41**(6): 679-692.
Herndler-Brandstetter, D., H. Ishigame, R. Shinnakasu, V. Plajer, C. Stecher, J. Zhao, M. Lietzenmayer, L. Kroehling, A. Takumi and K. Kometani (2018). "KLRG1+ effector CD8+ T cells lose KLRG1, differentiate into all memory T cell lineages, and convey enhanced protective immunity." Immunity **48**(4): 716-729. e718.
Hocaoglu-Emre, F. S., D. Saribal, G. Yenmis and G. Guvenen (2017). "Vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and cluster of differentiation 146 levels in patients with Type 2 diabetes with complications." Endocrinology and Metabolism **32**(1): 99-105.
Hong, J., R. E. Stubbins, R. R. Smith, A. E. Harvey and N. P. Núñez (2009). "Differential susceptibility to obesity between male, female and ovariectomized female mice." Nutrition journal **8**(1): 1-5.
Hotamisligil, G. S., N. S. Shargill and B. M. Spiegelman (1993). "Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance." Science **259**(5091): 87-91.
Hotta, K., T. Funahashi, Y. Arita, M. Takahashi, M. Matsuda, Y. Okamoto, H. Iwahashi, H. Kuriyama, N. Ouchi and K. Maeda (2000). "Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients." Arteriosclerosis, thrombosis, and vascular biology **20**(6): 1595-1599.

Huang, B. W., M. T. Chiang, H. T. Yao and W. Chiang (2004). "The effect of high-fat and high-fructose diets on glucose tolerance and plasma lipid and leptin levels in rats." Diabetes, Obesity and Metabolism **6**(2): 120-126.
Huang, S. and M. P. Czech (2007). "The GLUT4 glucose transporter." Cell metabolism **5**(4): 237-252.
Huang, S., Z. Liu, H. Liu, D. Lee, J. Wang, R. Yuan and B. Li (2019). "Nepeta angustifolia attenuates responses to vascular inflammation in high glucose-induced human umbilical vein endothelial cells through heme oxygenase-1 induction." Journal of ethnopharmacology **231**: 187-196.
Huang, Z.-y., Y. Wu, N. Hedrick and D. H. Gutmann (2003). "T-cadherin-mediated cell growth regulation involves G2 phase arrest and requires p21CIP1/WAF1 expression." Molecular and cellular biology **23**(2): 566-578.
Hubbard, S. R. (2013). "The insulin receptor: both a prototypical and atypical receptor tyrosine kinase." Cold Spring Harbor perspectives in biology **5**(3): a008946.
Hundal, R. S., M. Krssak, S. Dufour, D. Laurent, V. Lebon, V. Chandramouli, S. E. Inzucchi, W. C. Schumann, K. F. Petersen and B. R. Landau (2000). "Mechanism by which metformin reduces glucose production in type 2 diabetes." Diabetes **49**(12): 2063-2069.
Huo, Y. and K. Ley (2001). "Adhesion molecules and atherogenesis." Acta Physiologica Scandinavica **173**(1): 35-43.
Hurrle, S. and W. H. Hsu (2017). "The etiology of oxidative stress in insulin resistance." Biomedical journal **40**(5): 257-262.
Hyduk, S. J., J. R. Chan, S. T. Duffy, M. Chen, M. D. Peterson, T. K. Waddell, G. C. Digby, K. Szaszi, A. Kapus and M. I. Cybulsky (2007). "Phospholipase C, calcium, and calmodulin are critical for α4β1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants." Blood **109**(1): 176-184.
Hyun, E., R. Ramachandran, M. D. Hollenberg and N. Vergnolle (2011). "Mechanisms behind the anti-inflammatory actions of insulin." Critical Reviews™ in Immunology **31**(4).
Iliaki, E., V. Poulaki, N. Mitsiades, C. S. Mitsiades, J. W. Miller and E. S. Gragoudas (2009). "Role of α4 integrin (CD49d) in the pathogenesis of diabetic retinopathy." Investigative ophthalmology & visual science **50**(10): 4898-4904.
Inaishi, J. and Y. Saisho (2020). "Beta-cell mass in obesity and type 2 diabetes, and its relation to pancreas fat: a mini-review." Nutrients **12**(12): 3846.
Isobe, M., J.-i. Suzuki, S. Yamazaki, Y. Yazaki, S. Horie, Y. Okubo, K. Maemura, Y. Yazaki and M. Sekiguchi (1997). "Regulation by differential development of Th1 and Th2 cells in peripheral tolerance to cardiac allograft induced by blocking ICAM-1/LFA-1 adhesion." Circulation **96**(7): 2247-2253.
Isoda, K., J. L. Young, A. Zirlik, L. A. MacFarlane, N. Tsuboi, N. Gerdes, U. Schonbeck and P. Libby (2006). "Metformin inhibits proinflammatory responses and nuclear factor-κB in human vascular wall cells." Arteriosclerosis, thrombosis, and vascular biology **26**(3): 611-617.
Ivanov, D., M. Philippova, J. Antropova, F. Gubaeva, O. Iljinskaya, E. Tararak, V. Bochkov, P. Erne, T. Resink and V. Tkachuk (2001). "Expression of cell adhesion molecule T-cadherin in the human vasculature." Histochemistry and cell biology **115**(3): 231-242.
Iwabu, M., T. Yamauchi, M. Okada-Iwabu, K. Sato, T. Nakagawa, M. Funata, M. Yamaguchi, S. Namiki, R. Nakayama and M. Tabata (2010). "Adiponectin and AdipoR1 regulate PGC-1α and mitochondria by Ca2+ and AMPK/SIRT1." Nature **464**(7293): 1313-1319.
Iwasaki, A. and R. Medzhitov (2010). "Regulation of adaptive immunity by the innate immune system." science **327**(5963): 291-295.
Jagannathan, M., M. McDonnell, Y. Liang, H. Hasturk, J. Hetzel, D. Rubin, A. Kantarci, T. Van Dyke, L. Ganley-Leal and B. Nikolajczyk (2010). "Toll-like receptors regulate B cell cytokine production in patients with diabetes." Diabetologia **53**(7): 1461-1471.

Jager, J., T. Grémeaux, M. Cormont, Y. Le Marchand-Brustel and J.-F. Tanti (2007). "Interleukin-1β-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression." Endocrinology **148**(1): 241-251.
Jing, Y., F. Wu, D. Li, L. Yang, Q. Li and R. Li (2018). "Metformin improves obesity-associated inflammation by altering macrophages polarization." Molecular and cellular endocrinology **461**: 256-264.
Joshi, M. B., M. Philippova, D. Ivanov, R. Allenspach, P. Erne and T. J. Resink (2005). "T-cadherin protects endothelial cells from oxidative stress-induced apoptosis." The FASEB Journal **19**(12): 1737-1739.
Kadowaki, T. and T. Yamauchi (2011). "Adiponectin receptor signaling: a new layer to the current model." Cell metabolism **13**(2): 123-124.
Kadowaki, T., T. Yamauchi, N. Kubota, K. Hara, K. Ueki and K. Tobe (2006). "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome." The Journal of clinical investigation **116**(7): 1784-1792.
Kahn, S. E., R. L. Hull and K. M. Utzschneider (2006). "Mechanisms linking obesity to insulin resistance and type 2 diabetes." Nature **444**(7121): 840.
Kansas, G. S. (1996). "Selectins and their ligands: current concepts and controversies."
Katsiki, N., C. Mantzoros and D. P. Mikhailidis (2017). "Adiponectin, lipids and atherosclerosis." Current opinion in lipidology **28**(4): 347-354.
Katsumata, K., J. Ishihara, K. Fukunaga, A. Ishihara, E. Yuba, E. Budina and J. A. Hubbell (2019). "Conferring extracellular matrix affinity enhances local therapeutic efficacy of anti-TNF-α antibody in a murine model of rheumatoid arthritis." Arthritis research & therapy **21**(1): 1-10.
Kawanishi, N., H. Yano, Y. Yokogawa and K. Suzuki (2010). "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." Exercise immunology review **16**.
Kay, E., L. Gomez-Garcia, A. Woodfin, R. S. Scotland and J. R. Whiteford (2015). "Sexual dimorphisms in leukocyte trafficking in a mouse peritonitis model." Journal of leukocyte biology **98**(5): 805-817.
Khalfaoui, T., G. Lizard and A. Ouertani-Meddeb (2008). "Adhesion molecules (ICAM-1 and VCAM-1) and diabetic retinopathy in type 2 diabetes." Journal of molecular histology **39**(2): 243-249.
Kim, C., S. Lee, Y. Kim, B. Kim, H. Choi, T. Kawada, B. Kwon and R. Yu (2008). "Visceral fat accumulation induced by a high-fat diet causes the atrophy of mesenteric lymph nodes in obese mice." Obesity **16**(6): 1261-1269.
Kim, M., C. V. Carman and T. A. Springer (2003). "Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins." Science **301**(5640): 1720-1725.
Kindt, T. J., R. A. Goldsby, B. A. Osborne and J. Kuby (2007). Kuby immunology, Macmillan.
Kiran, S., V. Kumar, E. A. Murphy, R. T. Enos and U. P. Singh (2021). "High fat diet-induced CD8+ T cells in adipose tissue mediate macrophages to sustain low-grade chronic inflammation." Frontiers in Immunology: 2357.
Kiran, S., V. Kumar, E. A. Murphy, R. T. Enos and U. P. Singh (2021). "High Fat Diet-Induced CD8+ T Cells in Adipose Tissue Mediate Macrophages to Sustain Low-Grade Chronic Inflammation." Frontiers in Immunology **12**.
Koeppen, B. M. and B. A. Stanton (2017). Berne and levy physiology e-book, Elsevier Health Sciences.
Krysiak, R. and B. Okopien (2012). "Lymphocyte-suppressing and systemic anti-inflammatory effects of high-dose metformin in simvastatin-treated patients with impaired fasting glucose." Atherosclerosis **225**(2): 403-407.
Krysiak, R. and B. Okopien (2013). "The effect of metformin on monocyte secretory function in simvastatin-treated patients with impaired fasting glucose." Metabolism **62**(1): 39-43.
Kubo, M. and Y. Motomura (2012). "Transcriptional regulation of the anti-inflammatory cytokine IL-10 in acquired immune cells." Frontiers in immunology **3**: 275.

Kuboki, K., Z. Y. Jiang, N. Takahara, S. W. Ha, M. Igarashi, T. Yamauchi, E. P. Feener, T. P. Herbert, C. J. Rhodes and G. L. King (2000). "Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo: a specific vascular action of insulin." Circulation **101**(6): 676-681.
Kumar, P., K. Natarajan and N. Shanmugam (2014). "High glucose driven expression of pro-inflammatory cytokine and chemokine genes in lymphocytes: molecular mechanisms of IL-17 family gene expression." Cellular signalling **26**(3): 528-539.
Kummer, D. and K. Ebnet (2018). "Junctional adhesion molecules (JAMs): The JAM-integrin connection." Cells **7**(4): 25.
Kuwano, Y., O. Spelten, H. Zhang, K. Ley and A. Zarbock (2010). "Rolling on E-or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils." Blood, The Journal of the American Society of Hematology **116**(4): 617-624.
Lamson, G., L. C. Giudice and R. G. Rosenfeld (1991). "Insulin-like growth factor binding proteins: structural and molecular relationships." Growth factors **5**(1): 19-28.
Lau, E., E. Carroll, L. Callender, G. Hood, V. Berryman, M. Pattrick, S. Finer, G. Hitman, G. Ackland and S. Henson (2019). "Type 2 diabetes is associated with the accumulation of senescent T cells." Clinical & Experimental Immunology **197**(2): 205-213.
Leahy, J. L. (2005). "Pathogenesis of type 2 diabetes mellitus." Archives of medical research **36**(3): 197-209.
Lei, Y., S. K. Devarapu, M. Motrapu, C. Cohen, M. T. Lindenmeyer, S. Moll, S. V. R. Kumar and H.-J. Anders (2019). "Interleukin-1β inhibition for chronic kidney disease in obese mice with type 2 diabetes." Frontiers in immunology **10**: 1223.
Lew, E. A. and E. M. Stoffel (2003). "Natalizumab for active Crohn's disease." The New England journal of medicine **348**(16): 1599; author reply 1599.
Ley, K. (2007). Adhesion molecules: function and inhibition, Springer Science & Business Media.
Ley, K., C. Laudanna, M. I. Cybulsky and S. Nourshargh (2007). "Getting to the site of inflammation: the leukocyte adhesion cascade updated." Nature Reviews Immunology **7**(9): 678-689.
Li, X., F. Zhang, N. Wu, D. Ye, Y. Wang, X. Zhang, Y. Sun and Y.-A. Zhang (2020). "A critical role of foxp3a-positive regulatory T cells in maintaining immune homeostasis in zebrafish testis development." Journal of Genetics and Genomics **47**(9): 547-561.
Liu, H.-F., H.-J. Zhang, Q.-X. Hu, X.-Y. Liu, Z.-Q. Wang, J.-Y. Fan, M. Zhan and F.-L. Chen (2012). "Altered polarization, morphology, and impaired innate immunity germane to resident peritoneal macrophages in mice with long-term type 2 diabetes." Journal of Biomedicine and Biotechnology **2012**.
Liu, L., J. Hu, Y. Wang, H. Lei and D. Xu (2021). "The role and research progress of the balance and interaction between regulatory T cells and other immune cells in obesity with insulin resistance." Adipocyte **10**(1): 66-79.
Liu, M., J. Wright, H. Guo, Y. Xiong and P. Arvan (2014). "Proinsulin entry and transit through the endoplasmic reticulum in pancreatic beta cells." Vitamins & Hormones **95**: 35-62.
Liu, Y., X. Lu, X. Li, P. Du and G. Qin (2020). "High-fat diet triggers obesity-related early infiltration of macrophages into adipose tissue and transient reduction of blood monocyte count." Molecular immunology **117**: 139-146.
Lo, C. G., Y. Xu, R. L. Proia and J. G. Cyster (2005). "Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit." The Journal of experimental medicine **201**(2): 291-301.
Lo, W.-Y., W.-K. Yang, C.-T. Peng, W.-Y. Pai and H.-J. Wang (2018). "MicroRNA-200a/200b modulate high glucose-induced endothelial inflammation by targeting O-linked N-acetylglucosamine transferase expression." Frontiers in physiology **9**: 355.

Lontchi-Yimagou, E., E. Sobngwi, T. E. Matsha and A. P. Kengne (2013). "Diabetes mellitus and inflammation." Current diabetes reports **13**(3): 435-444.
Lu, J.-Y., K.-C. Huang, L.-C. Chang, Y.-S. Huang, Y.-C. Chi, T.-C. Su, C.-L. Chen and W.-S. Yang (2008). "Adiponectin: a biomarker of obesity-induced insulin resistance in adipose tissue and beyond." Journal of biomedical science **15**(5): 565-576.
Luster, A. D., R. Alon and U. H. von Andrian (2005). "Immune cell migration in inflammation: present and future therapeutic targets." Nature immunology **6**(12): 1182-1190.
Luu, N. T., G. E. Rainger and G. B. Nash (2000). "Differential ability of exogenous chemotactic agents to disrupt transendothelial migration of flowing neutrophils." The Journal of Immunology **164**(11): 5961-5969.
Ma, J., Y. Nakagawa, I. Kojima and H. Shibata (2014). "Prolonged insulin stimulation down-regulates GLUT4 through oxidative stress-mediated retromer inhibition by a protein kinase CK2-dependent mechanism in 3T3-L1 adipocytes." Journal of Biological Chemistry **289**(1): 133-142.
Madonna, R., A. Pandolfi, M. Massaro, A. Consoli and R. De Caterina (2004). "Insulin enhances vascular cell adhesion molecule-1 expression in human cultured endothelial cells through a pro-atherogenic pathway mediated by p38 mitogen-activated protein-kinase." Diabetologia **47**(3): 532-536.
Malone, J. I. and B. C. Hansen (2019). "Does obesity cause type 2 diabetes mellitus (T2DM)? Or is it the opposite?" Pediatric diabetes **20**(1): 5-9.
Mariño, E. and S. T. Grey (2012). "B cells as effectors and regulators of autoimmunity." Autoimmunity **45**(5): 377-387.
Martinez-Pomares, L., D. M. Reid, G. D. Brown, P. R. Taylor, R. J. Stillion, S. A. Linehan, S. Zamze, S. Gordon and S. Y. Wong (2003). "Analysis of mannose receptor regulation by IL-4, IL-10, and proteolytic processing using novel monoclonal antibodies." Journal of Leukocyte Biology **73**(5): 604-613.
Mather, K. J., S. Verma and T. J. Anderson (2001). "Improved endothelial function with metformin in type 2 diabetes mellitus." Journal of the American College of Cardiology **37**(5): 1344-1350.
Matsubara, H., Y. Shimizu, M. Arai, A. Yamagata, S. Ito, T. Imakiire, M. Tsunoda, H. Kumagai and N. Oshima (2020). "PEPITEM/Cadherin 15 Axis Inhibits T Lymphocyte Infiltration and Glomerulonephritis in a Mouse Model of Systemic Lupus Erythematosus." The Journal of Immunology **204**(8): 2043-2052.
Matsuda, K., Y. Fujishima, N. Maeda, T. Mori, A. Hirata, R. Sekimoto, Y. Tsushima, S. Masuda, M. Yamaoka and K. Inoue (2015). "Positive feedback regulation between adiponectin and T-cadherin impacts adiponectin levels in tissue and plasma of male mice." Endocrinology **156**(3): 934-946.
Matsumoto, K., Y. Sera, H. Nakamura, Y. Ueki and S. Miyake (2002). "Serum concentrations of soluble adhesion molecules are related to degree of hyperglycemia and insulin resistance in patients with type 2 diabetes mellitus." Diabetes research and clinical practice **55**(2): 131-138.
McEver, R. P. (2015). "Selectins: initiators of leucocyte adhesion and signalling at the vascular wall." Cardiovascular research **107**(3): 331-339.
McGettrick, H. M., K. Hunter, P. A. Moss, C. D. Buckley, G. Rainger and G. B. Nash (2009). "Direct observations of the kinetics of migrating T cells suggest active retention by endothelial cells with continual bidirectional migration." Journal of leukocyte biology **85**(1): 98-107.
McGettrick, H. M., E. Smith, A. Filer, S. Kissane, M. Salmon, C. D. Buckley, G. E. Rainger and G. B. Nash (2009). "Fibroblasts from different sites may promote or inhibit recruitment of flowing lymphocytes by endothelial cells." European journal of immunology **39**(1): 113-125.
McLeod, D. S., D. J. Lefer, C. Merges and G. A. Lutty (1995). "Enhanced expression of intracellular adhesion molecule-1 and P-selectin in the diabetic human retina and choroid." The American journal of pathology **147**(3): 642.
Meigs, J. B., F. B. Hu, N. Rifai and J. E. Manson (2004). "Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus." Jama **291**(16): 1978-1986.

Melmed, S., K. S. Polonsky, P. R. Larsen and H. M. Kronenberg (2015). Williams textbook of endocrinology E-Book, Elsevier Health Sciences.
Mittelbrunn, M., A. Molina, M. M. Escribese, M. Yáñez-Mó, E. Escudero, Á. Ursa, R. Tejedor, F. Mampaso and F. Sánchez-Madrid (2004). "VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses." Proceedings of the National Academy of Sciences **101**(30): 11058-11063.
Miyachi, Y., K. Tsuchiya, C. Komiya, K. Shiba, N. Shimazu, S. Yamaguchi, M. Deushi, M. Osaka, K. Inoue and Y. Sato (2017). "Roles for cell-cell adhesion and contact in obesity-induced hepatic myeloid cell accumulation and glucose intolerance." Cell reports **18**(11): 2766-2779.
Molema, G. (2010). Heterogeneity in endothelial responsiveness to cytokines, molecular causes, and pharmacological consequences. Seminars in thrombosis and hemostasis, © Thieme Medical Publishers.
Monickaraj, F., S. R. Oruganti, P. McGuire and A. Das (2020). "A potential novel therapeutic target in diabetic retinopathy: a chemokine receptor (CCR2/CCR5) inhibitor reduces retinal vascular leakage in an animal model." Graefe's Archive for Clinical and Experimental Ophthalmology: 1-8.
Moraes-Vieira, P. M., A. Saghatelian and B. B. Kahn (2016). "GLUT4 expression in adipocytes regulates de novo lipogenesis and levels of a novel class of lipids with antidiabetic and anti-inflammatory effects." Diabetes **65**(7): 1808-1815.
Moran, B., Y. Abdel-Wahab, P. Flatt and A. McKillop (2014). "Evaluation of the insulin-releasing and glucose-lowering effects of GPR120 activation in pancreatic β-cells." Diabetes, Obesity and Metabolism **16**(11): 1128-1139.
Morigi, M., S. Angioletti, B. Imberti, R. Donadelli, G. Micheletti, M. Figliuzzi, A. Remuzzi, C. Zoja and G. Remuzzi (1998). "Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion." The Journal of clinical investigation **101**(9): 1905-1915.
Morris, D. L., K. W. Cho, J. L. DelProposto, K. E. Oatmen, L. M. Geletka, G. Martinez-Santibanez, K. Singer and C. N. Lumeng (2013). "Adipose tissue macrophages function as antigen-presenting cells and regulate adipose tissue CD4+ T cells in mice." Diabetes **62**(8): 2762-2772.
Mosser, D. M. (2003). "The many faces of macrophage activation." Journal of leukocyte biology **73**(2): 209-212.
Muller, W. A. (2011). "Mechanisms of leukocyte transendothelial migration." Annual Review of Pathology: Mechanisms of Disease **6**: 323-344.
Munir, H. and H. M. McGettrick (2015). "Mesenchymal stem cell therapy for autoimmune disease: risks and rewards." Stem cells and development **24**(18): 2091-2100.
Mutgan, A. C., K. Jandl and G. Kwapiszewska (2020). "Endothelial Basement Membrane Components and Their Products, Matrikines: Active Drivers of Pulmonary Hypertension?" Cells **9**(9): 2029.
Nguyen, D. V., L. Shaw and M. Grant (2012). "Inflammation in the pathogenesis of microvascular complications in diabetes." Frontiers in endocrinology **3**: 170.
Nishikawa, T., D. Kukidome, K. Sonoda, K. Fujisawa, T. Matsuhisa, H. Motoshima, T. Matsumura and E. Araki (2007). "Impact of mitochondrial ROS production in the pathogenesis of insulin resistance." Diabetes research and clinical practice **77**(3): S161-S164.
Nishimura, S., I. Manabe, M. Nagasaki, K. Eto, H. Yamashita, M. Ohsugi, M. Otsu, K. Hara, K. Ueki and S. Sugiura (2009). "CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity." Nature medicine **15**(8): 914-920.
Noshita, N., T. Sugawara, T. Hayashi, A. Lewén, G. Omar and P. H. Chan (2002). "Copper/zinc superoxide dismutase attenuates neuronal cell death by preventing extracellular signal-regulated kinase activation after transient focal cerebral ischemia in mice." Journal of Neuroscience **22**(18): 7923-7930.
Nunemaker, C. S., H. G. Chung, G. M. Verrilli, K. L. Corbin, A. Upadhye and P. R. Sharma (2014). "Increased serum CXCL1 and CXCL5 are linked to obesity, hyperglycemia, and impaired islet function." Journal of Endocrinology **222**(2): 267-276.

Obata, Y., S. Kita, Y. Koyama, S. Fukuda, H. Takeda, M. Takahashi, Y. Fujishima, H. Nagao, S. Masuda and Y. Tanaka (2018). "Adiponectin/T-cadherin system enhances exosome biogenesis and decreases cellular ceramides by exosomal release." JCI insight **3**(8).
Oguntibeju, O. O. (2019). "Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links." International journal of physiology, pathophysiology and pharmacology **11**(3): 45.
Okouchi, M., N. Okayama, S. Imai, H. Omi, M. Shimizu, T. Fukutomi and M. Itoh (2002). "High insulin enhances neutrophil transendothelial migration through increasing surface expression of platelet endothelial cell adhesion molecule-1 via activation of mitogen activated protein kinase." Diabetologia **45**(10): 1449-1456.
Okouchi, M., N. Okayama, M. Shimizu, H. Omi, T. Fukutomi and M. Itoh (2002). "High insulin exacerbates neutrophil-endothelial cell adhesion through endothelial surface expression of intercellular adhesion molecule-1 via activation of protein kinase C and mitogen-activated protein kinase." Diabetologia **45**(4): 556-559.
Oliver, E., F. McGillicuddy, C. Phillips, S. Toomey and H. M. Roche (2010). "Postgraduate Symposium. The role of inflammation and macrophage accumulation in the development of obesity-induced type 2 diabetes mellitus and the possible therapeutic effects of long-chain n-3 PUFA." Proceedings of the Nutrition Society **69**(2): 232-243.
Olszewski, W. L. (2003). "The lymphatic system in body homeostasis: physiological conditions." Lymphatic research and biology **1**(1): 11-24.
Østergaard, M., N. K. Mishra and K. J. Jensen (2020). "The ABC of insulin: the organic chemistry of a small protein." Chemistry–A European Journal **26**(38): 8341-8357.
Ouchi, N., S. Kihara, Y. Arita, K. Maeda, H. Kuriyama, Y. Okamoto, K. Hotta, M. Nishida, M. Takahashi and T. Nakamura (1999). "Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin." Circulation **100**(25): 2473-2476.
Ouedraogo, R., Y. Gong, B. Berzins, X. Wu, K. Mahadev, K. Hough, L. Chan, B. J. Goldstein and R. Scalia (2007). "Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo." The Journal of clinical investigation **117**(6): 1718-1726.
Pang, T. T. and P. Narendran (2008). "The distribution of adiponectin receptors on human peripheral blood mononuclear cells." Annals of the New York Academy of Sciences **1150**(1): 143-145.
Paquot, N., M. J. Castillo, P. J. Lefèbvre and A. J. Scheen (2000). "No increased insulin sensitivity after a single intravenous administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin-resistant patients." The Journal of Clinical Endocrinology & Metabolism **85**(3): 1316-1319.
Parker-Duffen, J. L., K. Nakamura, M. Silver, R. Kikuchi, U. Tigges, S. Yoshida, M. S. Denzel, B. Ranscht and K. Walsh (2013). "T-cadherin is essential for adiponectin-mediated revascularization." Journal of Biological Chemistry **288**(34): 24886-24897.
Peraldi, P. and B. Spiegelman (1998). "TNF-α and insulin resistance: summary and future prospects." Molecular and cellular biochemistry **182**(1): 169-175.
Pezhman, L., A. Tahrani and M. Chimen (2021). "Dysregulation of leukocyte trafficking in type 2 diabetes: mechanisms and potential therapeutic avenues." Frontiers in cell and developmental biology **9**: 624184.
Philippova, M., Y. Suter, S. Toggweiler, A. W. Schoenenberger, M. B. Joshi, E. Kyriakakis, P. Erne and T. J. Resink (2011). "T-cadherin is present on endothelial microparticles and is elevated in plasma in early atherosclerosis." European heart journal **32**(6): 760-771.
Polonsky, K., B. Given and E. Van Cauter (1988). "Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects." The Journal of clinical investigation **81**(2): 442-448.

Powers, D. C., J. E. Morley and J. F. Flood (1992). "Age-related changes in LFA-1 expression, cell adhesion, and PHA-induced proliferation by lymphocytes from senescence-accelerated mouse (SAM)-P/8 and SAM-R/1 substrains." Cellular immunology **141**(2): 444-456.
Pratipanawatr, W., T. Pratipanawatr, K. Cusi, R. Berria, J. M. Adams, C. P. Jenkinson, K. Maezono, R. A. DeFronzo and L. J. Mandarino (2001). "Skeletal muscle insulin resistance in normoglycemic subjects with a strong family history of type 2 diabetes is associated with decreased insulin-stimulated insulin receptor substrate-1 tyrosine phosphorylation." Diabetes **50**(11): 2572-2578.
Qiu, D., L. Zhang, J. Zhan, Q. Yang, H. Xiong, W. Hu, Q. Ji and J. Huang (2020). "Hyperglycemia Decreases Epithelial Cell Proliferation and Attenuates Neutrophil Activity by Reducing ICAM-1 and LFA-1 Expression Levels." Frontiers in Genetics: 1604.
Ranscht, B. and M. T. Dours-Zimmermann (1991). "T-cadherin, a novel cadherin cell adhesion molecule in the nervous system lacks the conserved cytoplasmic region." Neuron **7**(3): 391-402.
Rehfeld, A., M. Nylander and K. Karnov (2017). The Immune System and the Lymphatic Organs. Compendium of Histology, Springer**:** 379-409.
Reinehr, T., B. Karges, T. Meissner, S. Wiegand, B. Stoffel-Wagner, R. W. Holl and J. Woelfle (2016). "Inflammatory markers in obese adolescents with type 2 diabetes and their relationship to hepatokines and adipokines." The Journal of pediatrics **173**: 131-135.
Rieu, I. and S. J. Powers (2009). "Real-time quantitative RT-PCR: design, calculations, and statistics." The Plant Cell **21**(4): 1031-1033.
Ruan, H. and L. Q. Dong (2016). "Adiponectin signaling and function in insulin target tissues." Journal of molecular cell biology **8**(2): 101-109.
Rutledge, N. S. and W. A. Muller (2020). "Understanding Molecules that Mediate Leukocyte Extravasation." Current Pathobiology Reports **8**(2): 25-35.
Rutter, G. A., T. J. Pullen, D. J. Hodson and A. Martinez-Sanchez (2015). "Pancreatic β-cell identity, glucose sensing and the control of insulin secretion." Biochemical Journal **466**(2): 203-218.
Saisho, Y. (2015). "Metformin and inflammation: its potential beyond glucose-lowering effect." Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders) **15**(3): 196-205.
Saisho, Y., A. E. Butler, E. Manesso, D. Elashoff, R. A. Rizza and P. C. Butler (2013). "β-cell mass and turnover in humans: effects of obesity and aging." Diabetes care **36**(1): 111-117.
Salani, B., C. Marini, A. D. Rio, S. Ravera, M. Massollo, A. M. Orengo, A. Amaro, M. Passalacqua, S. Maffioli and U. Pfeffer (2013). "Metformin impairs glucose consumption and survival in Calu-1 cells by direct inhibition of hexokinase-II." Scientific reports **3**(1): 1-8.
Saravia, J., N. M. Chapman and H. Chi (2019). "Helper T cell differentiation." Cellular & molecular immunology **16**(7): 634-643.
Sayyed, S. G., M. Ryu, O. P. Kulkarni, H. Schmid, J. Lichtnekert, S. Grüner, L. Green, P. Mattei, G. Hartmann and H.-J. Anders (2011). "An orally active chemokine receptor CCR2 antagonist prevents glomerulosclerosis and renal failure in type 2 diabetes." Kidney international **80**(1): 68-78.
Scheepers, A., H.-G. Joost and A. Schurmann (2004). "The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function." Journal of parenteral and enteral nutrition **28**(5): 364-371.
Schenkel, A. R., Z. Mamdouh and W. A. Muller (2004). "Locomotion of monocytes on endothelium is a critical step during extravasation." Nature immunology **5**(4): 393-400.
Schieber, M. and N. S. Chandel (2014). "ROS function in redox signaling and oxidative stress." Current biology **24**(10): R453-R462.
Shamri, R., V. Grabovsky, J.-M. Gauguet, S. Feigelson, E. Manevich, W. Kolanus, M. K. Robinson, D. E. Staunton, U. H. von Andrian and R. Alon (2005). "Lymphocyte arrest requires instantaneous induction of

an extended LFA-1 conformation mediated by endothelium-bound chemokines." Nature immunology **6**(5): 497-506.
Shanaki, M., M. Khosravi, A. Khoshdooni-Farahani, A. Dadashi, M. F. Heydari, M. Delfan, H. Jafary and S. Gorgani-Firuzjaee (2020). "High-Intensity Interval Training Reversed High-Fat Diet–Induced M1-Macrophage Polarization in Rat Adipose Tissue via Inhibition of NOTCH Signaling." Journal of Inflammation Research **13**: 165.
Shaw, R. J., K. A. Lamia, D. Vasquez, S.-H. Koo, N. Bardeesy, R. A. DePinho, M. Montminy and L. C. Cantley (2005). "The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin." Science **310**(5754): 1642-1646.
Shen, Y. Y., J. A. Charlesworth, J. J. Kelly, K. W. Loi and P. W. Peake (2007). "Up-regulation of adiponectin, its isoforms and receptors in end-stage kidney disease." Nephrology Dialysis Transplantation **22**(1): 171-178.
Shendurse, A. and C. Khedkar (2016). "Glucose: properties and analysis." Encyclopedia of Food and Health **3**: 239-247.
Shirakawa, K., X. Yan, K. Shinmura, J. Endo, M. Kataoka, Y. Katsumata, T. Yamamoto, A. Anzai, S. Isobe and N. Yoshida (2016). "Obesity accelerates T cell senescence in murine visceral adipose tissue." The Journal of clinical investigation **126**(12): 4626-4639.
Sloan-Lancaster, J., E. Abu-Raddad, J. Polzer, J. W. Miller, J. C. Scherer, A. De Gaetano, J. K. Berg and W. H. Landschulz (2013). "Double-blind, randomized study evaluating the glycemic and anti-inflammatory effects of subcutaneous LY2189102, a neutralizing IL-1β antibody, in patients with type 2 diabetes." Diabetes care **36**(8): 2239-2246.
Smith, U., M. Axelsen, E. Carvalho, B. Eliasson, P. A. JANSSON and C. Wesslau (1999). "Insulin signaling and action in fat cells: associations with insulin resistance and type 2 diabetes." Annals of the New York Academy of Sciences **892**(1): 119-126.
Sorice, A., E. Guerriero, F. Capone, G. Colonna, G. Castello and S. Costantini (2014). "Ascorbic acid: its role in immune system and chronic inflammation diseases." Mini reviews in medicinal chemistry **14**(5): 444-452.
Stienstra, R., L. A. Joosten, T. Koenen, B. Van Tits, J. A. Van Diepen, S. A. Van Den Berg, P. C. Rensen, P. J. Voshol, G. Fantuzzi and A. Hijmans (2010). "The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity." Cell metabolism **12**(6): 593-605.
Stögerer, T. and S. Stäger (2020). "Innate immune sensing by cells of the adaptive immune system." Frontiers in immunology **11**: 1081.
Stulc, T., R. Ceska, I. Marinov and J. Skrha (2012). "The effect of simvastatin and fenofibrate on the expression of leukocyte adhesion molecules and lipopolysaccharide receptor CD14 in type 2 diabetes mellitus." Neuro Endocrinol Lett **33**(Suppl 2): 73-77.
Stumvoll, M., N. Nurjhan, G. Perriello, G. Dailey and J. E. Gerich (1995). "Metabolic effects of metformin in non-insulin-dependent diabetes mellitus." New England Journal of Medicine **333**(9): 550-554.
Sumagin, R., E. Lomakina and I. H. Sarelius (2008). "Leukocyte-endothelial cell interactions are linked to vascular permeability via ICAM-1-mediated signaling." American Journal of Physiology-Heart and Circulatory Physiology **295**(3): H969-H977.
Sundararajan, S., I. Jayachandran, M. Balasubramanyam, V. Mohan, B. Venkatesan and N. Manickam (2019). "Sestrin2 regulates monocyte activation through AMPK-mTOR nexus under high-glucose and dyslipidemic conditions." Journal of cellular biochemistry **120**(5): 8201-8213.
Taguchi, K., K. Sakata, W. Ohashi, T. Imaizumi, J. Imura and Y. Hattori (2014). "Tonic Inhibition by G protein–coupled receptor kinase 2 of Akt/endothelial nitric-oxide synthase signaling in human vascular endothelial cells under conditions of hyperglycemia with high insulin levels." Journal of Pharmacology and Experimental Therapeutics **349**(2): 199-208.

Tajima, K., J. Shirakawa, T. Okuyama, M. Kyohara, S. Yamazaki, Y. Togashi and Y. Terauchi (2017). "Effects of metformin on compensatory pancreatic β-cell hyperplasia in mice fed a high-fat diet." American Journal of Physiology-Endocrinology and Metabolism **313**(3): E367-E380.
Takeuchi, T., Y. Adachi, Y. Ohtsuki and M. Furihata (2007). "Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease." Medical Molecular Morphology **40**(3): 115-120.
Takeuchi, T., A. Misaki, S. B. Liang, A. Tachibana, N. Hayashi, H. Sonobe and Y. Ohtsuki (2000). "Expression of T-cadherin (CDH13, H-Cadherin) in human brain and its characteristics as a negative growth regulator of epidermal growth factor in neuroblastoma cells." Journal of neurochemistry **74**(4): 1489-1497.
Tang, L.-L., Q. Liu, S.-Z. Bu, L.-T. Xu, Q.-W. Wang, Y.-F. Mai and S.-W. Duan (2013). "The effect of environmental factors and DNA methylation on type 2 diabetes mellitus." Yi Chuan= Hereditas **35**(10): 1143-1152.
Todoric, J., M. Löffler, J. Huber, M. Bilban, M. Reimers, A. Kadl, M. Zeyda, W. Waldhäusl and T. Stulnig (2006). "Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n−3 polyunsaturated fatty acids." Diabetologia **49**(9): 2109-2119.
Tsai, H.-C. and M. H. Han (2016). "Sphingosine-1-phosphate (S1P) and S1P signaling pathway: therapeutic targets in autoimmunity and inflammation." Drugs **76**(11): 1067-1079.
Tschen, S.-I., S. Dhawan, T. Gurlo and A. Bhushan (2009). "Age-dependent decline in β-cell proliferation restricts the capacity of β-cell regeneration in mice." Diabetes **58**(6): 1312-1320.
Tsuchida, A., T. Yamauchi, Y. Ito, Y. Hada, T. Maki, S. Takekawa, J. Kamon, M. Kobayashi, R. Suzuki and K. Hara (2004). "Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity." Journal of Biological Chemistry **279**(29): 30817-30822.
Uotila, L. M., F. Jahan, L. S. Hinojosa, E. Melandri, M. Grönholm and C. G. Gahmberg (2014). "Specific phosphorylations transmit signals from leukocyte β2 to β1 integrins and regulate adhesion." Journal of Biological Chemistry **289**(46): 32230-32242.
Vara-Ciruelos, D., M. Dandapani, F. M. Russell, K. M. Grzes, A. Atrih, M. Foretz, B. Viollet, D. J. Lamont, D. A. Cantrell and D. G. Hardie (2019). "Phenformin, but not metformin, delays development of T cell acute lymphoblastic leukemia/lymphoma via cell-autonomous AMPK activation." Cell reports **27**(3): 690-698. e694.
Vessby, B., M. Uusitupa, K. Hermansen, G. Riccardi, A. A. Rivellese, L. C. Tapsell, C. Nälsén, L. Berglund, A. Louheranta and B. Rasmussen (2001). "Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study." Diabetologia **44**(3): 312-319.
Voehringer, D., C. Blaser, P. Brawand, D. H. Raulet, T. Hanke and H. Pircher (2001). "Viral infections induce abundant numbers of senescent CD8 T cells." The Journal of Immunology **167**(9): 4838-4843.
von Andrian, U. H. and B. Engelhardt (2003). α4 integrins as therapeutic targets in autoimmune disease, Mass Medical Soc.
Wang, L., X. Zhou, Y. Yin, Y. Mai, D. Wang and X. Zhang (2019). "Hyperglycemia induces neutrophil extracellular traps formation through an NADPH oxidase-dependent pathway in diabetic retinopathy." Frontiers in immunology **9**: 3076.
Wang, Q., J. Cai, J. Wang, C. Xiong, L. Yan, Z. Zhang, Y. Fang and J. Zhao (2014). "Down-regulation of adiponectin receptors in osteoarthritic chondrocytes." Cell biochemistry and biophysics **70**(1): 491-497.
Wen, Y., J. Skidmore, M. Porter-Turner, C. Rea, M. Khokher and B. Singh (2002). "Relationship of glycation, antioxidant status and oxidativestress to vascular endothelial damage in diabetes." Diabetes, obesity and metabolism **4**(5): 305-308.
Winer, D. A., S. Winer, L. Shen, P. P. Wadia, J. Yantha, G. Paltser, H. Tsui, P. Wu, M. G. Davidson and M. N. Alonso (2011). "B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies." Nature medicine **17**(5): 610-617.

Winer, S., Y. Chan, G. Paltser, D. Truong, H. Tsui, J. Bahrami, R. Dorfman, Y. Wang, J. Zielenski and F. Mastronardi (2009). "Normalization of obesity-associated insulin resistance through immunotherapy." Nature medicine **15**(8): 921-929.
Wondmkun, Y. T. (2020). "Obesity, insulin resistance, and type 2 diabetes: associations and therapeutic implications." Diabetes, metabolic syndrome and obesity: targets and therapy **13**: 3611.
Wright, R. D. and D. Cooper (2014). "Glycobiology of leukocyte trafficking in inflammation." Glycobiology **24**(12): 1242-1251.
Wu, H., S. Ghosh, X. D. Perrard, L. Feng, G. E. Garcia, J. L. Perrard, J. F. Sweeney, L. E. Peterson, L. Chan and C. W. Smith (2007). "T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity." Circulation **115**(8): 1029-1038.
Xiu, F., M. Stanojcic, L. Diao and M. G. Jeschke (2014). "Stress hyperglycemia, insulin treatment, and innate immune cells." International journal of endocrinology **2014**.
Xu, H. E., M. H. Lambert, V. G. Montana, K. D. Plunket, L. B. Moore, J. L. Collins, J. A. Oplinger, S. A. Kliewer, R. T. Gampe and D. D. McKee (2001). "Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors." Proceedings of the National Academy of Sciences **98**(24): 13919-13924.
Xue, C.-B., A. Wang, Q. Han, Y. Zhang, G. Cao, H. Feng, T. Huang, C. Zheng, M. Xia and K. Zhang (2011). "Discovery of INCB8761/PF-4136309, a potent, selective, and orally bioavailable CCR2 antagonist." ACS medicinal chemistry letters **2**(12): 913-918.
Yago, T., B. Shao, J. J. Miner, L. Yao, A. G. Klopocki, K. Maeda, K. M. Coggeshall and R. P. McEver (2010). "E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin αLβ2-mediated slow leukocyte rolling." Blood **116**(3): 485-494.
Yamagata, K., C. Tagawa, H. Matsufuji and M. Chino (2012). "Dietary apigenin regulates high glucose and hypoxic reoxygenation-induced reductions in apelin expression in human endothelial cells." The Journal of nutritional biochemistry **23**(8): 929-936.
Yanaba, K., J. D. Bouaziz, T. Matsushita, C. M. Magro, E. W. St. Clair and T. F. Tedder (2008). "B-lymphocyte contributions to human autoimmune disease." Immunological reviews **223**(1): 284-299.
Yao, L., O. Herlea-Pana, J. Heuser-Baker, Y. Chen and J. Barlic-Dicen (2014). "Roles of the chemokine system in development of obesity, insulin resistance, and cardiovascular disease." Journal of immunology research **2014**: 81450.
Yaribeygi, H., F. R. Farrokhi, A. E. Butler and A. Sahebkar (2019). "Insulin resistance: Review of the underlying molecular mechanisms." Journal of cellular physiology **234**(6): 8152-8161.
Yates, C. M., S. P. Tull, J. Madden, P. C. Calder, R. F. Grimble, G. B. Nash and G. E. Rainger (2011). "Docosahexaenoic acid inhibits the adhesion of flowing neutrophils to cytokine stimulated human umbilical vein endothelial cells." The Journal of nutrition **141**(7): 1331-1334.
Yoon, H. J., S. W. Cho, B. W. Ahn and S. Y. Yang (2010). "Alterations in the activity and expression of endothelial NO synthase in aged human endothelial cells." Mechanisms of Ageing and Development **131**(2): 119-123.
Yuseff, M.-I., P. Pierobon, A. Reversat and A.-M. Lennon-Duménil (2013). "How B cells capture, process and present antigens: a crucial role for cell polarity." Nature Reviews Immunology **13**(7): 475-486.
Zaccardi, F., D. R. Webb, T. Yates and M. J. Davies (2016). "Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective." Postgraduate medical journal **92**(1084): 63-69.
Zand, H., N. Morshedzadeh and F. Naghashian (2017). "Signaling pathways linking inflammation to insulin resistance." Diabetes & Metabolic Syndrome: Clinical Research & Reviews **11**: S307-S309.
Zha, J., X.-w. Chi, X.-l. Yu, X.-m. Liu, D.-q. Liu, J. Zhu, H. Ji and R.-t. Liu (2016). "Interleukin-1β-targeted vaccine improves glucose control and β-cell function in a diabetic KK-Ay mouse model." PloS one **11**(5): e0154298.

Zhang, J., P. Alcaide, L. Liu, J. Sun, A. He, F. W. Luscinskas and G.-P. Shi (2011). "Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils." PloS one **6**(1): e14525.
Zhang, L., L. Zhang, H. Gu, Y. Chen and G. Z. Chen (2021). "Honeycombed activated carbon with greatly increased specific surface by direct activation of glucose for supercapacitors." Journal of Alloys and Compounds **883**: 160907.
Zhang, P., Y. Wang, Y. Fan, Z. Tang and N. Wang (2009). "Overexpression of adiponectin receptors potentiates the antiinflammatory action of subeffective dose of globular adiponectin in vascular endothelial cells." Arteriosclerosis, thrombosis, and vascular biology **29**(1): 67-74.
Zhao, H.-y., M. Zhao, T.-n. Yi and J. Zhang (2011). "Globular adiponectin protects human umbilical vein endothelial cells against apoptosis through adiponectin receptor 1/adenosine monophosphate-activated protein kinase pathway." Chinese Medical Journal **124**(16): 2540-2547.
Zhou, G., R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber and N. Fujii (2001). "Role of AMP-activated protein kinase in mechanism of metformin action." The Journal of clinical investigation **108**(8): 1167-1174.
Zhou, T., Z. Hu, S. Yang, L. Sun, Z. Yu and G. Wang (2018). "Role of adaptive and innate immunity in type 2 diabetes mellitus." Journal of diabetes research **2018**: 1-9.
Zierath, J., A. Krook and H. Wallberg-Henriksson (2000). "Insulin action and insulin resistance in human skeletal muscle." Diabetologia **43**(7): 821-835.
Zou, Y., S. Yoon, K. J. Jung, C. H. Kim, T. G. Son, M.-S. Kim, Y. J. Kim, J. Lee, B. P. Yu and H. Y. Chung (2006). "Upregulation of aortic adhesion molecules during aging." The Journals of Gerontology Series A: Biological Sciences and Medical Sciences **61**(3): 232-244.

8 Appendix

8.1 Published review

frontiers in Cell and Developmental Biology

REVIEW
published: 22 February 2021
doi: 10.3389/fcell.2021.624184

Dysregulation of Leukocyte Trafficking in Type 2 Diabetes: Mechanisms and Potential Therapeutic Avenues

*Laleh Pezhman[1], Abd Tahrani[2,3,4] and Myriam Chimen[1]**

[1] Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [2] Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, United Kingdom, [3] Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, United Kingdom, [4] University Hospitals Birmingham NHS Foundation Trust, Birmingham, United Kingdom

OPEN ACCESS

Edited by:
Hao Sun,
University of California, San Diego, United States

Reviewed by:
ChangDong Lin,
Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China
Sirpa Jalkanen,
University of Turku, Finland
Eroboghene Ubogu,
University of Alabama at Birmingham, United States

***Correspondence:**
Myriam Chimen
m.chimen@bham.ac.uk;
chimenm@bham.ac.uk

Specialty section:
This article was submitted to Cell Adhesion and Migration, a section of the journal Frontiers in Cell and Developmental Biology

Received: 30 October 2020
Accepted: 04 February 2021
Published: 22 February 2021

Citation:
Pezhman L, Tahrani A and Chimen M (2021) Dysregulation of Leukocyte Trafficking in Type 2 Diabetes: Mechanisms and Potential Therapeutic Avenues. Front. Cell Dev. Biol. 9:624184. doi: 10.3389/fcell.2021.624184

Type 2 Diabetes Mellitus (T2DM) is a chronic inflammatory disorder that is characterized by chronic hyperglycemia and impaired insulin signaling which in addition to be caused by common metabolic dysregulations, have also been associated to changes in various immune cell number, function and activation phenotype. Obesity plays a central role in the development of T2DM. The inflammation originating from obese adipose tissue develops systemically and contributes to insulin resistance, beta cell dysfunction and hyperglycemia. Hyperglycemia can also contribute to chronic, low-grade inflammation resulting in compromised immune function. In this review, we explore how the trafficking of innate and adaptive immune cells under inflammatory condition is dysregulated in T2DM. We particularly highlight the obesity-related accumulation of leukocytes in the adipose tissue leading to insulin resistance and beta-cell dysfunction and resulting in hyperglycemia and consequent changes of adhesion and migratory behavior of leukocytes in different vascular beds. Thus, here we discuss how potential therapeutic targeting of leukocyte trafficking could be an efficient way to control inflammation as well as diabetes and its vascular complications.

Keywords: therapies, inflammation, type 2 diabetes, obesity, trafficking, leukocyte

INTRODUCTION

Inflammation is a protective response against harmful stimuli such as injuries, infections and toxins, which requires the trafficking of leukocytes from the blood stream to the site of inflammation within damaged tissues. This process is critical for elimination of harmful stimuli and for tissue repair and is tightly regulated by a variety of mediators such as cell adhesion molecules, cytokines and chemokines (Wright and Cooper, 2014). Leukocyte behavior and their recruitment to tissues is modified under inflammatory conditions such as in Type 2 diabetes (T2DM) (Wu et al., 2011). T2DM is a chronic inflammatory disorder characterized by hyperglycemia and impaired insulin signaling and production (Calle and Fernandez, 2012). Components of the immune system are altered in T2DM, with the most apparent changes occurring in the adipose tissue, the pancreatic islets, the vasculature and in circulating leukocytes (Donath and Shoelson, 2011; Mraz and Haluzik, 2014; Eguchi and Nagai, 2017). Indeed, cellular stresses, such as oxidative stress and

lipotoxicity cause insulin resistance and pancreatic islets dysfunction, can induce an inflammatory response and also be exacerbated by local inflammation. Patients with T2D have elevated levels of inflammatory cytokines (such as IL-1β and IL-6) and chemokines (such as CCL2, CXCL8) (Donath and Shoelson, 2011). This rise in inflammatory mediators completely modifies leukocyte behavior and recruitment into tissues, which in turn contributes to maintenance of insulin resistance, loss of insulin secretion and accelerates development of micro and macrovascular complications (Wu et al., 2011). Clinical studies show that targeting inflammation using small molecules or biological agents to turn off pro-inflammatory cytokines, deplete immune cells or block regulatory surface receptors, results in improved blood glucose levels and insulin sensitivity (Larsen et al., 2007; Donath and Shoelson, 2011). Obesity, particularly visceral adiposity, is a major risk factor for T2DM (Donath and Shoelson, 2011) and the adipose tissue of patients with T2DM and/or obese patients appears to be a major site of inflammation (Lontchi-Yimagou et al., 2013). Both innate and adaptive immune cells present in the adipose tissue have critical roles in the regulation of metabolic homeostasis. Dysregulated trafficking of leukocytes is observed in the adipose tissue and is associated with a shift in cell populations from an anti-inflammatory to a pro-inflammatory profile in obesity (Esser et al., 2014). This switch to a production of pro-inflammatory cytokines leads to the development of systemic low-grade inflammation, which results in impaired insulin signaling, beta-cell dysfunction and subsequent insulin resistance (Esser et al., 2014). Hyperglycemia rises as the pancreas fails to compensate insulin resistance (Donath and Shoelson, 2011). Hyperglycemia then leads to oxidative stress and induces systemic inflammation by stimulating the production of inflammatory cytokines (Noshita et al., 2002; Dimayuga et al., 2007) and chemokines, resulting in endothelial dysfunction (Nguyen et al., 2012). This review will summarize how T2DM-related changes in the expression of adhesion molecules and chemokine receptors/ligands drive endothelial dysfunction and dysregulates leukocyte trafficking under inflammatory conditions which ultimately leads to diabetic complications (**Figure 1**).

THE MOLECULAR PROCESSES OF LEUKOCYTE RECRUITMENT IN INFLAMMATION

Recruitment of leukocytes is triggered by the local release of inflammatory mediators such as histamine, tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) in inflamed tissue (Liu et al., 2004; Zhang et al., 2011; Muller, 2013). These mediators induce the expression of specific adhesion receptors such as selectins, adhesion molecules and chemokines, which are expressed or presented at the surface of endothelial cells (ECs) and bind to integrins or chemokine receptors located at the surface of leukocytes (Zhang et al., 2011). Selectins interaction with their ligands results in leukocyte tethering and rolling from rapidly flowing blood (Kansas, 1996; Harjunpää et al., 2019). Selectins are transmembrane calcium-dependent lectins that consist of three functionally different adhesion molecules expressed by ECs (E-selectin), platelets and ECs (P-selectin) and leukocytes (L-selectin) (McEver, 2015). L-selectin and P-selectin mediate fast rolling of leukocyte through sequential interactions with their ligands. However, slow rolling events are mediated by E-selectin on ECs binding to E-selectin ligand-1 (ESL-1) or P-selectin glycoprotein ligand 1 (PSGL-1) on leukocytes (Yago et al., 2010). Leukocyte arrest and migration on the endothelium depends on chemokines (Luu et al., 2000). The shift from fast rolling to slow rolling allows G-protein-coupled receptors (GPCRs) on leukocytes to bind to the chemokines expressed on the surface of ECs, which induces leukocyte integrin activation and enables leukocyte firm adhesion to the vessel wall (Giagulli et al., 2004; Rutledge and Muller, 2020). Integrins are a family of transmembrane receptors expressed by leukocytes. Both $\alpha_4\beta_1$ integrin or very late antigen-4 (VLA-4) and $\alpha_M\beta_2$ integrin or lymphocyte function-associated antigen-1 (LFA-1) have been found to play a role in leukocyte arrest (Uotila et al., 2014). $\alpha_M\beta_2$ is expressed on leukocytes as a resting low affinity state. Conformational changes are required for integrins to switch from a low affinity state to an active high affinity state enabling them to bind to their ligands expressed on the endothelium (Kim et al., 2003; Ley et al., 2007; Fan and Ley, 2015). The activation of integrins is mediated by chemokines and results in integrin extension and changes in the cytoskeleton of leukocytes, enabling tight adhesion on the vessel wall (Rutledge and Muller, 2020). LFA-1 and VLA-4 on leukocytes, bind to their respective ligand ICAM-1 and VCAM-1 on ECs, mediate leukocyte adhesion (Hyduk et al., 2007; Kuwano et al., 2010) and induce intracellular signals to allow leukocyte arrest (Giagulli et al., 2006; Kummer and Ebnet, 2018). Leukocytes then extend lamellipodia and prepare for transendothelial migration (Rutledge and Muller, 2020). Additional downstream signals such as prostaglandin-D2 (PGD2) signals are required for shape change and dynamic transmigration of activated leukocytes (Ahmed et al., 2011). Macrophage integrin-1 (Mac-1) or CD11b/CD18, also known as $\alpha_M\beta_2$, interaction with ICAM-1 expressed by the inflamed endothelium is required for the transition of leukocytes from tight adhesion to crawling along the vessel wall (leukocyte locomotion) to then find a suitable site for transendothelial migration (Schenkel et al., 2004). Leukocytes either transmigrate (diapedesis) through the endothelial junctions (paracellular route), or through the body of the ECs (transcellular route) (Ley et al., 2007). In the paracellular route, the distribution of junctional molecules is modified in inflamed ECs in a way that facilitates transendothelial cell migration (Shaw et al., 2001). Junctional molecules such as platelet/endothelial-cell adhesion molecule 1 (PECAM-1) and junctional adhesion molecule A (JAM-A) may support leukocyte migration through mobilizing to the luminal surface where they can bind to their ligands expressed on leukocytes and guide them to the junctions (Muller, 2003). JAM-A interaction with $\alpha_L\beta_2$ allows strong adhesion of activated leukocyte to the endothelium (Kummer and Ebnet, 2018). PECAM-1 is expressed at EC junctions and leukocytes (Newman et al., 1990). When leukocytes reach the site of transmigration, the homophilic interactions between PECAM-1 on leukocytes with PECAM-1 on the endothelium causes a transient increase in cytosolic calcium in ECs which guide

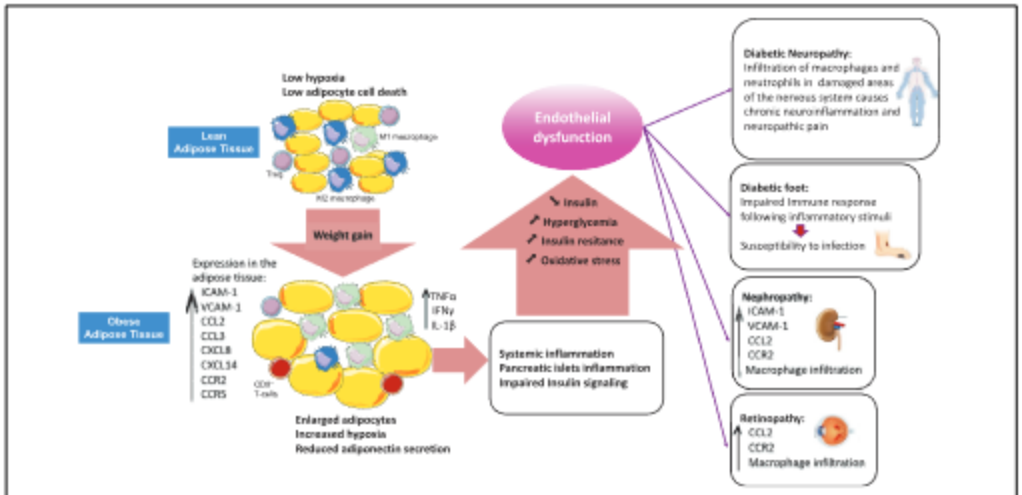


FIGURE 1 | Obesity-related dysregulation of leukocyte trafficking in T2DM. Adipose tissue from lean individuals is characterized by increased numbers of anti-inflammatory M2 macrophages and higher presence of regulatory T-cells (Tregs). Obesity induces adipose tissue enrichment of cytotoxic $CD8^+$ T-cells and pro-inflammatory M1 macrophages with a shift from an anti-inflammatory to a pro-inflammatory state. In obesity, the imbalance among leukocytes results in production of inflammatory cytokines (TNF-α, IFN-γ, IL-1β) and chemokines which promote systemic inflammation and peripheral insulin resistance as well as beta-cell dysfunction. Hyperglycemia occurs as a result of beta-cells failure to compensate for insulin resistance. Hyperglycemia exacerbates systemic inflammation by inducing pro-inflammatory cytokines production and modulating leukocyte trafficking through alteration of the expression of adhesion molecules, chemokines and chemokine receptors in different vascular beds, which ultimately results in vascular complications.

the leukocytes to the junctions (Ley, 2007; Muller, 2011). Like PECAM-1, CD99 (Schenkel et al., 2002) and CD99L2, expressed at EC junctions and on leukocytes, rely on a homophilic interactions to promote leukocyte extravasation (Schenkel et al., 2002; Seelige et al., 2013; Rutledge et al., 2015). In ECs, the major functional component of the adherent junction is VE-cadherin. During transendothelial migration, there is a transient gap in VE-cadherin at the site of the transmigrating leukocytes, which facilitates transmigration (Dejana and Giampietro, 2012). This gap has been shown to be triggered by constitutive shedding of VE-cadherin by ADAM-10, which is promoted by Tspan5 and Tspan17 and therefore allows T-cell diapedesis (Reyat et al., 2017).

Transcellular leukocyte migration only occurs for a minority of emigrating cells and mostly takes place in thin parts of the endothelium (Carman and Springer, 2004). In this route, leukocyte migration occurs through membrane-associated channels, which act as a gateway for leukocytes through the body of ECs. In fact, the preferred route of leukocyte transmigration depends on the leukocyte type and also the type of vascular bed (Dvorak and Feng, 2001). The endothelium is heterogeneous in different vascular beds, depending on the size and the organ considered (Aird, 2007). ECs in the arterioles of the vascular system have lower permeability to leukocytes, whereas those in the venules are thinner and permissive allowing leukocytes to easily transmigrate particularly in postcapillary venules (Dejana et al., 2009). In addition, the endothelial barrier can be even more permissive such as in lymphoid organs (Miyasaka and Tanaka, 2004), or very tight such as in the central nervous system (Wolburg and Lippoldt, 2002).

To reach inflamed tissues, leukocytes need to migrate through the basement membrane. The basement membrane is made up of laminin and collagen type IV (Mutgan et al., 2020). In murine models, leukocytes cross the basement membrane at areas with low expression of laminin (Song et al., 2017). PECAM-1, CD99, and CD99L2 are required for homophilic interactions between leukocyte and ECs to initiate the migration through the basement membrane (Thompson et al., 2001; Bixel et al., 2010; Sullivan et al., 2016). Leukocytes express several membrane-type matrix metalloproteinases (MMP) including the secreted- and membrane-anchored MT-MMPs, which develop an appropriate proteolytic reaction (Marco et al., 2013) and enable cells to cross this structural barrier. However, the exact mechanisms still remain to be fully characterized. Finally, following a successful diapedesis, leukocytes utilize a chemokine gradient to migrate toward the site of infection upon their entry into inflamed tissues (Ley et al., 2007).

EXPRESSION OF ADHESION MOLECULES IN T2DM

Cell adhesion molecules (CAMs) are glycoproteins expressed on the surface of various cell types such as ECs and leukocytes

(Galkina and Ley, 2007). Upon inflammation, vascular ECs express or up-regulate the expression of CAMs that increase the attachment of leukocytes to the endothelium (Cook-Mills et al., 2011). Selectins, ICAM-1 and VCAM-1 are the major CAMs responsible for leukocyte adhesion (Huo and Ley, 2001). Soluble form of CAMs (sCAMs) in the circulation indirectly reflect the rate of endothelial expression and activation of CAMs since they are shed from the surface of ECs and lymphocytes after being activated (Abe et al., 1998). Elevated expression and activity of CAMs are therefore indicative of inflammation and endothelial dysfunction (Cook-Mills et al., 2011).

Soluble CAMs and Selectins

The baseline levels of circulating E-selectin, ICAM-1, and VCAM-1 were found higher in patients with T2DM compared to healthy controls (Meigs et al., 2004). A study on 150 Japanese patients with T2DM showed that higher serum concentrations of sVCAM-1 and sE-selectin were positively correlated with fasting plasma glucose levels, and negatively correlated with insulin sensitivity (Matsumoto et al., 2002). Higher levels of circulating CAMs in T2DM is linked to an increased production of advanced glycosylation end products (AGEs) and oxidative stress occurring under hyperglycemic conditions (Hadi and Al Suwaidi, 2007). The interaction of AGEs with the vessel wall components increases the generation of reactive oxygen species, which results in an increase in the surface expression of CAMs on activated ECs (Wen et al., 2002; Basta et al., 2004; Farhangkhoee et al., 2006).

Hyperinsulinemia can also directly affect the surface expression of adhesion molecules on ECs from healthy volunteers and from patients with non-insulin-dependent diabetes *in vitro* (Okouchi et al., 2002). Culture of ECs in insulin-rich medium (over 50 microUnit/ml) for 24 h increased the surface expression of PECAM-1 but not of ICAM-1, P-selectin or E-selectin (Okouchi et al., 2002). This was clearly associated with an increase in neutrophil adhesion therefore suggesting that high insulin conditions promote vascular injury and therefore T2DM-associated macro- and micro-vascular complications.

However, a recent study on 58 patients with T2DM on insulin therapy and displaying microvascular complications found lower levels of serum sICAM-1 in these patients irrespective of the type of diabetic complication when compared to age-matched healthy controls (Hocaoglu-Emre et al., 2017). Since the participants with T2DM were also receiving angiotensin-converting enzyme (ACE)-inhibitor agents, decreased levels of sICAM-1 might have resulted from the combination of insulin and ACE-inhibitor therapies. Indeed, both insulin and ACE inhibitors can individually inhibit CAMs surface expression on ECs as well as their circulating levels (Drexler et al., 1995; Aljada et al., 2000). However much remain to be clarified in this area as the effect of both insulin and ACE inhibitors seems to vary in different patient cohorts. Some studies have hypothesized that these discrepancies between cohorts are linked to the degree of complications and the bigger impact seems to occur in late-stage diabetic complications when other treatments such as ACE-inhibitors are used. The conflicting evidence about the levels of sCAMs in the circulation suggests that different CAMs may play different roles in the different stages of vascular complications in T2DM (Hocaoglu-Emre et al., 2017).

Surface Expression of CAMs on the Endothelium

Variations in the expression of adhesion molecules on the vasculature play a role in microangiopathy in patients with T2DM by enhancing leukocyte adhesion in the vasculature and causing capillary obstruction (McLeod et al., 1995). This has been observed in different vascular beds and is linked to a majority of complications such as diabetic nephropathy (Gu et al., 2013) and retinopathy (McLeod et al., 1995). A study on 40 patients with T2DM showed a significant increase in the expression of ICAM-1 and VCAM-1 on vascular ECs from the conjunctiva in comparison with conjunctiva from healthy controls (Khalfaoui et al., 2008). Confocal microscopy imaging of retina vessels in streptozotocin (STZ)-induced hyperglycemic mice reported increased VCAM-1 protein levels after 8 weeks, and this was synchronized with the expression of the inflammatory cytokines TNFα, IL-6 and interleukin 1 beta (IL-1β) in the retina (Gustavsson et al., 2010). However, not many studies have performed in depth study of leukocyte recruitment in patients and in mice models to show that defects in leukocyte recruitment could mean leukocyte trafficking is impaired despite upregulation of endothelial CAMs.

Vascular adhesion protein-1 (VAP-1) is an enzyme and an adhesion molecule mainly expressed by ECs, smooth muscle, and the adipose tissue (Kuo et al., 2019). Endothelial VAP-1 supports leukocyte rolling, firm adhesion, and transmigration (Salmi and Jalkanen, 2019). The catalytic activity of VAP-1 can be damaging and cytotoxic to ECs via the generation of AGEs, which are involved in the pathogenesis of diabetic complications such as retinopathy, nephropathy, neuropathy, and atherosclerosis (Stolen et al., 2004).

Vascular adhesion protein-1 expressed on the vessels in the pancreatic islet of Non-Obese Diabetic (NOD) mice (model of T1DM) was associated with high degree of lymphocyte infiltration into the islets (Bono et al., 1999). Invalidation of the gene encoding VAP-1, using a null mutation in the amine oxidase copper-containing-3 (AOC3), decreased infiltration of T-cells, macrophages and NK cells in both epididymal and inguinal white adipose tissue of AOC3-KO mice compared to age-matched wild-type controls. This reduction in leukocyte infiltration was associated to a reduced capacity of leukocyte extravasation normally enabled via VAP-1 (Jargaud et al., 2020). Serum VAP-1 levels are higher in patients with T2DM and in patients with chronic kidney disease, which makes serum VAP-1, a good predictor of end-stage renal disease in diabetic patients and a useful biomarker to improve risk stratification of patients with T2DM (Li et al., 2016). Many *in vivo* studies further validate the role of VAP-1 as an anti-inflammatory target (Xu et al., 2006; Foot et al., 2013; Carpéné et al., 2019). Indeed, administration of a long-lasting VAP-1 and 2 inhibitor, PXS-4681A, in a mouse model of LPS-induced lung inflammation attenuated neutrophil migration into the lungs (Foot et al., 2013). PXS-4681A is a promising drug candidate as it ensures

complete and long-lasting inhibition of the enzymes after a single low dose *in vivo* and could also be tested in other models of chronic inflammation.

Surface Expression of CAMs on Leukocytes

Leukocyte adhesion to the endothelium is mediated by surface integrins expressed on leukocytes such as macrophage integrin-1 (Mac-1) or CD11b/CD18, also known as $\alpha_M\beta_2$ (Carlos and Harlan, 1994). In a study comparing patients with T2DM to age-matched healthy controls, basal expression of CD11b on monocytes and neutrophils did not differ between the groups (Sampson et al., 2002). However, higher expression of CD11b was observed on monocytes after a glucose load in both T2DM and control groups. This suggests, an association between hyperglycemia and monocyte-endothelial interactions through increased expression of monocytic CD11b (Sampson et al., 2002). Higher expression of CD11b in both diabetic and control groups after a glucose load could be due to the translocation of stored CD11b to the cell surface in response to any acute or sudden change in glucose levels regardless of basal plasma glucose (Sampson et al., 2002). However, another group later found higher monocyte expression of CD11b at baseline in patients with obesity, which may suggest higher degree of leukocyte activation with increased adhesive properties in this group compared to lean participants (Van Oostrom et al., 2004). This was not limited to monocytes as later de Vries et al. (2015) also found up-regulation of CD66b (glycosylated antigen implicated in adhesion to E-selectin) on neutrophils from patients with T2DM. Change in CD66 can be implicated in aberrant neutrophil recruitment to the vasculature but also indicates neutrophil activation and degranulation (Weber, 2003), which confirms how hyperglycemia can lead to exacerbated inflammatory responses.

Dipeptidyl peptidase-4 (DPP-4) or CD26 is an amino-peptidase expressed in numerous tissues including the vasculature and in immune cells (Mentlein, 1999; Lambeir et al., 2003). DPP-4 cleaves dipeptides from the N-terminus of many chemokines and cytokines, usually after a penultimate proline or an alanine (Broxmeyer et al., 2016). DPP-4 controls glucose homeostasis through regulating bioactivity of the incretin hormone and glucagon-like peptide 1 (Augustyns et al., 2010). Involvement of DPP-4 in metabolic control raises the possibility that it may play a role in metabolic diseases such as diabetes and obesity (Mulvihill and Drucker, 2014; Omar and Ahrén, 2014). Given its various roles and its altered expression and activity, DPP-4 has been implicated in several pathological processes, including inflammation, viral entry and immune-mediated diseases (Lambeir et al., 2003; Yu et al., 2010).

Plasma levels of DPP-4 and circulating DPP-4 activity both increase with obesity and this correlates with insulin resistance (Mulvihill and Drucker, 2014; Ahmed et al., 2017). Numerous studies have detailed the effects of DPP-4 inhibitors on insulin and/or glucagon secretion, but little evidence indicates that DPP-4 inhibitors directly improve chronic inflammation. The effects of DPP-4 inhibition was investigated in diet-induced adipose tissue inflammation using ($Gck^{+/2}$) diabetic mice, an animal model of non-obese T2DM (Shirakawa et al., 2011). DPP-4 inhibition in this model led to a significant reduction in adipose tissue infiltration of $CD8^+$ T-cells and M1 macrophages which was associated to decreased mRNA expression levels of TNF-α and MCP-1 in the adipose tissue (Shirakawa et al., 2011). Because DPP-4 mediates the cleavage of many chemokines and adipokines, inhibition of DPP-4 may cause off-target side effects and further research is needed to clarify this (Shirakawa et al., 2011).

In addition, Zhuge et al. (2016) found that the expression of DPP-4 was mainly detected and up-regulated on $F4/80^+$ macrophages in the white adipose tissue of HFD-induced obese mice). Oral administration of the DPP-4 inhibitor, linagliptin, caused an anti-inflammatory macrophage polarization with a dynamic M2 shift of macrophages within the adipose tissue of HFD-induced obese mice compared to non-treated obese mice, and this contributed to the attenuation of whole-body insulin resistance (Zhuge et al., 2016). In this study, the anti-inflammatory effects of DPP-4 inhibition were due to a decreased in ROS generation and an attenuation of oxidative stress in the white adipose tissue (Zhuge et al., 2016). Loss of macrophage inflammatory protein-1α (MIP-1α), a chemokine and potential DPP-4 substrate, abrogated the M2 macrophage-polarizing and insulin-sensitizing effects of linagliptin in MIP-$1\alpha^{-/-}$ mice on HFD. This suggests that MIP-1α may be a substrate for DPP-4 and contributes to the regulation of macrophage polarization in obesity models (Zhuge et al., 2016).

Altogether the *in vivo* studies highlight a potential role of DPP-4 in the modulation of leukocyte migration by affecting chemokines. However, this has not been extensively investigated in the studies presented here as not all studies seem to have linked this to migration despite the important role of DPP-4 at inhibiting chemokines. The approved DPP4 inhibitors being used in clinic, such as sitagliptin and vildagliptin are based on the ability of DPP4 to lower glucose rather than its regulatory effects on inflammation (Deacon, 2018).

Similar to studies investigating endothelial expression of adhesion molecules in T2DM, data showing changes in adhesion molecules/integrins on leukocytes have not performed analysis of leukocyte recruitment in targeted tissues, instead this has been extrapolated from knowledge on adhesion molecule profiles and function in "normal" inflammatory responses, in other models and/or diseases.

EXPRESSION OF CHEMOKINES AND CHEMOKINE RECEPTORS IN T2DM

Chemokines and their receptors play a central role in leukocyte trafficking and are involved in the pathophysiology of T2DM (Buraczynska et al., 2012). Production and release of cytokines and chemokines including CC-chemokine ligand 2 (CCL2), CCL3 and CXC-chemokine ligand 8 (CXCL8) is increased in the adipose tissue and pancreatic islets of patients with T2DM (Donath and Shoelson, 2011; Xu et al., 2015). This is associated with an increased recruitment of macrophages in these tissues, and thus contributes to tissue inflammation

(Donath and Shoelson, 2011; Xu et al., 2015). CCL2 (also known as MCP-1) is known to regulate monocyte recruitment by directing monocytes migration from the bone marrow to inflamed tissues (Crane et al., 2009; Boels et al., 2017). The CCL2-CCR2 axis has a key role in diabetes-related complications such as retinopathy, nephropathy and neuropathy (White et al., 2009; Zhu et al., 2014; Moreno et al., 2018; Monickaraj et al., 2020). Indeed, contribution of CCL2-CCR2 axis to leukocytes recruitment into the injured nerve was shown using a preclinical model of peripheral neuropathic pain induced by chronic nerve constriction (CCI) in Sprague–Dawley rats (Van Steenwinckel et al., 2015). This study shows that CCI-induced mechanical hypersensitivity in rats upregulated the expression of CCL2 and increased local macrophage infiltration in the sciatic nerve compared to the sham group (Van Steenwinckel et al., 2015). In contrast, in the same study, CCL2-deficient mice presented attenuated CCI-induced mechanical hypersensitivity as well as decreased number of macrophages infiltrating the injured sciatic nerve. Altogether, these findings suggest that CCL2 could play an important role in mediating neuropathic pain by increasing leukocyte infiltration in nerves but also by mediating neuro-immune interactions during inflammation-induced pain (reviewed in White et al., 2009).

In addition, upregulation of the CCL2 gene was also demonstrated in retinas of STZ-induced diabetic rats, and this was coincident with the trafficking and infiltration of numerous monocytes into the retina (Rangasamy et al., 2014). Furthermore, using CCL2 knockout ($Ccl2^{-/-}$) mice, the same authors found significantly decreased monocyte/macrophage trafficking into diabetic $Ccl2^{-/-}$ retinas, indicating that this chemokine may be essential for the alteration of the blood-retinal barrier (Rangasamy et al., 2014). The role of CCL2-CCR2 axis is also widely investigated in diabetic renal injuries. Indeed, accumulation of macrophages in the kidney was reduced in $Ccl2^{-/-}$ *db/db* double knock-out diabetic mice compared to $Ccl2^{+/+}$ *db/db* animals (Chow et al., 2007). Immunohistochemistry results from kidney biopsies of patients with T2DM showed overexpression of CCL2 and CCR2 in the glomeruli of these patients compare to healthy kidneys (Tarabra et al., 2009). This was accompanied with reduced nephrin expression in cultured podocytes from T2DM patients (Tarabra et al., 2009). Moreover, in the STZ- model of diabetes, induction of diabetes increased albuminuria in $CCL2^{+/+}$ mice, which was significantly reduced in CCL2-deficient mice. Together these studies suggest a pathogenic role of the CCL2/CCR2 axis in the development of diabetic nephropathy (Tarabra et al., 2009) and targeting this pathway could be a potential therapeutic avenue to reduce common diabetic complications associated with aberrant infiltration of leukocytes.

Other chemokines and their receptors are also changed in T2DM. CX3CL1 was found at higher levels in the subcutaneous adipose tissue of patients with T2DM and in obese people when compared to patients with normal weight and also was shown to modulate monocyte adhesion to adipocytes (Shah et al., 2011). CCL5 (RANTES) was also found at higher levels in the circulation of 236 patients with T2DM, 242 individuals with impaired glucose tolerance (IGT) but not in 244 individuals with normal glycemic control (Herder et al., 2005). However, higher circulating levels of CCL5 were not significantly associated with other inflammatory variables and metabolic parameters, suggesting more studies are required to evaluate the novel hypothesis that CCL5 is a risk factor for T2DM.

Infiltration of inflammatory leukocytes in adipose tissue plays an important role in the development of insulin resistance. The inflammatory cytokines secreted by these cells interfere with insulin signaling and decrease glucose uptake in peripheral tissues (Asghar and Sheikh, 2017). Studies looking at ways to down-regulate aberrant recruitment of leukocytes as a mean to control inflammation and therefore restore insulin sensitivity have targeted chemokine receptors such as CCR5 and CCR2. CCR5 a chemokine receptor expressed by T-cells and macrophages, plays a critical role in recruitment and polarization of macrophage in inflammation. Kitade et al. (2012) showed that a higher gene expression of CCR5 in the white adipose tissue of High Fat Diet (HFD)-induced obese mice was concomitant to an accumulation of macrophages in this tissue. Importantly, mice deficient for CCR5 in their myeloid lineage did not develop insulin resistance and diabetes normally induced by HFD. Loss of CCR5 was associated with a reduction in total adipose tissue macrophage content and polarization of macrophages toward and an anti-inflammatory M2-dominant phenotype in the adipose tissue (Kitade et al., 2012).

The administration of a dual CCR2/CCR5 antagonist has shown to improve obesity-associated insulin resistance and glucose intolerance via reducing macrophages and $CD8^+$ T-cell numbers in the white adipose tissue of HFD-fed mice, indicating that blocking both CCR2 and CCR5 has potential to maintain both metabolic and immune homeostasis in obesity-induced inflammation (Huh et al., 2018).

Overall, these studies clearly demonstrate how up-regulation of chemokine pathways, which in turn leads to aberrant recruitment of leukocytes in different tissues is contributing to some of the common diabetic complications and targeting this pathophysiological process could be a promising therapeutic advance. However, further research is required to fully characterize the interactions and redundancy of chemokine with chemokine receptors in T2DM.

INNATE AND ADAPTIVE IMMUNE CELL IN T2DM

In this section, we will explore how the recruitment of innate and adaptive immune cells changes in obesity and T2DM as summarized in **Table 1**. Obesity-induced inflammatory events originating from the adipose tissue such as production of local inflammatory molecules are responsible for the activation of immune responses and progression of systemic inflammation in patients with T2DM (Richardson et al., 2013).

Innate Immune Cells

Monocytes and Macrophages

Innate immune cells play a critical role in the early stage of adipose tissue inflammation in T2DM (Weisberg et al., 2003).

TABLE 1 | A summary of studies discussed in this review assessing obesity/T2DM-related changes to the trafficking of innate and adaptive immune cells.

	Effect of obesity/T2DM on cell recruitment	Potential mechanisms	References
Monocyte/ macrophage	↑ recruitment of macrophages in retinas of STZ-induced diabetic rats	CCL2-CCR2 axis	Rangasamy et al., 2014
	↑ recruitment of macrophages in kidney of STZ-induced diabetic mice	CCL2-CCR2 axis	Tarabra et al., 2009
	↑ adhesion of monocyte to adipocytes in the subcutaneous adipose tissue of patients with T2DM and obesity **(Human study)**	↑ CX3CL1 expression in adipose tissue	Shah et al., 2011
	↑ recruitment of macrophages in white adipose tissue of HFD-induced obese mice	↑ CCR5 expression in white adipose tissue	Kitade et al., 2012
	↑ levels of F4/80 and CD11c mRNA expression in adipose tissue of HFD fed mice	↑ Adipose tissue expression of ICAM-1, VCAM-1, CCL2, CXCL14	Kawanishi et al., 2010
	↑ expression of F4/80 in adipose tissue of HFD fed mice	↑ Adipose tissue expression of mRNA for ICAM-1	Brake et al., 2006
	↓ RPMs in db/db mice ↑ Increased M2 polarization	Ongoing chronic inflammation in the peritoneal cavity in db/db mice	Liu et al., 2012
	In vitro ↓ adhesion and phagocytosis capacity of RPMs from in *db/db* mice	abnormal microenvironment in db/db mouse	Liu et al., 2012)
	↓ monocyte/macrophages in lung tissue of diabetic $DPP4^{I/M}$ mice following infection by *MERS-CoV*	↓ lung tissue expression of *Ccl2* and *Cxcl10*	Kulcsar et al., 2019
DCs	↑ $CD11c^{high}F4/80^{low}$ DCs in mice visceral adipose tissue in HFD-induced obese mice	Not described	Bertola et al., 2012.
	↑ $CD11c^{+}CD1c^{+}$ cDCs in obese human subcutaneous adipose tissue **(Human study)**	Not described	Bertola et al., 2012.
	↑ $CD11c^{+}$ cDCs numbers in the adipose tissue of HFD-fed mice	Not described	Chen et al., 2014
Neutrophils	↑ trafficking of neutrophil into mice visceral adipose tissue after 3 days on HFD	↑ CD11b surface expression on neutrophils	Elgazar-Carmon et al., 2008
	↑ recruitment of neutrophils into the adipose tissue of HFD-fed mice after 3 days on HFD (sustained infiltration for up to 90 days on HFD)	↑ expression and activity of neutrophil-secreted elastase in the adipose tissue of HFD-fed mice	Talukdar et al., 2012
$CD8^{+}$ T-cells	↑ infiltrated $CD8^{+}$ effector T-cells in visceral adipose tissue in HFD-fed mice	Activation of $CD8^{+}$ T cells by endogenous stimuli localized in the adipose tissue	Nishimura et al., 2009.
	↓ lower numbers of $CD8^{+}$ T-cells in their brains in *db/db* mice following infection with *West Nile virus*	↓ expression of E-selectin and ICAM-1 in db/db brains	Kumar et al., 2014
$CD4^{+}$ T-cells	↓ $CD4^{+}$ T-cells in lung tissue of diabetic $DPP4^{I/M}$ mice following infection by *MERS-CoV*	↓ lung tissue expression of *Ccl2* and *Cxcl10*	Kulcsar et al., 2019
Tregs	↓ regulatory T-cells in visceral adipose tissue of HFD-fed mice	Not described	Nishimura et al., 2009.
	↓ $CD4^{+}$ Tregs HFD-fed mice	↓ levels of adiponectin in obese fat	Feuerer et al., 2009; Ilan et al., 2010.
B-cells	↑ number of B-cells in the bone marrow of HFD-fed mice	Not described	Trottier et al., 2012
	↓ number of B-cells in the bone marrow of HFD-fed mice	lower expression of Pax5 in the bone marrow of HFD-fed mice	Chan et al., 2012
Granulocytes	↓ granulocytes in the alveolar airspace of stz-induced diabetic mice following infection by *Klebsiella pneumoniae*	↓ levels of (CXCL1, CXCL2) and (IL-1β, TNF-α) in lung tissue	Martinez et al., 2016

Monocytes derived from the bone marrow remain in the circulatory system for 1–2 days before they migrate into peripheral tissues, where they turn into fully mature resident macrophages (Martinez-Pomares et al., 2003) either with a M1 pro-inflammatory or M2 anti-inflammatory phenotype in response to local factors (Mosser, 2003). In a recent *in vivo* study, an acute decrease in number of circulating monocytes was synchronized with a concomitant infiltration of macrophages into visceral adipose tissue, therefore demonstrating dynamic changes in blood monocyte trafficking at the early stages of HFD-induced diabetes (Liu et al., 2020). Higher levels of F4/80 and CD11c mRNA expression in adipose tissue of HFD fed mice, representative of a M1 polarization, were attributed to an elevated gene expression of ICAM-1, VCAM-1, CCL2 and CXCL14 in this tissue, which facilitates macrophage trafficking (Kawanishi et al., 2010). Similarly, Brake et al., found higher levels of mRNA for ICAM-1 in mice on a 3-week HFD, as well as an increased expression of F4/80 in their adipose tissue

(Brake et al., 2006). The activation of M1 macrophages is associated with release of pro-inflammatory mediators, such as TNFα and IL-6 (Biswas and Mantovani, 2010). Using an HFD-induced rat model of T2DM, Shanaki et al. reported increased serum and adipose tissue levels of IL-6 and TNFα in T2DM rats compared to control animals. This pro-inflammatory shift was linked to higher fasting plasma glucose levels and insulin resistance in the diabetic animals (Shanaki et al., 2020). The impact of long-term diabetes on the functions of macrophages was investigated using a 5-month old *db/db* mice model of T2DM (Liu et al., 2012). In this study, the authors focused on resident peritoneal macrophages (RPMs) to investigate potential changes of their function, phenotype and migratory capacity. RPMs numbers were reduced in *db/db* mice compared to C57BL/6 control mice and most were preferentially polarized toward a M2 phenotype. Since M2 macrophages exhibit immunosuppressive functions and therefore contribute to the resolution of harmful inflammation, their increased proportion in the *db/db* mice may be caused by an effort to control the chronic low-grade inflammation observed in the peritoneal cavity of these mice (Liu et al., 2012). Furthermore, in this study, *in vitro* assessment of *db/db* RPMs highlighted their decreased adhesion capacity along with a decreased phagocytosis ability compared to wild-type RPMs. These observations validate that diabetes and obesity are associated with immune dysfunction which predispose these patients to an increased susceptibility to infections (Liu et al., 2012).

Human studies also indicate a role of macrophages in mediating insulin resistance. Indeed, higher numbers of circulating leukocytes are found in patients with T2DM along with higher Free Fatty Acid (FFA) and IL-6 circulating levels (van Beek et al., 2014). Elevated number of total leukocytes counts in patients with T2DM was also characterized in other studies (Kizilgul et al., 2018; Palella et al., 2020) and were often indicative of macro- and micro-vascular complications and diabetes duration (Papazafiropoulou et al., 2010; Moradi et al., 2012). In particular, Wouters et al. demonstrated higher numbers of circulating classical monocytes and this was associated with higher presence of M1 macrophages in the white adipose tissue of individuals with obesity compared to individuals with normal weight (Wouters et al., 2017). In addition, impaired glucose tolerance was associated with the presence of crown-like structures (marker of adipose inflammation) in the adipose tissue of patients with obesity and T2DM compare to patients with obesity and normal glucose tolerance (van Beek et al., 2014).

Most of the current observations seem to indicate a crucial role of macrophage infiltration in the adipose tissue of patients with obesity and T2DM. However, it is not entirely clear what mechanisms mediate recruitment of macrophages in these tissues and more research is needed to understand whether macrophage infiltration could be limited to improve insulin sensitivity and glucose tolerance and therefore prevent diabetic complications.

Neutrophils

Neutrophils are the most abundant subsets of leukocytes of the innate immune system in humans and are the first to infiltrate inflamed tissue and promote subsequent recruitment of other leukocytes such as monocytes (Soehnlein et al., 2008). The number of circulating neutrophils in patients with T2DM increases in comparison to age- and gender-matched healthy controls (Huang et al., 2019). However, neutrophils isolated from patients with T2DM displayed normal migratory capacity and phagocytic rate (Huang et al., 2019). Neutrophil trafficking into murine visceral adipose tissue was reported after 3 days on HFD. The same study reported an absence of neutrophils in the adipose tissue after 7 days on HFD and that neutrophil adhesion to mouse adipocytes depends on their activation state (Elgazar-Carmon et al., 2008). Here, the degree of CD11b surface expression on neutrophils correlated with their capacity to adhere. However, the mechanisms responsible for the absence of neutrophils in the adipose tissue after 7 days on HFD was not explained in this study (Elgazar-Carmon et al., 2008). Similarly, Talukdar et al. (2012) found an early recruitment of neutrophils into the adipose tissue of HFD-fed mice after 3 days. Here, the time course of neutrophil infiltration into the adipose tissue showed a sustained infiltration in the adipose tissue for up to 90 days on HFD (Talukdar et al., 2012). According to this study, the trafficking of neutrophils was associated with an elevated expression and activity of neutrophil-secreted elastase in the adipose tissue of HFD-fed mice which increased after only 3 days of HFD and remained high after 12 weeks. Elastase is a neutrophil-specific protease which can promote inflammatory responses (Pham, 2006) and inhibition of neutrophil elastase in HFD-induced obese mice improved glucose tolerance and reduced trafficking of neutrophils into the adipose tissue (Talukdar et al., 2012).

Dendritic Cells

Dendritic cells (DCs) are antigen-presenting immune cells that mediate lymphocytes polarization into effector cells (Yu and Martin-Gayo, 2019). In humans, two major subtypes of DCs are identified according to their markers expression: conventional or myeloid DCs (cDCs) ($CD11c^{+}$ $CD1c^{+}$ $CD141^{+}$) and plasmacytoid DCs (pDCs) ($CD11c^{-}$ $CD123^{+}$) (Tamura et al., 2005; Gilliet et al., 2008; Sundara Rajan and Longhi, 2016). In mice, two major DC subsets have also been described: $CD11c^{low}B220^{+}$ (pDCs) and $CD11c^{high}B220^{-}$ cells that include cDCs (O'Keeffe et al., 2002). The number of circulating cDCs increases in obese post-menopausal women with T2DM compared to age-matched healthy women and a smaller increase was observed for pDCs (Musilli et al., 2011). This change in DCs numbers in the circulation suggests that DCs might contribute to pathological vascular remodeling (Musilli et al., 2011). Mráz et al. reported a decrease in the number of total DCs in the subcutaneous adipose tissue from patients with T2DM compared to non-diabetic individuals. In contrast, the number of pDCs was increased in the subcutaneous adipose tissue of the T2DM group. These differences suggest a potential role of pDCs in the development of T2DM-associated adipose tissue low-grade inflammation (Mráz et al., 2019). Bertola et al. investigated the role of DCs in the regulation of adipose tissue inflammation in a murine HFD-induced obesity model and in two cohorts of obese subjects (Bertola et al., 2012). The authors in this study only found $CD11c^{high}B220^{-}$ DCs in the visceral adipose tissue of lean mice. In contrast, obesity

was associated with the presence of $CD11c^{high}F4/80^{low}$ DCs in murine visceral adipose tissue, and $CD11c^{+}CD1c^{+}$ cDCs in human subcutaneous adipose tissue (Bertola et al., 2012). $CD11c^{high}B220^{-}$ DCs resident in the adipose tissue of lean mice participate in the differentiation of naive $CD4^{+}$ T-cells into effector T-cells with a predominance of Th1 cells over Th17 cells. In contrast, the adipose tissue of HFD-induced insulin resistant mice displayed a switch from Th1 towards a Th17 phenotype (Bertola et al., 2012). In addition, the presence of $CD11c^{+}CD1c^{+}$ cDCs in the subcutaneous adipose tissue of obese subjects correlated with CD1c expression and a concomitant skew towards a Th17 T-cell phenotype. Finally, CD1c expression strongly correlated with insulin resistance in patients with a high Body Mass Index (BMI) (Bertola et al., 2012). Altogether, these observations suggests an important role of inflammatory DCs in obese and diabetic adipose tissue inflammation by switching T-cell responses toward Th17 responses, which is associated with insulin resistance (Bertola et al., 2012). These observations were confirmed by another group who showed that $CD11c^{+}$ cDCs infiltrated the adipose tissue of HFD-fed mice and also secrete high levels of IL-6 and IL-23, which promoted a Th17 T-cell phenotype (Chen et al., 2014). The presence of DCs in the visceral adipose tissue of HFD-mice can also induce the formation of crown-like structures which enclose macrophages and were linked to adipose tissue inflammation and insulin resistance (Stefanovic-Racic et al., 2012). Depletion of DCs results in loss of adipose tissue macrophage infiltration, and this could be restored by DC replacement in DC-null mice (Stefanovic-Racic et al., 2012). All together, these studies show the importance of DCs in determining the immune phenotype of lean versus obese or diabetic adipose tissue.

Adaptive Immune Cells

The mucosal immune system is a compartment of the adaptive immune system which is located near the surface, where most pathogens invade, providing the first line of defense (Janeway et al., 2001). The gut microbiota is key to the development and modulation of mucosal immune responses and maintains perfect balance between commensal flora and pathogens, as well as the microbiota and the immune system (Wang and Li, 2015). The alteration of such balance is called dysbiosis (Pagliari et al., 2018). Given that the pancreas does not have its own microbial collection, the gut microbiota may be involved in the pathogenesis of pancreatic disorders such as pancreatitis (Signoretti et al., 2017). Recently, Guo et al. (2017) demonstrated that HFD was able to alter gut microbial communities and increase circulating pro-inflammatory cytokines, such as TNFα, IL-6 and IL-1β. In addition, recent evidence demonstrated that intestinal dysbiosis may also cause alterations in the Th17 cells/Tregs balance which are responsible for the development inflammatory disease including obesity-related T2DM (Luo et al., 2017).Thus, understanding the mechanisms responsible for this alteration will allow to develop novel translational therapeutic targets to potentially treat these inflammatory diseases (Pagliari et al., 2018). In this section, we mostly focus on T- and B-cells as very little is known on mucosa-associated homing mechanisms in the context of T2DM.

T-Cells

Although, earlier studies focused on the role of innate immunity (macrophages) as the major cause of chronic low-grade inflammation in T2DM, the adaptive immune system also plays a role in progression of T2DM (Zhou et al., 2018). Evidence shows that B-cells are the first to infiltrate the adipose tissue of mice on HFD, quickly followed by T-cells and finally an accumulation of macrophages leading to insulin resistance, but no changes in resident macrophages populations (Duffaut et al., 2009). Nishimura et al. (2009) found larger number of infiltrated $CD8^{+}$ effector T-cells along with reduced numbers of $CD4^{+}$ helper and regulatory T-cells in visceral adipose tissue of HFD fed mice). In these conditions, a majority of $CD8^{+}$ T-cells infiltrate the adipose tissue thereby promoting recruitment and activation of macrophages. This sequential accumulation of leukocytes is clearly linked with glucose intolerance and a decrease in insulin sensitivity in wild-type animals (Duffaut et al., 2009). Studies in the lymphocyte-deficient $RAG2^{-/-}$ knockout mouse provide strong evidence for the role of lymphocytes in HFD-mediated adipose tissue inflammation. Unexpectedly, lymphocyte-deficient animals displayed striking accumulation of macrophages and NK cells in the adipose tissue compared to wild-type mice (Duffaut et al., 2009). This accumulation of NK cells highlights an overreaction of the innate immunity in absence of the adaptive immune system. Indeed, NK cells have potent cytotoxic effector functions and produce chemokines and cytokines that can recruit macrophages (Duffaut et al., 2009). The exaggerated recruitment of macrophages in lymphocyte-deficient mice demonstrates that early lymphocyte infiltration could be considered a protective process to decrease adipose tissue inflammation and suggests adipose tissue as a site of dynamic innate and adaptive immune system during diet-induced obesity and insulin resistance (Duffaut et al., 2009).

According to recent reports, the changes in quantity and polarization of adipose tissue T-cells during weight gain is a key regulator of systemic insulin sensitivity (Deng et al., 2017). HFD is associated with increased numbers of Th1 $CD4^{+}$ T-cells and decreased numbers of $CD4^{+}$ Tregs in the adipose tissue (Feuerer et al., 2009; Ilan et al., 2010). The depletion of Tregs using diphtheria toxin in mice leads to an induction of genes encoding inflammatory cytokines such as TNFα and IL-6 in visceral adipose tissue and enhance levels of fasting insulin (Feuerer et al., 2009). On the other hand, in the same study, increasing the quantity Tregs in the adipose tissue using a Tregs-enriched HFD-fed mice model, improved insulin resistance and glucose tolerance (Feuerer et al., 2009). Since, the gene expression of IL-10 was increased in the adipose tissue of Tregs–enriched animals, the metabolic changes observed can be attributed to IL-10 synthesis by Tregs in the adipose tissue of these mice (Feuerer et al., 2009). These findings suggest a strong therapeutic potential for Tregs to suppress inflammation and improve insulin action in obesity and T2DM. It is therefore key to understand how Tregs migratory capacity and infiltration in the adipose tissue

could be manipulated in patients. In addition, further studies are required to fully characterize the involvement of different T-cell subsets and characterize the intricate balance between innate and adaptive immune regulations.

B-Cells

B-cells have been shown to play important roles in many chronic inflammatory and autoimmune conditions (Yanaba et al., 2008; Mariño and Grey, 2012) but only recently has their role been revealed in obesity-associated insulin resistance. B-cells infiltrate the white adipose tissue in diet-induced obesity models and contribute to insulin resistance (Winer et al., 2011). The data on the development of B-cells in the bone marrow of obese models is controversial. Indeed, B-cells numbers in the bone marrow of mice on HFD were significantly increased after 90 days (Trottier et al., 2012). However, number of B-cells in the bone marrow were reduced in C57BL/6 mice on HFD for 210 days in another study (Chan et al., 2012). This reduction was associated to a lower expression of Pax5 in the bone marrow of HFD-fed mice (Chan et al., 2012). Differences between these studies may be due the type and duration of HFD. B-cell infiltration in the white adipose tissue of C57BL/6 mice peaks at around 3–4 weeks of HFD (Duffaut et al., 2009). The importance of B-cells in adipose tissue inflammation has been studied *in vivo* using B-cell null mice (μMT mice). Attenuated inflammation in visceral adipose tissue of μMT mice was shown by a reduced infiltration of macrophages. In this study, following 15 weeks of HFD, serum glucose levels were unchanged in obese μMT mice while fasting glucose levels increased in the control wild-type group (DeFuria et al., 2013). These data support the conclusion that B-cells regulate macrophage infiltration into the adipose tissue during inflammation and this contributes to insulin resistance.

IMMUNE RESPONSE FOLLOWING INFLAMMATORY STIMULUS IN T2DM

Hyperglycemia in diabetes can impair immune response to pathogens such as, fungi, bacterial and viral infections (Hostetter, 1990; Javid et al., 2016; Kulcsar et al., 2019). As a result, patients with diabetes are more susceptible to infections (Berbudi et al., 2020). Although the plasma concentration of sCAMs tends to be generally higher in patients with T2DM compared to healthy controls, their expression is differently affected in response to inflammatory pathogens in patients with T2DM. Indeed, plasma levels of sE-selectin, sVCAM-1 and sICAM-1 after intravenous injection of *E.coli* lipopolysaccharide (LPS) were lower in patients with T2DM compared to healthy volunteers. This study revealed that patients responded with an attenuated up-regulation of sCAMs even though the basal plasma concentration of these adhesion molecules were generally higher in diabetic individuals compared to healthy controls (Andreasen et al., 2010). Also, in this study, T2DM was associated with less pronounced LPS-induced cytokine responses. This weaker cytokine response following inflammatory stimuli in T2DM is also shown *in vitro* with cultures of peripheral blood mononuclear cells (PBMCs). Stimulation of PBMC from patients with diabetes with LPS and *Burkholderia pseudomallei* lead to a lower production of IL-1β and IFNγ respectively, compared to PBMCs from healthy donors (Mooradian et al., 1991; Tan et al., 2012). All together these findings may explain the immune dysfunction and increased risk of infections associated with T2DM (Andreasen et al., 2010). However, the evidence demonstrating changes in leukocyte recruitment following pathogen invasion in T2DM is rare and mostly limited to animal experimental studies. Accordingly, *db/db* mice infected with West Nile virus, had lower numbers of $CD45^+$ leukocytes and $CD8^+$ T-cells in their brains compared to wild-type mice (Kumar et al., 2014). Effector $CD8^+$ T-cells are necessary to limit viral load and therefore a lack of $CD8^+$ T-cells infiltration, which may be caused by a decreased migratory capacity or changes in the brain vasculature, lead to an increased viral burden in the brain of infected mice (Shrestha and Diamond, 2004; Kumar et al., 2014). This defect in leukocyte recruitment was in fact due to a reduced expression of E-selectin and ICAM-1 in *db/db* brains which failed to properly support the $CD8^+$ T-cells adhesion and migration (Kumar et al., 2014). Furthermore, impaired recruitment of leukocytes is not only due to changes on leukocytes themselves and in the local environment but can also be attributed to attenuated cytokine and chemokine production in diabetic mice. In STZ-diabetic mice infected by *Klebsiella pneumoniae*, which causes pneumonia (Bengoechea and Sa Pessoa, 2019) lower numbers of granulocytes were found in the alveolar airspace along with attenuated chemokines (CXCL1, CXCL2) and cytokines (IL-1β, TNFα) levels in lung tissue when compared to control mice (Martinez et al., 2016). Moreover, a recent study investigated the impact of T2DM on respiratory infection caused by Middle East respiratory syndrome coronavirus (MERS-CoV) (Kulcsar et al., 2019). In this study, humanized DDP4 mice were susceptible to MERS-CoV and T2DM was induced by HFD (Kulcsar et al., 2019). Following infection with MERS-CoV, diabetic $DPP4^{H/M}$ mice displayed weight loss and had a longer phase of severe disease with delayed recovery. Importantly, lung tissue analysis in the diabetic mice showed a decreased number of macrophages, $CD4^+$ T-cells, and lower expression of TNFα and IL-6 in the HFD group, compared to control $DPP4^{H/M}$ mice following infection (Kulcsar et al., 2019). These results suggest that MERS-CoV infection in patients with T2DM diabetes may develop more severe disease as a result of a dysregulated immune response targeting not only migratory capacities of leukocytes but the local environment by modulating secreted factors and expression of adhesion molecules. Maximal endothelial cell adhesion molecule expression or chemokine/cytokine receptor downregulation/internalization in chronic inflammatory conditions could theoretically cause an impaired immune response to an infection. In addition to alternative mechanisms, it is also plausible that insulin could have agonist and antagonist effects on the same signaling pathway based on its concentration and receptor expression state (i.e., the "U-shaped" biologic dose-response curve) (Calabrese and Baldwin, 2001). In general, it seems that high glucose levels are associated with impaired expression of adhesion molecules, cytokines and the chemokines supporting efficient leukocyte migration during an inflammatory response to infections in T2DM conditions. This is in contrast

with studies mentioned above which show how basal expression of adhesion molecules and chemokines/chemokine receptors is increased in different vascular beds and leukocytes in T2DM conditions. The defect in cytokine response to pathogen in T2DM is partially related to insulin deficiency and recently has been discussed by Tessaro and Martins (Tessaro et al., 2017). In their study, insulin increased TNFα and IL-6 release by bone marrow-derived macrophages from diabetic C57BL/6 mice following LPS stimulation. This finding supports the idea that insulin is crucial to induce a proper immune reaction in response to inflammatory stimuli. However, in the same study insulin inhibited LPS-induced pro-inflammatory cytokine secretion by peritoneal macrophages from diabetic mice (Tessaro et al., 2017). Together findings suggest that beyond its glucose modulatory effects, insulin also has distinctly immunomodulatory effects in macrophages and these urgently require to be fully understood.

THERAPEUTIC TARGETING OF LEUKOCYTE TRAFFICKING IN T2DM

Given that T2DM is a chronic inflammatory disorder, characterizing and targeting possible inflammatory pathways could be efficient to prevent and control diabetes and its vascular complications.

Chemokine and Cytokine Inhibition

Chemokine and cytokine pathways are known to govern the trafficking of leukocytes into peripheral tissues (Yao et al., 2014). Potential antagonists targeting chemokine receptors and drugs blocking inflammatory cytokines have been developed and tested in many inflammatory conditions, but little is known about their efficacy in obesity or T2DM and associated complications. In this section, we will discuss some of these targets.

CC-chemokine ligand 2 and CCL5 are key mediators of monocyte recruitment induced by high glucose levels via their receptor CCR2 and CCR5 (Nunemaker et al., 2014). Oral administration of RO5234444, a CCR2 antagonist, to *db/db* mice, reduced infiltration of monocytes in the glomerulus, resulted in preservation of glomeruli podocytes numbers and reduced albuminuria (Sayyed et al., 2011). Similarly, intraperitoneal administration of TAK-779, a dual inhibitor of chemokine receptors CCR2 and CCR5, reduced macrophage infiltration and expression of ICAM-1 in the retinas of STZ-diabetic mice (Monickaraj et al., 2020). These studies clearly indicate that blocking key recruitment chemokine receptors such as CCR2 and/or CCR5 is a promising therapeutic avenue to ameliorate common diabetic complications such as nephropathy and retinopathy. Those compounds are currently pursued in human clinical trials as well as others such as INCB8761/PF-413630, which are a new series of CCR2 antagonists that are orally bioavailable (Xue et al., 2011).

Due to their pro-inflammatory nature, IL-1β and TNFα actions have been widely studied in many inflammatory conditions and their blockade resulted in improvements in T2DM-related conditions such as chronic kidney disease (Lei et al., 2019) and pancreatic islet inflammation (Zha et al., 2016). However, it remains unsure whether targeting these cytokines affects leukocyte infiltration in peripheral tissues in T2DM. Circulating levels of IL-1β are higher in T2DM (Reinehr et al., 2016) and macrophages are the primary source of IL-1β in obesity-induced inflammation (Gao et al., 2014). Administration of LY2189102, a neutralizing IL-1β antibody, in patients with T2DM, improved glycemic control and demonstrated significant anti-inflammatory effects by lowering circulating IL-6 levels when compared with placebo treatment (Sloan-Lancaster et al., 2013). Moreover, islets from IL-1β-deficient mice exposed to high glucose *in vitro*, produced lower IL-6 and chemokines compared to wild-type islets (Ehses et al., 2009). Thus, efficacy of IL-1β blocking on inflammatory biomarkers and glycemic control raises the possibility of its use as a treatment in T2DM and other inflammatory conditions.

Although, anti-TNFα is an approved medication for some patients with rheumatoid arthritis (Katsumata et al., 2019), the evidence showing the efficacy of this drug in T2DM is conflicting. Indeed, anti-TNFα treatment using recombinant soluble TNFα receptor-immuno-globulin G increased insulin sensitivity in obese rodents (Hotamisligil et al., 1993). However, treatment with Ro 45-2081, a TNFα antagonist (recombinant fusion protein that consists of the soluble TNF-receptor linked to the Fc portion of human IgG1), had no effect on blood glucose levels and insulin-mediated glucose uptake in patients with T2DM (Paquot et al., 2000). The inefficiency of Ro 45-2081 to control blood glucose in patients with T2DM suggest that in addition its endocrine action, TNFα may also act through an autocrine or paracrine route. Consequently, sole neutralization of circulating TNFα might not be enough to observe improvements on insulin action (Paquot et al., 2000).

Anti-integrins

The importance of integrins in leukocyte adhesion and arresting clearly offer potential therapeutical avenues in relapsing inflammatory conditions such as rheumatoid arthritis (von Andrian and Engelhardt, 2003) and multiple sclerosis (Chaudhuri and Behan, 2003). Neutralization of integrins has been tested in murine experimental models of diabetes, particularly for the treatment and/or prevention of diabetic complications (Barouch et al., 2000; Iliaki et al., 2009; Miyachi et al., 2017). However, data showing efficacy in human clinical trials is not available in the context of T2DM.

The expression of CD11a, CD11b, and CD18 integrins is increased on the surface of neutrophils from STZ-induced diabetic rats (Barouch et al., 2000). This increase was associated with an enhanced adhesion of diabetic neutrophils to rat EC monolayers *in vitro*. Pre-treatment of leukocytes with either anti-CD11b or anti-CD18 antibodies significantly lowered the proportion of adherent diabetic neutrophils (Barouch et al., 2000). In the same study, systemic administration of anti-CD18 $F(ab')_2$ fragments to STZ-induced diabetic rats significantly decreased diabetic retinal leukostasis (Barouch et al., 2000). Furthermore, intraperitoneal administration of anti-α_4 integrin neutralizing antibody to a STZ-induced rat model of diabetes retinopathy attenuated leukocyte adhesion to the retina and suppressed TNFα expression and NF-κB activity in the retina

of treated rats (Iliaki et al., 2009). Notably, blockage of VLA-4 using a neutralizing antibody in HFD-fed mice attenuated myeloid cell accumulation in the liver and improved systemic glucose tolerance in treated mice compared to control HFD-fed mice (Miyachi et al., 2017). Overall, all these animal data identify the integrins as functional adhesive molecule in diabetic complications and provides a potential target for the prevention and/or treatment of the disease.

A humanized anti–VLA-4 monoclonal antibody (Natalizumab) was clinically beneficial in the treatment of Crohn's disease (Lew and Stoffel, 2003), and rheumatoid arthritis (von Andrian and Engelhardt, 2003). However, VLA-4 also has key regulatory roles in immune response, including the formation of the immune synapse (Mittelbrunn et al., 2004) and the differentiation of Th1 T-cells (Isobe et al., 1997). Obviously, the long-term administration of anti-integrins drugs to patients with inflammatory conditions may have undesirable or unexpected effects and more studies are required to fully understand their action.

Fatty Acids

Long-chain *n*–3 polyunsaturated fatty acid (*n*–3 PUFA) such as eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA), found in marine fish oils, are able to down-regulate the activity of NF-kB directly and therefore reduce the production of inflammatory cytokines (Xu et al., 2001). *n*–3 PUFA can also control leukocyte recruitment during inflammation and display immunomodulatory properties (Yates et al., 2011). Inclusion of *n*–3 PUFA in HFD prevented macrophage infiltration into the adipose tissue of *db/db* mice (Todoric et al., 2006). Interestingly, *n*–3 PUFA efficiently prevented the HFD-induced downregulation of adiponectin circulating levels normally observed in *db/db* (Todoric et al., 2006). These data therefore suggest that the beneficial effects of *n*–3 PUFA on diabetes could be mediated by their effect on adipose tissue inflammation, which could in turn contribute to improving insulin sensitivity (Oliver et al., 2010). However, much of the human evidence examining the beneficial effects of n–3 PUFA in patients with T2DM have limited follow-up periods (Vessby et al., 2001). As a result, it is unclear whether *n*–3 PUFA supplementation has long lasting beneficial effects and the exact mechanisms of *n*–3 PUFA action and how they modulate the adipose tissue, macrophages and even T-cells remain unknown.

Insulin

Several studies suggest a direct anti-inflammatory action of insulin irrespectively of its glucose modulating capacity as mentioned here in previous sections (Hyun et al., 2011). Insulin was found to attenuates the activity of NF-κB and MCP-1 on human aortic ECs *in vitro* (Aljada et al., 2001). Insulin infusion in individuals with obesity reduced plasma sICAM-1 and CCL2 levels (Dandona et al., 2001). In addition, treatment of human monocytes with insulin *in vitro* promoted the secretion of IL-8 (CXCL8), a potent chemoattractant for neutrophils (Dandona et al., 2001). All together, these findings suggest a possible regulatory effect of insulin on leukocyte trafficking via regulating chemokine and cytokine secretion as well as modulating adhesion molecules shedding. However, more studies are required to clarify the effect of insulin on leukocyte migration by including *in vitro* models taking into account the high glucose levels observed in some patients with T2DM, their associated complications and BMI to accurately determine the mechanism of insulin action and lift the controversy in this field.

Adiponectin and PEPITEM

Since obesity is a key feature in T2DM, studies demonstrate that changes in adipose tissue induce dysregulation in adipokines such as adiponectin and receptors involved in lipid metabolism such as Proliferator–activated receptor γ (PPAR-γ) (Bermudez et al., 2010). Adiponectin is well characterized as an insulin-sensitizing hormone as well as an anti-inflammatory adipokine (Ruan and Dong, 2016). Circulating levels of adiponectin are decreased in patients with obesity and T2DM (Hotta et al., 2000; Engeli et al., 2003), suggesting that dysregulation of adiponectin may be relevant to obesity-linked endothelial dysfunction in these individuals (Cho et al., 2002; Katsiki et al., 2017). In addition, adiponectin is also a regulator of adhesion molecules on ECs (Ouchi et al., 1999). Treating ECs with adiponectin inhibited the expression of adhesion molecules such as VCAM-1, E-selectin, and ICAM-1 *in vitro* (Ouchi et al., 1999) and *in vivo* (Ouedraogo et al., 2007). We also know that adiponectin is able to regulate T-cell trafficking during inflammation in a novel pathway characterized in our laboratory a few years ago (Chimen et al., 2015). In this novel pathway, adiponectin induces the release of the novel PEPtide Inhibitor of Trans-endothelial Migration (PEPITEM) by B-cells via signaling through the adiponectin receptors (AdipoR1/2). PEPITEM induces the release of sphingosine-1-phosphate (S1P) by ECs via binding with endothelial cadherin-15 (CDH15). In turn, S1P inhibits T-cell transmigration by preventing integrin activation (Chimen et al., 2015). The PEPITEM/Adiponectin pathway is dysregulated in inflammatory conditions such as Type 1 Diabetes Melitus, Rheumatoid Arthritis and in older adults. Dysregulation of this pathway is caused by a lack of AdipoR1/2 expression on B-cells in these patient groups leading to insufficient secretion of PEPITEM and consequently allowing aberrant T-cell trafficking. PEPITEM shows great therapeutic potential since it is an endogenous peptide and developing strategies to restore this pathway could help regain control on chronic inflammation. However, more work is needed to understand the full profile of T-cell subsets recruitment in adipose and pancreatic tissues in T2DM as this would allow to characterize whether PEPITEM could be used as a therapeutic avenue in T2DM.

PPAR Agonists

Proliferator–activated receptor-γ is a member of the PPAR family of nuclear receptors (Braissant et al., 1996) and its activation is associated with the induction of glucoregulatory molecules and enhanced insulin sensitivity (Olefsky, 2000). Pioglitazone is a PPARγ agonist that enhances the action of insulin mainly by promoting glucose utilization in peripheral tissues (Yki-Järvinen, 2004). Treatment of patients with T2DM with pioglitazone rapidly reduced systemic inflammation in those patients who were also receiving angiotensin II receptor blockers. This trial

also showed decreased CRP levels accompanied by a reduction in sICAM-1 and sVCAM. These observations uncovered anti-atherogenic effects of the PPAR-γ agonist which may also contribute to a reduction of cardiovascular events in patients at risk such as those with T2DM (Takase et al., 2007).

Current drugs such as IL-1β blockers and thiazolidinediones targeting the metabolic side of T2DM and aiming to restore glucose tolerance have shown to also have anti-inflammatory effects (Yki-Järvinen, 2004; Sloan-Lancaster et al., 2013). However, the evidence showing a potential effect of these drugs on leukocyte recruitment as a way to control inflammation are limited and this area needs further investigations. Blocking cytokines such as TNF- α may reduce inflammation but also renders the host susceptible to infection by silencing the danger signals, which are necessary for adequate immune cell activation (Rider et al., 2016) and maybe even to cancer (reviewed in Dinarello, 2005). On the other hand, anti-cytokine therapy has little or no organ toxicity or gastrointestinal disturbances and so is well tolerated. Therefore, new site-restricted biologics which block inflammatory cytokines only at sites of inflammation are needed (Rider et al., 2016). In addition, it seems that targeting inflammation in T2DM and obesity is promising but still only show partial reduction of disease. This could be explained by the fact that diabetes and obesity-mediated inflammation involve multiple mechanisms and are not necessarily always linked to hyperglycemia. This clearly highlights the need to identify how anti-inflammatory treatments modulate glucose tolerance and the complications associated with T2DM, but also an urgent necessity to understand how inflammation is dysregulated in T2DM and in obesity. More work is clearly required to fully characterize the mechanisms behind the changes in leukocyte phenotype and in local environments which directly influence leukocyte migration. Finally, more specialistic studies from leukocyte trafficking groups are needed to entirely determine the changes in leukocyte migratory capacity and behaviors *in vitro* and *in vivo* to complete the findings showing changes in expression profiles such as those of adhesion molecules.

AUTHOR CONTRIBUTIONS

LP wrote the first draft of the manuscript. MC and AT contributed to the manuscript revision, read and approved the submitted version. All authors contributed to the article and approved the submitted version.

FUNDING

LP was supported by a Rosetrees Trust award (M814) and MC was supported by a Royal Society Dorothy Hodgkin Research fellowship (DH160044).

REFERENCES

Abe, Y., El-Masri, B., Kimball, K. T., Pownall, H., Reilly, C. F., Osmundsen, K., et al. (1998). Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion. *Arterioscler. Thromb. Vasc. Biol.* 18, 723–731. doi: 10.1161/01.atv.18.5.723

Ahmed, R. H., Huri, H. Z., Muniandy, S., Al-Hamodi, Z., Al-absi, B., Alsalahi, A., et al. (2017). Altered circulating concentrations of active glucagon-like peptide (GLP-1) and dipeptidyl peptidase 4 (DPP4) in obese subjects and their association with insulin resistance. *Clin. Biochem.* 50, 746–749. doi: 10.1016/j.clinbiochem.2017.03.008

Ahmed, S. R., McGettrick, H. M., Yates, C. M., Buckley, C. D., Ratcliffe, M. J., Nash, G. B., et al. (2011). Prostaglandin D2 regulates CD4+ memory T cell trafficking across blood vascular endothelium and primes these cells for clearance across lymphatic endothelium. *J. Immunol.* 187, 1432–1439. doi: 10.4049/jimmunol.1100299

Aird, W. C. (2007). Phenotypic heterogeneity of the endothelium: I. Structure function, and mechanisms. *Circul. Res.* 100, 158–173. doi: 10.1161/01.res.0000255691.76142.4a

Aljada, A., Ghanim, H., Saadeh, R., and Dandona, P. (2001). Insulin inhibits NFκB and MCP-1 expression in human aortic endothelial cells. *J. Clin. Endocrinol. Metab.* 86, 450–453. doi: 10.1210/jc.86.1.450

Aljada, A., Saadeh, R., Assian, E., Ghanim, H., and Dandona, P. (2000). Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothelial cells through stimulation of nitric oxide. *J. Clin. Endocrinol. Metab.* 85, 2572–2575. doi: 10.1210/jc.85.7.2572

Andreasen, A. S., Pedersen-Skovsgaard, T., Berg, R. M., Svendsen, K. D., Feldt-Rasmussen, B., and Pedersen, B. K. (2010). Type 2 diabetes mellitus is associated with impaired cytokine response and adhesion molecule expression in human endotoxemia. *Intensive Care Med.* 36, 1548–1555. doi: 10.1007/s00134-010-1845-1

Asghar, A., and Sheikh, N. (2017). Role of immune cells in obesity induced low grade inflammation and insulin resistance. *Cell Immunol.* 315, 18–26. doi: 10.1016/j.cellimm.2017.03.001

Augustyns, K., Bal, G., Thonus, G., Belyaev, A., Zhang, X., Bollaert, W., et al. (2010). ChemInform abstract: the unique properties of dipeptidyl-peptidase IV (DPP IV/CD26) and the therapeutic potential of DPP IV inhibitors". *ChemInform* 30,

Barouch, F. C., Miyamoto, K., Allport, J. R., Fujita, K., Bursell, S. E., Aiello, L. P., et al. (2000). Integrin-mediated neutrophil adhesion and retinal leukostasis in diabetes. *Invest. Ophthalmol. Vis. Sci.* 41, 1153–1158.

Basta, G., Del, S. T., and De, R. C. (2004). Advanced glycation endproducts: implications for accelerated atherosclerosis in diabetes. *Recenti Prog. Med.* 95, 67–80.

Bengoechea, J. A., and Sa Pessoa, J. (2019). *Klebsiella pneumoniae* infection biology: living to counteract host defences. *FEMS Microbiol. Rev.* 43, 123–144. doi: 10.1093/femsre/fuy043

Berbudi, A., Rahmadika, N., Tjahjadi, A. I., and Ruslami, R. (2020). Type 2 diabetes and its impact on the immune system. *Curr. Diabetes Rev.* 16:442. doi: 10.2174/1573399815666191024085838

Bermudez, V., Finol, F., Parra, N., Parra, M., Pérez, A., Penaranda, L., et al. (2010). PPAR-γ agonists and their role in type 2 diabetes mellitus management. *Am. J. Ther.* 17, 274–283. doi: 10.1097/mjt.0b013e3181c08081

Bertola, A., Ciucci, T., Rousseau, D., Bourlier, V., Duffaut, C., Bonnafous, S., et al. (2012). Identification of adipose tissue dendritic cells correlated with obesity-associated insulin-resistance and inducing Th17 responses in mice and patients. *Diabetes* 61, 2238–2247. doi: 10.2337/db11-1274

Biswas, S. K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* 11, 889–896. doi: 10.1038/ni.1937

Bixel, M. G., Li, H., Petri, B., Khandoga, A. G., Khandoga, A., Zarbock, A., et al. (2010). CD99 and CD99L2 act at the same site as, but independently of, PECAM-1 during leukocyte diapedesis. *Blood* 116, 1172–1184. doi: 10.1182/blood-2009-12-256388

Boels, M. G., Koudijs, A., Avramut, M. C., Sol, W. M., Wang, G., van Oeveren-Rietdijk, A. M., et al. (2017). Systemic monocyte chemotactic protein-1 inhibition modifies renal macrophages and restores glomerular endothelial

glycocalyx and barrier function in diabetic nephropathy. *Am. J. Pathol.* 187, 2430–2440. doi: 10.1016/j.ajpath.2017.07.020

Bono, P., Jalkanen, S., and Salmi, M. (1999). Mouse vascular adhesion protein 1 is a sialoglycoprotein with enzymatic activity and is induced in diabetic insulitis. *Am. J. Pathol.* 155, 1613–1624. doi: 10.1016/s0002-9440(10)65477-6

Braissant, O., Foufelle, F., Scotto, C., Dauça, M., and Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha,-beta, and-gamma in the adult rat. *Endocrinology* 137, 354–366. doi: 10.1210/endo.137.1.8536636

Brake, D. K., Smith, E. O. B., Mersmann, H., Smith, C. W., and Robker, R. L. (2006). ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice. *Am. J. Physiol. Cell Physiol.* 291, C1232–C1239.

Broxmeyer, H. E., Capitano, M., Campbell, T. B., Hangoc, G., and Cooper, S. (2016). Modulation of hematopoietic chemokine effects in vitro and in vivo by DPP-4/CD26. *Stem Cells Dev.* 25, 575–585. doi: 10.1089/scd.2016.0026

Buraczynska, M., Zukowski, P., Wacinski, P., Berger-Smyka, B., Dragan, M., and Mezosi, S. (2012). Chemotactic cytokine receptor 5 gene polymorphism: relevance to microvascular complications in type 2 diabetes. *Cytokine* 58, 213–217. doi: 10.1016/j.cyto.2012.01.007

Calabrese, E. J., and Baldwin, L. A. (2001). U-shaped dose-responses in biology, toxicology, and public health. *Annu. Rev. Public Health* 22, 15–33. doi: 10.1146/annurev.publhealth.22.1.15

Calle, M., and Fernandez, M. (2012). Inflammation and type 2 diabetes. *Diabetes Metab.* 38, 183–191. doi: 10.1016/j.diabet.2011.11.006

Carlos, T. M., and Harlan, J. M. (1994). Leukocyte-endothelial adhesion molecules. *Blood* 84, 2068–2101. doi: 10.1182/blood.v84.7.2068.bloodjournal8472068

Carman, C. V., and Springer, T. A. (2004). A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J. Cell Biol.* 167, 377–388. doi: 10.1083/jcb.200404129

Carpéné, C., Boulet, N., Chaplin, A., and Mercader, J. (2019). Past, present and future anti-obesity effects of flavin-containing and/or copper-containing amine oxidase inhibitors. *Medicines* 6:9. doi: 10.3390/medicines6010009

Chan, M. E., Adler, B. J., Green, D. E., and Rubin, C. T. (2012). Bone structure and B-cell populations, crippled by obesity, are partially rescued by brief daily exposure to low-magnitude mechanical signals. *FASEB J.* 26, 4855–4863. doi: 10.1096/fj.12-209841

Chaudhuri, A., and Behan, P. O. (2003). Natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.* 348:1598. doi: 10.1056/nejm200304173481614

Chen, Y., Tian, J., Tian, X., Tang, X., Rui, K., Tong, J., et al. (2014). Adipose tissue dendritic cells enhances inflammation by prompting the generation of Th17 cells. *PLoS One* 9:e92450. doi: 10.1371/journal.pone.0092450

Chimen, M., McGettrick, H. M., Apta, B., Kuravi, S. J., Yates, C. M., Kennedy, A., et al. (2015). Homeostatic regulation of T cell trafficking by a B cell–derived peptide is impaired in autoimmune and chronic inflammatory disease. *Nat. Med.* 21:467. doi: 10.1038/nm.3842

Cho, E., Rimm, E. B., Stampfer, M. J., Willett, W. C., and Hu, F. B. (2002). The impact of diabetes mellitus and prior myocardial infarction on mortality from all causes and from coronary heart disease in men. *J. Am. Coll. Cardiol.* 40, 954–960. doi: 10.1016/s0735-1097(02)02044-2

Chow, F., Nikolic-Paterson, D. J., Ma, F., Ozols, E., Rollins, B., and Tesch, G. H. (2007). Monocyte chemoattractant protein-1-induced tissue inflammation is critical for the development of renal injury but not type 2 diabetes in obese db/db mice. *Diabetologia* 50, 471–480. doi: 10.1007/s00125-006-0497-8

Cook-Mills, J. M., Marchese, M. E., and Abdala-Valencia, H. (2011). Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. *Antioxid. Redox Signal.* 15, 1607–1638. doi: 10.1089/ars.2010.3522

Crane, M. J., Hokeness-Antonelli, K. L., and Salazar-Mather, T. P. (2009). Regulation of inflammatory monocyte/macrophage recruitment from the bone marrow during murine cytomegalovirus infection: role for type I interferons in localized induction of CCR2 ligands. *J. Immunol.* 183, 2810–2817. doi: 10.4049/jimmunol.0900205

Dandona, P., Aljada, A., Mohanty, P., Ghanim, H., Hamouda, W., Assian, E., et al. (2001). Insulin inhibits intranuclear nuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect?". *J. Clin. Endocrinol. Metab.* 86, 3257–3265. doi: 10.1210/jc.86.7.3257

de Vries, M. A., Alipour, A., Klop, B., van de Geijn, G. J. M., Janssen, H. W., Njo, T. L., et al. (2015). Glucose-dependent leukocyte activation in patients with type 2 diabetes mellitus, familial combined hyperlipidemia and healthy controls. *Metabolism* 64, 213–217. doi: 10.1016/j.metabol.2014.10.011

Deacon, C. F. (2018). Peptide degradation and the role of DPP-4 inhibitors in the treatment of type 2 diabetes. *Peptides* 100, 150–157. doi: 10.1016/j.peptides.2017.10.011

DeFuria, J., Belkina, A. C., Jagannathan-Bogdan, M., Snyder-Cappione, J., Carr, J. D., Nersesova, Y. R., et al. (2013). B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5133–5138. doi: 10.1073/pnas.1215840110

Dejana, E., and Giampietro, C. (2012). Vascular endothelial-cadherin and vascular stability. *Curr. Opin. Hematol.* 19, 218–223. doi: 10.1097/moh.0b013e3283523e1c

Dejana, E., Orsenigo, F., Molendini, C., Baluk, P., and McDonald, D. M. (2009). Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees. *Cell Tissue Res.* 335, 17–25. doi: 10.1007/s00441-008-0694-5

Deng, T., Liu, J., Deng, Y., Minze, L., Xiao, X., Wright, V., et al. (2017). Adipocyte adaptive immunity mediates diet-induced adipose inflammation and insulin resistance by decreasing adipose Treg cells. *Nat. Commun.* 8:15725.

Dimayuga, F. O., Wang, C., Clark, J. M., Dimayuga, E. R., Dimayuga, V. M., and Bruce-Keller, A. J. (2007). SOD1 overexpression alters ROS production and reduces neurotoxic inflammatory signaling in microglial cells. *J. Neuroimmunol.* 182, 89–99. doi: 10.1016/j.jneuroim.2006.10.003

Dinarello, C. A. (2005). Differences between anti-tumor necrosis factor-alpha monoclonal antibodies and soluble TNF receptors in host defense impairment. *J. Rheumatol. Suppl.* 74, 40–47.

Donath, M. Y., and Shoelson, S. E. (2011). Type 2 diabetes as an inflammatory disease. *Nat. Rev. Immunol.* 11, 98–107.

Drexler, H., Kurz, S., Jeserich, M., Münzel, T., and Hornig, B. (1995). Effect of chronic angiotensin-converting enzyme inhibition on endothelial function in patients with chronic heart failure. *Am. J. Cardiol.* 76, 13E–18E.

Duffaut, C., Galitzky, J., Lafontan, M., and Bouloumié, A. (2009). Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. *Biochem. Biophys. Res. Commun.* 384, 482–485. doi: 10.1016/j.bbrc.2009.05.002

Dvorak, A. M., and Feng, D. (2001). The vesiculo–vacuolar organelle (VVO): a new endothelial cell permeability organelle. *J. Histochem. Cytochem.* 49, 419–431. doi: 10.1177/002215540104900401

Eguchi, K., and Nagai, R. (2017). Islet inflammation in type 2 diabetes and physiology. *J. Clin. Invest.* 127, 14–23. doi: 10.1172/jci88877

Ehses, J., Lacraz, G., Giroix, M.-H., Schmidlin, F., Coulaud, J., Kassis, N., et al. (2009). IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13998–14003. doi: 10.1073/pnas.0810087106

Elgazar-Carmon, V., Rudich, A., Hadad, N., and Levy, R. (2008). Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *J. Lipid Res.* 49, 1894–1903. doi: 10.1194/jlr.m800132-jlr200

Engeli, S., Feldpausch, M., Gorzelniak, K., Hartwig, F., Heintze, U., Janke, J., et al. (2003). Association between adiponectin and mediators of inflammation in obese women. *Diabetes* 52, 942–947. doi: 10.2337/diabetes.52.4.942

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A. J., and Paquot, N. (2014). Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes. Res. Clin. Pract.* 105, 141–150. doi: 10.1016/j.diabres.2014.04.006

Fan, Z., and Ley, K. (2015). Leukocyte arrest: biomechanics and molecular mechanisms of β2 integrin activation. *Biorheology* 52, 353–377. doi: 10.3233/bir-15085

Farhangkhoee, H., Khan, Z. A., Kaur, H., Xin, X., Chen, S., and Chakrabarti, S. (2006). Vascular endothelial dysfunction in diabetic cardiomyopathy: pathogenesis and potential treatment targets. *Pharmacol. Ther.* 111, 384–399. doi: 10.1016/j.pharmthera.2005.10.008

Feuerer, M., Herrero, L., Cipolletta, D., Naaz, A., Wong, J., Nayer, A., et al. (2009). Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* 15, 930–939. doi: 10.1038/nm.2002

Foot, J. S., Yow, T. T., Schilter, H., Buson, A., Deodhar, M., Findlay, A. D., et al. (2013). PXS-4681A, a potent and selective mechanism-based inhibitor of SSAO/VAP-1 with anti-inflammatory effects in vivo. *J. Pharmacol. Exp. Ther.* 347, 365–374. doi: 10.1124/jpet.113.207613

Galkina, E., and Ley, K. (2007). Vascular adhesion molecules in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 27, 2292–2301. doi: 10.1161/atvbaha.107.149179

Gao, D., Madi, M., Ding, C., Fok, M., Steele, T., Ford, C., et al. (2014). Interleukin-1β mediates macrophage-induced impairment of insulin signaling in human primary adipocytes. *Am. J. Physiol. Endocrinol. Metab.* 307, E289–E304.

Giagulli, C., Ottoboni, L., Caveggion, E., Rossi, B., Lowell, C., Constantin, G., et al. (2006). The Src family kinases Hck and Fgr are dispensable for inside-out, chemoattractant-induced signaling regulating β2 integrin affinity and valency in neutrophils, but are required for β2 integrin-mediated outside-in signaling involved in sustained adhesion. *J. Immunol.* 177, 604–611. doi: 10.4049/jimmunol.177.1.604

Giagulli, C., Scarpini, E., Ottoboni, L., Narumiya, S., Butcher, E. C., Constantin, G., et al. (2004). RhoA and ζ PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte in vivo homing. *Immunity* 20, 25–35. doi: 10.1016/s1074-7613(03)00350-9

Gilliet, M., Cao, W., and Liu, Y. J. (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat. Rev. Immunol.* 8, 594–606. doi: 10.1038/nri2358

Gu, H. F., Ma, J., Gu, K. T., and Brismar, K. (2013). Association of intercellular adhesion molecule 1 (ICAM1) with diabetes and diabetic nephropathy. *Front. Endocrinol.* 3:179. doi: 10.3389/fendo.2012.00179

Guo, X., Li, J., Tang, R., Zhang, G., Zeng, H., Wood, R. J., et al. (2017). High fat diet alters gut microbiota and the expression of paneth cell-antimicrobial peptides preceding changes of circulating inflammatory cytokines. *Mediators Inflamm.* 2017:9474896.

Gustavsson, C., Agardh, C.-D., Zetterqvist, A. V., Nilsson, J., Agardh, E., and Gomez, M. F. (2010). Vascular cellular adhesion molecule-1 (VCAM-1) expression in mice retinal vessels is affected by both hyperglycemia and hyperlipidemia. *PLoS One* 5:e12699. doi: 10.1371/journal.pone.0012699

Hadi, H. A., and Al Suwaidi, J. (2007). Endothelial dysfunction in diabetes mellitus. *Vasc. Health Risk Manag.* 3, 853–876.

Harjunpää, H., Llort Asens, M., Guenther, C., and Fagerholm, S. C. (2019). Cell adhesion molecules and their roles and regulation in the immune and tumor microenvironment. *Front. Immunol.* 10:1078. doi: 10.3389/fimmu.2019.01078

Herder, C., Haastert, B., Müller-Scholze, S., Koenig, W., Thorand, B., Holle, R., et al. (2005). Association of systemic chemokine concentrations with impaired glucose tolerance and type 2 diabetes: results from the cooperative health research in the region of augsburg survey S4 (KORA S4). *Diabetes* 54(Suppl. 2), S11–S17.

Hocaoglu-Emre, F. S., Saribal, D., Yenmis, G., and Guvenen, G. (2017). Vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and cluster of differentiation 146 levels in patients with Type 2 diabetes with complications. *Endocrinol. Metab.* 32, 99–105. doi: 10.3803/enm.2017.32.1.99

Hostetter, M. K. (1990). Handicaps to host defense: effects of hyperglycemia on C3 and *Candida albicans*. *Diabetes* 39, 271–275. doi: 10.2337/diabetes.39.3.271

Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259, 87–91. doi: 10.1126/science.7678183

Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., et al. (2000). Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler. Thromb. Vasc. Biol.* 20, 1595–1599. doi: 10.1161/01.atv.20.6.1595

Huang, J., Xiao, Y., Zheng, P., Zhou, W., Wang, Y., Huang, G., et al. (2019). Distinct neutrophil counts and functions in newly diagnosed type 1 diabetes, latent autoimmune diabetes in adults, and type 2 diabetes. *Diabetes Metab. Res. Rev.* 35:e3064. doi: 10.1002/dmrr.3064

Huh, J. H., Kim, H. M., Lee, E. S., Kwon, M. H., Lee, B. R., Ko, H. J., et al. (2018). Dual CCR2/5 antagonist attenuates obesity-induced insulin resistance by regulating macrophage recruitment and M1/M2 status. *Obesity* 26, 378–386. doi: 10.1002/oby.22103

Huo, Y., and Ley, K. (2001). Adhesion molecules and atherogenesis. *Acta Physiol. Scand.* 173, 35–43. doi: 10.1046/j.1365-201x.2001.00882.x

Hyduk, S. J., Chan, J. R., Duffy, S. T., Chen, M., Peterson, M. D., Waddell, T. K., et al. (2007). Phospholipase C, calcium, and calmodulin are critical for α4β1 integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. *Blood* 109, 176–184. doi: 10.1182/blood-2006-01-029199

Hyun, E., Ramachandran, R., Hollenberg, M. D., and Vergnolle, N. (2011). Mechanisms behind the anti-inflammatory actions of insulin. *Crit. Rev. Immunol.* 31, 307–340. doi: 10.1615/critrevimmunol.v31.i4.30

Ilan, Y., Maron, R., Tukpah, A.-M., Maioli, T. U., Murugaiyan, G., Yang, K., et al. (2010). Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9765–9770. doi: 10.1073/pnas.0908771107

Iliaki, E., Poulaki, V., Mitsiades, N., Mitsiades, C. S., Miller, J. W., and Gragoudas, E. S. (2009). Role of α4 integrin (CD49d) in the pathogenesis of diabetic retinopathy. *Invest. Ophthalmol. Vis. Sci.* 50, 4898–4904. doi: 10.1167/iovs.08-2013

Isobe, M., Suzuki, J.-I., Yamazaki, S., Yazaki, Y., Horie, S., Okubo, Y., et al. (1997). Regulation by differential development of Th1 and Th2 cells in peripheral tolerance to cardiac allograft induced by blocking ICAM-1/LFA-1 adhesion. *Circulation* 96, 2247–2253. doi: 10.1161/01.cir.96.7.2247

Janeway, C. A. Jr., Travers, P., Walport, M., and Shlomchik, M. J. (2001). *The Mucosal Immune System. Immunobiology: The Immune System in Health and Disease*, 5th Edn. New York, NY: Garland Science.

Jargaud, V., Bour, S., Tercé, F., Collet, X., Valet, P., Bouloumié, A., et al. (2020). Obesity of mice lacking VAP-1/SSAO by Aoc3 gene deletion is reproduced in mice expressing a mutated vascular adhesion protein-1 (VAP-1) devoid of amine oxidase activity. *J. Physiol. Biochem.* doi: 10.1007/s13105-020-00756-y Online ahead of print

Javid, A., Zlotnikov, N., Pětrošová, H., Tang, T. T., Zhang, Y., Bansal, A. K., et al. (2016). Hyperglycemia impairs neutrophil-mediated bacterial clearance in mice infected with the Lyme disease pathogen. *PLoS One* 11:e0158019. doi: 10.1371/journal.pone.0158019

Kansas, G. S. (1996). Selectins and their ligands: current concepts and controversies. *Blood* 88, 3259–3287. doi: 10.1182/blood.v88.9.3259.bloodjournal8893259

Katsiki, N., Mantzoros, C., and Mikhailidis, D. P. (2017). Adiponectin, lipids and atherosclerosis. *Curr. Opin. Lipidol.* 28, 347–354. doi: 10.1097/mol.0000000000000431

Katsumata, K., Ishihara, J., Fukumaga, K., Ishihara, A., Yuba, E., Budina, E., et al. (2019). Conferring extracellular matrix affinity enhances local therapeutic efficacy of anti-TNF-α antibody in a murine model of rheumatoid arthritis. *Arthritis Res. Ther.* 21:298.

Kawanishi, N., Yano, H., Yokogawa, Y., and Suzuki, K. (2010). 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.* 16, 105–118.

Khalfaoui, T., Lizard, G., and Ouertani-Meddeb, A. (2008). Adhesion molecules (ICAM-1 and VCAM-1) and diabetic retinopathy in type 2 diabetes. *J. Mol. Histol.* 39, 243–249. doi: 10.1007/s10735-007-9159-5

Kim, M., Carman, C. V., and Springer, T. A. (2003). Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* 301, 1720–1725. doi: 10.1126/science.1084174

Kitade, H., Sawamoto, K., Nagashimada, M., Inoue, H., Yamamoto, Y., Sai, Y., et al. (2012). CCR5 plays a critical role in obesity-induced adipose tissue inflammation and insulin resistance by regulating both macrophage recruitment and M1/M2 status. *Diabetes* 61, 1680–1690. doi: 10.2337/db11-1506

Kizilgul, M., Sencar, E., Bekir, U., Beysel, S., Ozcelik, O., Ozbek, M., et al. (2018). Components of the complete blood count in type 2 diabetes mellitus with inadequate glycemic control. *Dicle Tip Dergisi* 45, 113–120.

Kulcsar, K. A., Coleman, C. M., Beck, S. E., and Frieman, M. B. (2019). Comorbid diabetes results in immune dysregulation and enhanced disease severity following MERS-CoV infection. *JCI Insight* 4:e131774.

Kumar, M., Roe, K., Nerurkar, P. V., Orillo, B., Thompson, K. S., Verma, S., et al. (2014). Reduced immune cell infiltration and increased pro-inflammatory mediators in the brain of Type 2 diabetic mouse model infected with West Nile virus. *J. Neuroinflammation* 11:80. doi: 10.1186/1742-2094-11-80

Kummer, D., and Ebnet, K. (2018). Junctional adhesion molecules (JAMs): the JAM-integrin connection. *Cells* 7:25. doi: 10.3390/cells7040025

Kuo, C.-H., Wei, J.-N., Yang, C.-Y., Ou, H.-Y., Wu, H.-T., Fan, K.-C., et al. (2019). Serum vascular adhesion protein-1 is up-regulated in hyperglycemia

and is associated with incident diabetes negatively. *Int. J. Obes.* 43, 512–522. doi: 10.1038/s41366-018-0172-4

Kuwano, Y., Spelten, O., Zhang, H., Ley, K., and Zarbock, A. (2010). Rolling on E-or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils. *Blood* 116, 617–624. doi: 10.1182/blood-2010-01-266122

Lambeir, A.-M., Durinx, C., Scharpé, S., and De Meester, I. (2003). Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Crit. Rev. Clin. Lab. Sci.* 40, 209–294. doi: 10.1080/713609354

Larsen, C. M., Faulenbach, M., Vaag, A., Vølund, A., Ehses, J. A., Seifert, B., et al. (2007). Interleukin-1–receptor antagonist in type 2 diabetes mellitus. *N. Engl. J. Med.* 356, 1517–1526.

Lei, Y., Devarapu, S. K., Motrapu, M., Cohen, C., Lindenmeyer, M. T., Moll, S., et al. (2019). Interleukin-1β inhibition for chronic kidney disease in obese mice with type 2 diabetes. *Front. Immunol.* 10:1223. doi: 10.3389/fimmu.2019.01223

Lew, E. A., and Stoffel, E. M. (2003). Natalizumab for active Crohn's disease. *N. Engl. J. Med.* 348:1599.

Ley, K. (2007). *Adhesion Molecules: Function and Inhibition*. Berlin: Springer Science & Business Media.

Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–689. doi: 10.1038/nri2156

Li, H.-Y., Lin, H.-A., Nien, F.-J., Wu, V.-C., Jiang, Y.-D., Chang, T.-J., et al. (2016). Serum vascular adhesion protein-1 predicts end-stage renal disease in patients with type 2 diabetes. *PLoS One* 11:e0147981. doi: 10.1371/journal.pone.0147981

Lin, H.-F., Zhang, H.-J., Hu, Q.-X., Liu, X.-Y., Wang, Z.-Q., Fan, J.-Y., et al. (2012). Altered polarization, morphology, and impaired innate immunity germane to resident peritoneal macrophages in mice with long-term type 2 diabetes. *J. Biomed. Biotechnol.* 2012:867023.

Liu, Y., Lu, X., Li, X., Du, P., and Qin, G. (2020). High-fat diet triggers obesity-related early infiltration of macrophages into adipose tissue and transient reduction of blood monocyte count. *Mol. Immunol.* 117, 139–146.

Liu, Y., Shaw, S. K., Ma, S., Yang, L., Luscinskas, F. W., and Parkos, C. A. (2004). Regulation of leukocyte transmigration: cell surface interactions and signaling events. *J. Immunol.* 172, 7–13.

Lontchi-Yimagou, E., Sobngwi, E., Matsha, T. E., and Kengne, A. P. (2013). Diabetes mellitus and inflammation. *Curr. Diab. Rep.* 13, 435–444.

Luo, A., Leach, S. T., Barres, R., Hesson, L. B., Grimm, M. C., and Simar, D. (2017). The microbiota and epigenetic regulation of T helper 17/regulatory T cells: in search of a balanced immune system. *Front. Immunol.* 8:417. doi: 10.3389/fimmu.2017.00417

Lum, N. T., Rainger, G. E., and Nash, G. B. (2000). Differential ability of exogenous chemotactic agents to disrupt transendothelial migration of flowing neutrophils. *J. Immunol.* 164, 5961–5969.

Marco, M., Fortin, C., and Pulop, T. (2013). Membrane-type matrix metalloproteinases: key mediators of leukocyte function. *J. Leukoc. Biol.* 94, 237–246.

Mariño, E., and Grey, S. T. (2012). B cells as effectors and regulators of autoimmunity. *Autoimmunity* 45, 377–387.

Martinez, N., Ketheesan, N., Martens, G. W., West, K., Lien, E., and Kornfeld, H. (2016). Defects in early cell recruitment contribute to the increased susceptibility to respiratory *Klebsiella pneumoniae* infection in diabetic mice. *Microbes Infect.* 18, 649–655.

Martinez-Pomares, L., Reid, D. M., Brown, G. D., Taylor, P. R., Stillion, R. J., Linehan, S. A., et al. (2005). Analysis of mannose receptor regulation by IL-4, IL-10, and proteolytic processing using novel monoclonal antibodies. *J. Leukoc. Biol.* 73, 604–613.

Matsumoto, K., Sera, Y., Nakamura, H., Ueki, Y., and Miyake, S. (2002). Serum concentrations of soluble adhesion molecules are related to degree of hyperglycemia and insulin resistance in patients with type 2 diabetes mellitus. *Diabetes. Res. Clin. Pract.* 55, 131–138.

McEver, R. P. (2015). Selectins: initiators of leucocyte adhesion and signalling at the vascular wall. *Cardiovasc. Res.* 107, 331–339.

McLeod, D. S., Lefer, D. J., Merges, C., and Lutty, G. A. (1995). Enhanced expression of intracellular adhesion molecule-1 and P-selectin in the diabetic human retina and choroid. *Am. J. Pathol.* 147:642.

Meigs, J. B., Hu, F. B., Rifai, N., and Manson, J. E. (2004). Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 291, 1978–1986.

Mentlein, R. (1999). Dipeptidyl peptidase IV (CD26)-role in the inactivation of regulatory peptides. *Regul. Pept.* 85, 9–24.

Mittelbrunn, M., Molina, A., Escribese, M. M., Yáñez-Mó, M., Escudero, E., Ursa, Á, et al. (2004). VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11058–11063.

Miyachi, Y., Tsuchiya, K., Komiya, C., Shiba, K., Shimazu, N., Yamaguchi, S., et al. (2017). Roles for cell-cell adhesion and contact in obesity-induced hepatic myeloid cell accumulation and glucose intolerance. *Cell Rep.* 18, 2766–2779.

Miyasaka, M., and Tanaka, T. (2004). Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. *Nat. Rev. Immunol.* 4, 360–370.

Monickaraj, F., Oruganti, S. R., McGuire, P., and Das, A. (2020). A potential novel therapeutic target in diabetic retinopathy: a chemokine receptor (CCR2/CCR5) inhibitor reduces retinal vascular leakage in an animal model. *Graefes Arch. Clin. Exp. Ophthalmol.* 259, 93–100.

Mooradian, A. D., Reed, R. L., Meredith, K. E., and Scuderi, P. (1991). Serum levels of tumor necrosis factor and IL-1α and IL-1β in diabetic patients. *Diabetes Care* 14, 63–65.

Moradi, S., Kerman, S. R. J., Rohani, F., and Salari, F. (2012). Association between diabetes complications and leukocyte counts in Iranian patients. *J. Inflamm. Res.* 5, 7–11.

Moreno, J. A., Gomez-Guerrero, C., Mas, S., Sanz, A. B., Lorenzo, O., Ruiz-Ortega, M., et al. (2018). Targeting inflammation in diabetic nephropathy: a tale of hope. *Expert Opin. Investig. Drugs* 27, 917–930.

Mosser, D. M. (2003). The many faces of macrophage activation. *J. Leukoc. Biol.* 73, 209–212.

Mráz, M., Cinkajzlová, A., Kloučková, J., Lacinová, Z., Kratochvílová, H., Lipš, M., et al. (2019). Dendritic cells in subcutaneous and epicardial adipose tissue of subjects with type 2 diabetes, obesity, and coronary artery disease. *Mediators Inflamm.* 2019:5481725.

Mraz, M., and Haluzik, M. (2014). The role of adipose tissue immune cells in obesity and low-grade inflammation. *J. Endocrinol.* 222, R113–R127.

Muller, W. A. (2003). Leukocyte–endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol.* 24, 326–333.

Muller, W. A. (2011). Mechanisms of leukocyte transendothelial migration. *Annu. Rev. Pathol.* 6, 323–344.

Muller, W. A. (2013). Getting leukocytes to the site of inflammation. *Vet. Pathol.* 50, 7–22.

Mulvihill, E. E., and Drucker, D. J. (2014). Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. *Endocr. Rev.* 35, 992–1019.

Musilli, C., Paccosi, S., Pala, L., Gerlini, G., Ledda, F., Mugelli, A., et al. (2011). Characterization of circulating and monocyte-derived dendritic cells in obese and diabetic patients. *Mol. Immunol.* 49, 234–238.

Mutgan, A. C., Jandl, K., and Kwapiszewska, G. (2020). Endothelial basement membrane components and their products, matrikines: active drivers of pulmonary hypertension?". *Cells* 9:2029.

Newman, P. J., Berndt, M. C., Gorski, J., White, G. C., Lyman, S., Paddock, C., et al. (1990). PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* 247, 1219–1222.

Nguyen, D. V., Shaw, L., and Grant, M. (2012). Inflammation in the pathogenesis of microvascular complications in diabetes. *Front. Endocrinol.* 3:170. doi: 10.3389/fendo.2012.00170

Nishimura, S., Manabe, I., Nagasaki, M., Eto, K., Yamashita, H., Ohsugi, M., et al. (2009). CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* 15, 914–920.

Noshita, N., Sugawara, T., Hayashi, T., Lewén, A., Omar, G., and Chan, P. H. (2002). Copper/zinc superoxide dismutase attenuates neuronal cell death by preventing extracellular signal-regulated kinase activation after transient focal cerebral ischemia in mice. *J. Neurosci.* 22, 7923–7930.

Nunemaker, C. S., Chung, H. G., Verrilli, G. M., Corbin, K. L., Upadhye, A., and Sharma, P. R. (2014). Increased serum CXCL1 and CXCL5 are linked to obesity, hyperglycemia, and impaired islet function. *J. Endocrinol.* 222, 267–276.

O'Keeffe, M., Hochrein, H., Vremec, D., Caminschi, I., Miller, J. L., Anders, E. M., et al. (2002). Mouse plasmacytoid cells: long-lived cells, heterogeneous

in surface phenotype and function, that differentiate into CD8+ dendritic cells only after microbial stimulus. *J. Exp. Med.* 196, 1307–1319.

Okouchi, M., Okayama, N., Imai, S., Omi, H., Shimizu, M., Fukutomi, T., et al. (2002). High insulin enhances neutrophil transendothelial migration through increasing surface expression of platelet endothelial cell adhesion molecule-1 via activation of mitogen activated protein kinase. *Diabetologia* 45, 1449–1456.

Olefsky, J. M. (2000). Treatment of insulin resistance with peroxisome proliferator–activated receptor γ agonists. *J. Clin. Invest.* 106, 467–472.

Oliver, E., McGillicuddy, F., Phillips, C., Toomey, S., and Roche, H. M. (2010). Postgraduate Symposium. The role of inflammation and macrophage accumulation in the development of obesity-induced type 2 diabetes mellitus and the possible therapeutic effects of long-chain n-3 PUFA. *Proc. Nutr. Soc.* 69, 232–243.

Omar, B., and Ahrén, B. (2014). Pleiotropic mechanisms for the glucose-lowering action of DPP-4 inhibitors. *Diabetes* 63, 2196–2202.

Ouchi, N., Kihara, S., Arita, Y., Maeda, K., Kuriyama, H., Okamoto, Y., et al. (1999). Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100, 2473–2476.

Ouedraogo, R., Gong, Y., Berzins, B., Wu, X., Mahadev, K., Hough, K., et al. (2007). Adiponectin deficiency increases leukocyte-endothelium interactions via upregulation of endothelial cell adhesion molecules in vivo. *J. Clin. Invest.* 117, 1718–1726.

Pagliari, D., Saviano, A., Newton, E., Serricchio, M., Dal Lago, A., Gasbarrini, A., et al. (2018). Gut microbiota-immune system crosstalk and pancreatic disorders. *Mediators Inflamm.* 2018:7946431.

Palella, E., Cimino, R., Pullano, S. A., Fiorillo, A. S., Gulletta, E., Brunetti, A., et al. (2020). Laboratory parameters of hemostasis, adhesion molecules, and inflammation in type 2 diabetes mellitus: correlation with glycemic control. *Int. J. Environ. Res. Public Health* 17:300.

Papazafiropoulou, A., Kardara, M., Sotiropoulos, A., Bousboulas, S., Stamataki, P., and Pappas, S. (2010). Plasma glucose levels and white blood cell count are related with ankle brachial index in type 2 diabetic subjects. *Hellenic J. Cardiol.* 51, 402–406.

Paquot, N., Castillo, M. J., Lefebvre, P. J., and Scheen, A. J. (2000). No increased insulin sensitivity after a single intravenous administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin-resistant patients. *J. Clin. Endocrinol. Metab.* 85, 1316–1319.

Pham, C. T. (2006). Neutrophil serine proteases: specific regulators of inflammation. *Nat. Rev. Immunol.* 6, 541–550.

Rangasamy, S., McGuire, P. G., Nitta, C. F., Monickaraj, F., Oruganti, S. R., and Das, A. (2014). Chemokine mediated monocyte trafficking into the retina: role of inflammation in alteration of the blood-retinal barrier in diabetic retinopathy. *PLoS One* 9:e108508. doi: 10.1371/journal.pone.0108508

Reinehr, T., Karges, B., Meissner, T., Wiegand, S., Stoffel-Wagner, B., Holl, R. W., et al. (2016). Inflammatory markers in obese adolescents with type 2 diabetes and their relationship to hepatokines and adipokines. *J. Pediatr.* 173, 131–135. doi: 10.1016/j.jpeds.2016.02.055

Reyat, J. S., Chimen, M., Noy, P. J., Szyroka, J., Rainger, G. E., and Tomlinson, M. G. (2017). ADAM10 interacting tetraspanins Tspan5 and Tspan17 regulate VE-cadherin expression and promote T lymphocyte transmigration. *J. Immunol.* 199, 666–676. doi: 10.4049/jimmunol.1600713

Richardson, V. R., Smith, K. A., and Carter, A. M. (2013). Adipose tissue inflammation: feeding the development of type 2 diabetes mellitus. *Immunobiology* 218, 1497–1504. doi: 10.1016/j.imbio.2013.05.002

Rider, P., Carmi, Y., and Cohen, I. (2016). Biologics for targeting inflammatory cytokines, clinical uses, and limitations. *Int. J. Cell Biol.* 2016:9259646. doi: 10.1155/2016/9259646

Ruan, H., and Dong, L. Q. (2016). Adiponectin signaling and function in insulin target tissues. *J. Mol. Cell Biol.* 8, 101–109. doi: 10.1093/jmcb/mjw014

Rutledge, N. S., and Muller, W. A. (2020). Understanding molecules that mediate leukocyte extravasation. *Curr. Pathobiol. Rep.* 8, 25–35. doi: 10.1007/s40139-020-00207-9

Rutledge, N. S., Weber, E. W., Winger, R., Tourtellotte, W. G., Park, S. H., and Muller, W. A. (2015). CD99-like 2 (CD99L2)-deficient mice are defective in the acute inflammatory response. *Exp. Mol. Pathol.* 99, 455–459. doi: 10.1016/j.yexmp.2015.08.011

Salmi, M., and Jalkanen, S. (2019). Vascular adhesion protein-1: a cell surface amine oxidase in translation. *Antioxid. Redox Signal.* 30, 314–332. doi: 10.1089/ars.2017.7418

Sampson, M., Davies, I., Brown, J., Ivory, K., and Hughes, D. (2002). Monocyte and neutrophil adhesion molecule expression during acute hyperglycemia and after antioxidant treatment in type 2 diabetes and control patients. *Arterioscler. Thromb. Vasc. Biol.* 22, 1187–1193. doi: 10.1161/01.ATV.0000021759.08060.63

Sayyed, S. G., Ryu, M., Kulkarni, O. P., Schmid, H., Lichtnekert, J., Grüner, S., et al. (2011). An orally active chemokine receptor CCR2 antagonist prevents glomerulosclerosis and renal failure in type 2 diabetes. *Kidney Int.* 80, 68–78. doi: 10.1038/ki.2011.102

Schenkel, A. R., Mamdouh, Z., Chen, X., Liebman, R. M., and Muller, W. A. (2002). CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat. Immunol.* 3, 143–150. doi: 10.1038/ni749

Schenkel, A. R., Mamdouh, Z., and Muller, W. A. (2004). Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat. Immunol.* 5, 393–400. doi: 10.1038/ni1051

Seelige, R., Natsch, C., März, S., Jing, D., Frye, M., Butz, S., et al. (2013). Cutting edge: endothelial-specific gene ablation of CD99L2 impairs leukocyte extravasation in vivo. *J. Immunol.* 190, 892–896. doi: 10.4049/jimmunol.1202721

Shah, R., Hinkle, C. C., Ferguson, J. F., Mehta, N. N., Li, M., Qu, L., et al. (2011). Fractalkine is a novel human adipochemokine associated with type 2 diabetes. *Diabetes* 60, 1512–1518. doi: 10.2337/db10-0956

Shanaki, M., Khosravi, M., Khoshdoozi-Farahani, A., Dadashi, A., Heydari, M. F., Delfan, M., et al. (2020). High-intensity interval training reversed high-fat diet–induced M1-macrophage polarization in rat adipose tissue via inhibition of NOTCH signaling. *J. Inflamm. Res.* 13, 165–174. doi: 10.2147/JIR.S237049

Shaw, S. K., Bamba, P. S., Perkins, B. N., and Luscinskas, F. W. (2001). Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. *J. Immunol.* 167, 2323–2330. doi: 10.4049/jimmunol.167.4.2323

Shirakawa, J., Fujii, H., Ohnuma, K., Sato, K., Ito, Y., Kaji, M., et al. (2011). Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. *Diabetes* 60, 1246–1257. doi: 10.2337/db10-1338

Shrestha, B., and Diamond, M. S. (2004). Role of CD8+ T cells in control of West Nile virus infection. *J. Virol.* 78, 8312–8321. doi: 10.1128/JVI.78.15.8312-8321.2004

Signoretti, M., Roggiolani, R., Stornello, C., Fave Delle, G., and Capurso, G. (2017). Gut microbiota and pancreatic diseases. *Minerva Gastroenterol. Dietol.* 63, 399–410.

Sloan-Lancaster, J., Abu-Raddad, E., Polzer, J., Miller, J. W., Scherer, J. C., De Gaetano, A., et al. (2013). Double-blind, randomized study evaluating the glycemic and anti-inflammatory effects of subcutaneous LY2189102, a neutralizing IL-1β antibody, in patients with type 2 diabetes. *Diabetes Care* 36, 2239–2246. doi: 10.2337/dc12-1835

Soehnlein, O., Zernecke, A., Eriksson, E. E., Rothfuchs, A. G., Pham, C. T., Herwald, H., et al. (2008). Neutrophil secretion products pave the way for inflammatory monocytes. *Blood* 112, 1461–1471. doi: 10.1182/blood-2008-02-139634

Song, J., Zhang, X., Buscher, K., Wang, Y., Wang, H., Di, J., et al. (2017). Endothelial basement membrane laminin 511 contributes to endothelial junctional tightness and thereby inhibits leukocyte transmigration. *Cell Rep.* 18, 1256–1269. doi: 10.1016/j.celrep.2016.12.092

Stefanovic-Racic, M., Yang, X., Turner, M. S., Mantell, B. S., Stolz, D. B., Sumpter, T. L., et al. (2012). Dendritic cells promote macrophage infiltration and comprise a substantial proportion of obesity-associated increases in CD11c+ cells in adipose tissue and liver. *Diabetes* 61, 2330–2339. doi: 10.2337/db11-1523

Stolen, M. C., Madanat, R., Marti, I., Kari, S., Yegutkin, G. G., Sariola, H., et al. (2004). Semicarbazide-sensitive amine oxidase overexpression has dual consequences: insulin mimicry and diabetes-like complications. *FASEB J.* 18, 702–704. doi: 10.1096/fj.03-0562fje

Sullivan, D. P., Watson, R. L., and Muller, W. A. (2016). 4D intravital microscopy uncovers critical strain differences for the roles of PECAM and CD99 in

leukocyte diapedesis. *Am. J. Physiol. Heart Circul. Physiol.* 311, H621–H632. doi: 10.1152/ajpheart.00289.2016

Sundara Rajan, S., and Longhi, M. P. (2016). Dendritic cells and adipose tissue. *Immunology* 149, 353–361. doi: 10.1111/imm.12653

Takase, H., Nakazawa, A., Yamashita, S., Toriyama, T., Sato, K., Ueda, R., et al. (2007). Pioglitazone produces rapid and persistent reduction of vascular inflammation in patients with hypertension and type 2 diabetes mellitus who are receiving angiotensin II receptor blockers. *Metabolism* 56, S59–S64. doi: 10.1016/j.metabol.2007.01.002

Talukdar, S., Bandyopadhyay, G., Li, D., Xu, J., McNelis, J., Lu, M., et al. (2012). Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nat. Med.* 18, 1407–1412. doi: 10.1038/nm.2885

Tamura, T., Tailor, P., Yamaoka, K., Kong, H. J., Tsujimura, H., O'Shea, J. J., et al. (2005). IFN regulatory factor-4 and-8 govern dendritic cell subset development and their functional diversity. *J. Immunol.* 174, 2573–2581. doi: 10.4049/jimmunol.174.5.2573

Tan, K. S., Lee, K. O., Low, K. C., Gamage, A. M., Liu, Y., Tan, G.-Y. G., et al. (2012). Glutathione deficiency in type 2 diabetes impairs cytokine responses and control of intracellular bacteria. *J. Clin. Invest.* 122, 2289–2300. doi: 10.1172/JCI57817

Tarabra, E., Giunti, S., Barutta, F., Salvidio, G., Burt, D., Deferrari, G., et al. (2009). Effect of the monocyte chemoattractant protein-1/CC chemokine receptor 2 system on nephrin expression in streptozotocin-treated mice and human cultured podocytes. *Diabetes* 58, 2109–2118. doi: 10.2337/db08-0895

Tessaro, F. H., Ayala, T. S., Nolasco, E. L., Bella, L. M., and Martins, J. O. (2017). Insulin influences LPS-Induced TNF-α and IL-6 release through distinct pathways in mouse macrophages from different compartments. *Cell Physiol. Biochem.* 42, 2093–2104. doi: 10.1159/000479904

Thompson, R. D., Noble, K. E., Larbi, K. Y., Dewar, A., Duncan, G. S., Mak, T. W., et al. (2001). Platelet-endothelial cell adhesion molecule-1 (PECAM-1)–deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane. *Blood* 97, 1854–1860. doi: 10.1182/blood.V97.6.1854

Todoric, J., Löffler, M., Huber, J., Bilban, M., Reimers, M., Kadl, A., et al. (2006). Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* 49, 2109–2119. doi: 10.1007/s00125-006-0300-x

Trottier, M. D., Naaz, A., Li, Y., and Fraker, P. J. (2012). Enhancement of hematopoiesis and lymphopoiesis in diet-induced obese mice. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7622–7629.

Uotila, L. M., Jahan, F., Hinojosa, L. S., Melandri, E., Grönholm, M., and Gahmberg, C. G. (2014). Specific phosphorylations transmit signals from leukocyte β2 to β1 integrins and regulate adhesion. *J. Biol. Chem.* 289, 32230–32242. doi: 10.1074/jbc.M114.588111

van Beek, L., Lips, M. A., Visser, A., Pijl, H., Ioan-Facsinay, A., Toes, R., et al. (2014). Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance. *Metabolism* 63, 492–501. doi: 10.1016/j.metabol.2013.12.002

Van Oostrom, A., Van Wijk, J., Sijmonsma, T., Rabelink, T., and Castro Cabezas, M. (2004). Increased expression of activation markers on monocytes and neutrophils in type 2 diabetes. *Neth. J. Med.* 62, 320–325.

Van Steenwinckel, J., Auvynet, C., Sapienza, A., Reaux-Le Goazigo, A., Combadière, C., and Parsadaniantz, S. M. (2015). Stromal cell-derived CCL2 drives neuropathic pain states through myeloid cell infiltration in injured nerve. *Brain Behav. Immun.* 45, 198–210. doi: 10.1016/j.bbi.2014.10.016

Vessby, B., Uusitupa, M., Hermansen, K., Riccardi, G., Rivellese, A. A., Tapsell, L. C., et al. (2001). Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: the Kanwu study. *Diabetologia* 44, 312–319. doi: 10.1007/s001250051620

von Andrian, U. H., and Engelhardt, B. (2003). α4 integrins as therapeutic targets in autoimmune disease*. *Mass Med. Soc.* doi: 10.1056/NEJMe020157

Wang, C., and Li, J. (2015). Pathogenic microorganisms and pancreatic cancer. *Gastrointest. Tumors* 2, 41–47. doi: 10.1159/000380896

Weber, C. (2003). Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules. *J. Mol. Med.* 81, 4–19. doi: 10.1007/s00109-002-0391-x

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., and Ferrante, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 112, 1796–1808. doi: 10.1172/JCI200319246

Wen, Y., Skidmore, J., Porter-Turner, M., Rea, C., Khokher, M., and Singh, B. (2002). Relationship of glycation, antioxidant status and oxidativestress to vascular endothelial damage in diabetes. *Diabetes. Obes. Metab.* 4, 305–308. doi: 10.1046/j.1463-1326.2002.00212.x

White, F. A., Feldman, P., and Miller, R. J. (2009). Chemokine signaling and the management of neuropathic pain. *Mol. Interv.* 9:188. doi: 10.1124/mi.9.4.7

Winer, D. A., Winer, S., Shen, L., Wadia, P. P., Yantha, J., Paltser, G., et al. (2011). B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. *Nat. Med.* 17, 610–617. doi: 10.1038/nm.2353

Wolburg, H., and Lippoldt, A. (2002). Tight junctions of the blood–brain barrier: development, composition and regulation. *Vascul. Pharmacol.* 38, 323–337. doi: 10.1016/S1537-1891(02)00200-8

Wouters, K., Gaens, K., Bijnen, M., Verboven, K., Jocken, J., Wetzels, S., et al. (2017). Circulating classical monocytes are associated with CD11c+ macrophages in human visceral adipose tissue. *Sci. Rep.* 7:42665. doi: 10.1038/srep42665

Wright, R. D., and Cooper, D. (2014). Glycobiology of leukocyte trafficking in inflammation. *Glycobiology* 24, 1242–1251. doi: 10.1093/glycob/cwu101

Wu, C.-C., Sytwu, H.-K., Lu, K.-C., and Lin, Y.-F. (2011). Role of T cells in type 2 diabetic nephropathy. *Exp. Diabetes Res.* 2011:514738. doi: 10.1155/2011/514738

Xu, H. E., Lambert, M. H., Montana, V. G., Plunket, K. D., Moore, L. B., Collins, J. L., et al. (2001). Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13919–13924. doi: 10.1073/pnas.241410198

Xu, H.-L., Salter-Cid, L., Linnik, M. D., Wang, E. Y., Paisansathan, C., and Pelligrino, D. A. (2006). Vascular adhesion protein-1 plays an important role in postischemic inflammation and neuropathology in diabetic, estrogen-treated ovariectomized female rats subjected to transient forebrain ischemia. *J. Pharmacol. Exp. Ther.* 317, 19–29. doi: 10.1124/jpet.105.096958

Xu, L., Kitade, H., Ni, Y., and Ota, T. (2015). Roles of chemokines and chemokine receptors in obesity-associated insulin resistance and nonalcoholic fatty liver disease. *Biomolecules* 5, 1563–1579. doi: 10.3390/biom5031563

Xue, C.-B., Wang, A., Han, Q., Zhang, Y., Cao, G., Feng, H., et al. (2011). Discovery of INCB8761/PF-4136309, a potent, selective, and orally bioavailable CCR2 antagonist. *ACS Med. Chem. Lett.* 2, 913–918. doi: 10.1021/ml200199c

Yago, T., Shao, B., Miner, J. J., Yao, L., Klopocki, A. G., Maeda, K., et al. (2010). E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin αLβ2-mediated slow leukocyte rolling. *Blood* 116, 485–494. doi: 10.1182/blood-2009-12-259556

Yanaba, K., Bouaziz, J. D., Matsushita, T., Magro, C. M., St. Clair, E. W., and Tedder, T. F. (2008). B-lymphocyte contributions to human autoimmune disease. *Immunol. Rev.* 223, 284–299. doi: 10.1111/j.1600-065X.2008.00646.x

Yao, L., Herlea-Pana, O., Heuser-Baker, J., Chen, Y., and Barlic-Dicen, J. (2014). Roles of the chemokine system in development of obesity, insulin resistance, and cardiovascular disease. *J. Immunol. Res.* 2014:81450. doi: 10.1155/2014/181450

Yates, C. M., Tull, S. P., Madden, J., Calder, P. C., Grimble, R. F., Nash, G. B., et al. (2011). Docosahexaenoic acid inhibits the adhesion of flowing neutrophils to cytokine stimulated human umbilical vein endothelial cells. *J. Nutr.* 141, 1331–1334. doi: 10.3945/jn.111.139287

Yki-Järvinen, H. (2004). Thiazolidinediones. *N. Engl. J. Med.* 351, 1106–1118. doi: 10.1056/NEJMra041001

Yu, D. M., Yao, T. W., Chowdhury, S., Nadvi, N. A., Osborne, B., Church, W. B., et al. (2010). The dipeptidyl peptidase IV family in cancer and cell biology. *FEBS J.* 277, 1126–1144. doi: 10.1111/j.1742-4658.2009.07526.x

Yu, X., and Martin-Gayo, E. (2019). Role of dendritic cells in natural immune control of HIV-1 infection. *Front. Immunol.* 10:1306. doi: 10.3389/fimmu.2019.01306

Zha, J., Chi, X.-W., Yu, X.-L., Liu, X.-M., Liu, D.-Q., Zhu, J., et al. (2016). Interleukin-1β-targeted vaccine improves glucose control and β-cell function in a diabetic KK-Ay mouse model. *PLoS One* 11:e0154298. doi: 10.1371/journal.pone.0154298

Zhang, J., Alcaide, P., Liu, L., Sun, J., He, A., Luscinskas, F. W., et al. (2011). Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils. *PLoS One* 6:e14525. doi: 10.1371/journal.pone.0014525

Zhou, T., Hu, Z., Yang, S., Sun, L., Yu, Z., and Wang, G. (2018). Role of adaptive and innate immunity in type 2 diabetes mellitus. *J. Diabetes Res.* 2018:7457269. doi: 10.1155/2018/7457269

Zhu, X., Cao, S., Zhu, M.-D., Liu, J.-Q., Chen, J.-J., and Gao, Y. J. (2014). Contribution of chemokine CCL2/CCR2 signaling in the dorsal root ganglion and spinal cord to the maintenance of neuropathic pain in a rat model of lumbar disc herniation. *J. Pain* 15, 516–526. doi: 10.1016/j.jpain.2014.01.492

Zhuge, F., Ni, Y., Nagashimada, M., Nagata, N., Xu, L., Mukaida, N., et al. (2016). DPP-4 inhibition by linagliptin attenuates obesity-related inflammation and insulin resistance by regulating M1/M2 macrophage polarization. *Diabetes* 65, 2966–2979. doi: 10.2337/db16-0317

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Pezhman, Tahrani and Chimen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.