Alterations of Scavenger Receptor CD163 in Diabetes Complications

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Statement of Originality

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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June 30th, 2023

Author Attribution Statement

Chapter 3 of this thesis is published as "**Siwan, E**., Twigg, S. M., & Min, D. (2022). Alterations of CD163 expression in the complications of diabetes: A systematic review. *Journal of Diabetes and its Complications*. 36(4): 108150"

I conceptualised the review with DM and ST and screened articles with DM. I extracted data and wrote the original draft manuscript. DM and ST revised and edited the manuscript.

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I performed the experiment, data analysis and wrote the first draft of the manuscript. SP assisted with the data analysis. KW acquired the clinical samples and clinical data. MM, TW and JW assisted with the recruitment. ST contributed to the study cohort design, interpretation of the results, and revision of the manuscript. DM contributed to study experiment design, interpretation of the results and writing and revision of the manuscript. All authors contributed to review/edit and approved the final version for submission.

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I performed the experiment, data analysis and drafted the manuscript. J.W. and B.B. acquired the clinical samples and clinical data. D.S. and C.B. assisted with mass cytometry and N.D. assisted with RNA-seq data analysis. S.M. assisted with the study cohort design. S.T. contributed to data interpretation, and revision of the manuscript. D.M. contributed to study design, performed part of experiment, supervision and revision of the manuscript. D.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to review/edit and approved the final version for submission.

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Other manuscripts in collaboration

Baker, C, J., Min, D., Marsh-Wakefield, F., **Siwan, E**., Gerofi, J., Wang, X., Hocking, S, L., Colagiuri, S., Johnson, N, A., Twigg, S, M. Circulating CD31+ Angiogenic T Cells are Reduced with Prediabetes and Improve with Combined Exercise Training (in preparation for submission).

Huang, C., **Siwan, E**., Baker, C.J., Shinko, D., McGuire, H., Twigg, S, M.& Min, D. Uncovering sex-related differences in skin macrophage polarization during wound healing in diabetes (under review).

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Awards

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List of Abbreviations

Abbreviation	Definition
ADAM17	A disintegrin and metalloproteinase
ADIPOR	Adiponectin receptor
AGE	Advanced glycation end product
APC	Antigen presenting cell
ATP	Adenosine triphosphate
AUC	Area under curve
BH	Benjamini-Hochberg
BMI	Body mass index
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Classical DC
CKD	Chronic kidney disease
CVD	Cardiovascular disease
CX3CR1	C-X3-C motif chemokine receptor 1
CXCR3	C-X-C motif chemokine receptor 3
CyTOF	Cytometry by time of flight
DAMPs	Damage associated molecular patterns
DAPI	Diamidino-2-phenylindole
DC	Dendritic cell
DEG	Differentially expressed genes
DFU	Diabetic foot ulcer
DKD	Diabetic kidney disease
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribose nucleic acid
DPN	Diabetic polyneuropathy
DR	Diabetic retinopathy
DRFU	Diabetes related foot ulcer
DSP	Distal symmetrical polyneuropathy
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
ESRD	End-stage renal disease
FACS	Flow cytometry staining buffer
FC	Fold change
FDR	False discovery rate
GM-CSF	Granulocyte macrophage colony stimulating factor
GO	Gene ontology
GOI	Genes of interest
Hb	Haemoglobin
HBA_{1c}	Glycated haemoglobin
Hb-Hp	Haemoglobin-haptoglobin
HbSR	Haemoglobin scavenger receptor

HDL-c	High density lipoprotein cholesterol
HLADR	Human leukocyte antigen DR
Hp	Haptoglobin
IL	Interleukin
INF	Interferon
infDC	Inflammatory DCs
IP	INF-ɣ inducible protein
IQR	Interquartile range
KEGG	Kyoto encyclopedia of genes and genomes
LDL-c	Low density lipoprotein cholesterol
LPS	Lipopolysaccharide
LSM	Liver stiffness measurement
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage stimulating factor
MDC	Macrophage derived chemokine
mDC	Myeloid DC
MDM	Monocyte-derived-macrophages
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
MT-MMPs	Membrane-type matrix metalloproteinases
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFS	NAFLD-fibrosis score
NK	Natural killer
NLR	Neutrophil-to-leukocyte ratio
NPDR	Non-proliferative DR
PAD	Peripheral artery disease
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid DC
PDR	Proliferative DR
PPI	Protein-protein interaction network
PRISMA	Preferred reporting items for systematic reviews and meta-analysis
PST	Proline-serine-threonine
RA	Rheumatoid arthritis
RANTES	Regulated upon activation, normal T cell expressed and presumable secreted
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
sCD163	Soluble CD163
SD	Standard deviation
SR	Scavenger receptors
SRCR	Scavenger receptor cysteine rich

STRING	Search tool for retrieval of interacting genes
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TE	Transient elastography
Th	T helper
TIMP	Tissue inhibitors of MMPs
TLB	Thaw lyse buffer
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TWEAK	TNF-like weak inducer of apoptosis
UACR	Urinary albumin creatinine ratio
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
VEGFα	Vascular endothelial growth factor alpha
VEGFR2	Vascular endothelial growth factor receptor 2
WBC	White Blood Cell

Abstract

Background: The global prevalence of diabetes mellitus is increasing at pandemic levels, as the population ages and less than healthy nutritional and activity lifestyles dominate, thus foreshadowing the rise of accompanying micro- and macro-vascular complications which are highly morbid and reduce the quality and quantity of life of individuals. This is because the complications are progressive in nature, leading often to irreversible damage of local tissues. Whilst inflammation plays a critical role in the pathophysiology of diabetes complications at a tissue level, low-grade systemic inflammation is also a feature of diabetes and its complications. The proportion of circulating anti-inflammatory haemoglobin-haptoglobin scavenger receptor, CD163+ monocytes has been reported significantly decreased in individuals with diabetes complications. However, the role of CD163 in diabetes complications and its regulation in fluids and tissues, remains incompletely understood. Therefore, this thesis aimed to investigate whether the levels of circulating CD163+ monocytes and sCD163 are impacted in diabetes complications and whether they can act as a mediator or biomarker for certain complication of diabetes and disease progression. In addition, the regulation of CD163 in diabetes complications has also been investigated.

Methods: Firstly, a systematic review was undertaken on literature surveying human studies of diabetes complications for the expression of CD163 published until 31st January 2022 in Scopus, Embase and Medline. Data extraction and assessment followed the PRISMA workflow. Secondly, blood samples were collected from adults with diabetes with and without complications from Diabetes Centre, Royal Prince Alfred Hospital. ELISA was used to quantify plasma sCD163 level and mass cytometry was conducted to immunophenotype CD163+ monocytes, as well as other circulating white blood cells. Additionally, RNA-sequencing was utilised to profile the transcriptome of CD163+ cells isolated from

individuals with and without diabetes complications. Finally multiplex immunoassay was utilised to quantify the levels of circulating cytokines, chemokines and matrix metalloproteinases (MMPs) in individuals with and without diabetes complications. Statistical analysis of all data was performed on GraphPad Prism.

Results: Systematic analysis revealed that sCD163 was increased in diabetes complications in various body fluids including plasma and serum and was implicated in the progression of those complications. However, the count of CD163+ cells was found to change differently across various diabetes complications. In our study with a cohort of individuals with diabetes complications as well as non-alcoholic steatohepatitis (NAFLD), sCD163 was increased in individuals with microvascular complications, specifically diabetes kidney disease. Furthermore, sCD163 was increased in individuals with advanced non-alcoholic steatohepatitis (NASH) fibrosis and implicated in the progression of NASH fibrosis with clinical utility. RNA-seq analysis revealed differentially expressed genes in the CD163+ monocytes in diabetes complications. Genes relating to 'regulation of centrosome cycle' were up-regulated and pro-apoptotic miRNAs were increased in individuals with diabetes complications. RNA-seq data in combination with mass cytometry immunophenotyping of the CD163+ monocytes revealed alterations in the CD163+ monocytes pertaining to: reduced capacity for trans-endothelial migration, reduced monocyte activation and dysregulated interaction with the adaptive immune system. Function and activation markers of circulating white blood cells were also different between individuals with and without diabetes complications, specifically, reduced adhesion markers on classical, intermediate and non-classical monocytes, and dysregulated T and B cell subsets. Plasma cytokines IL-10 and IL-15 were found increased in individuals with complications, but not correlated with the circulating level of CD163+ monocytes or sCD163. However, plasma MMP-2, -7 and -10 were correlated with sCD163 levels in individuals with diabetes complications.

Conclusion: The work in this thesis shows that the decreased circulating CD163+ monocytes in diabetes complications are associated with and may be caused by the up-regulation of 'centrosome cycle' genes and specific microRNAs in the transcriptome of the CD163+ monocytes; and also by the proteolytic action of MMP-2, -7 and -10. The consequential increase in plasma sCD163 is a useful indicator of disease severity, especially NASH fibrosis severity in individuals with diabetes. The phenotypes of the CD163+ monocytes and other circulating white blood cells are also altered in diabetes complications, such that immune cells favour the progression of chronic complications. This thesis research highlights the intricate relationship between the immune system and inflammatory processes in diabetes complications. Further investigation is warranted to address inflammatory pathways in diabetes complications.

Chapter 1: Literature Review and Aims

1.1 Diabetes and its Complications

Diabetes mellitus (DM) is a chronic condition with a disorder of glucose metabolism characterised by a state of hyperglycaemia in either fasting or postprandial state. The global incidence of diabetes is rising dramatically, from an estimated 382 million people in 2013 to 592 million by 2035 (1). The International Diabetes Federation has named diabetes as the largest global epidemic of the 21st century (2). There are two main types of diabetes, type 1 diabetes (T1DM) and T2DM (T2DM). T1DM is an autoimmune disorder whereby the immune cells in the body result in the destruction of insulin-producing pancreatic β-cells (3). It comprises up to 10 % of the total prevalence of diabetes; however incidence rates are also rising (4). T2DM is characterised by a combination of dysfunctional β-cells and insulin resistance. In recent decades the ongoing global obesity pandemic has played an active part in the increased prevalence of diabetes world-wide, especially T2DM which accounts for more than 85% of the total diabetes prevalence (5). The growing incidence of diabetes foreshadows the rise of chronic diabetes-related microvascular complications such as diabetic retinopathy (DR), polyneuropathy (DPN) and nephropathy (DN) and macrovascular complications including atherosclerosis, stroke and peripheral artery disease (PAD) (Figure 1.1). The ongoing and persisting nature of these vascular complications ultimately result in the failure of organs and tissues including the retina, kidney, nerves, heart and blood vessels (6). As such diabetes complications present as a modern medical health challenge as they are associated with premature morbidity, mortality, reduced life expectancy, financial and social costs to the patient with diabetes, their carers, family as well as the health service.

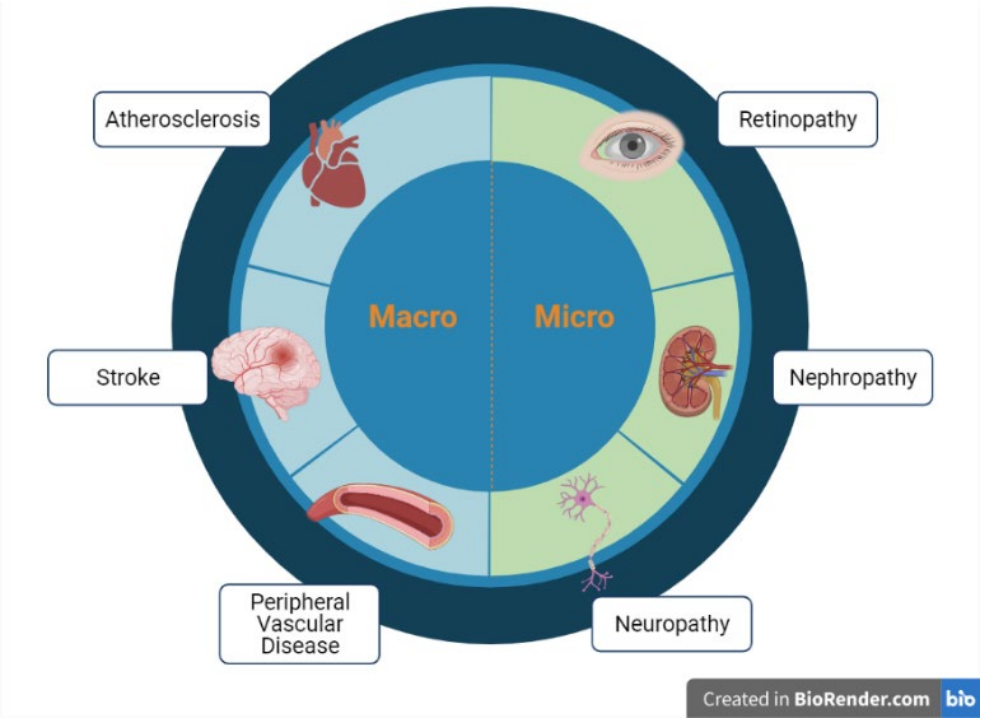


Figure 1.1: Microvascular and macrovascular complications of diabetes. Macrovascular complications are indicated on the left-hand side and include atherosclerosis, stroke and peripheral vascular disease. Microvascular complications are indicated on the right-hand side and include retinopathy, nephropathy and neuropathy. The image was created in BioRender.com.

1.1.1 Microvascular Complications of Diabetes

Diabetic Retinopathy

Microvascular complications of diabetes include DR, DPN and DN. The most common microvascular complication of diabetes is DR, which causes damage to the blood vessels in the light-sensitive tissue of the retina (4). DR can affect individuals with both T1DM or T2DM diabetes and the risk of its incidence increases with longer duration of diabetes and higher HbA1c which is indicative of their poor glucose control (7). DR is classified into two stages, non-proliferative DR (NPDR) and proliferative DR (PDR) (8). At the initial stages DR is characterised by inflammation, microglial activation, loss of endothelial cells, neuronal

cells and pericytes leading to subsequent vision loss, furthermore local ischemia can lead to vision-threatening PDR (9). The burden of DR is a result of the progression from mild and asymptomatic to severe and vision-threatening, which has resulted in DR being the leading cause of adult-onset blindness world-wide (10). It is estimated that the number of individuals with DR will increase from 127 million in 2010 to 191 million by 2030 (11). Early stage therapies are based around blood glucose control, whilst advanced stage treatments can minimize loss of vision (9) but there are still minimal therapeutics designed to halt the progression of the retinopathy (12).

Diabetic Polyneuropathy

DM is the most common cause of neuropathy worldwide. The prevalence of neuropathy is estimated to be about 8% in newly diagnosed diabetes and greater than 50% in diabetes with long-standing disease (13). DPN is a neurodegenerative disease of the peripheral nervous system that preferentially targets sensory axons, autonomic axons and motor axons, though the mechanisms by which diabetes targets sensory neurons remains debated (14). The most common manifestation of DPN is distal symmetrical polyneuropathy (DSP) (15). Symptoms include numbness, tingling, pain, weakness beginning in the feet and spreading proximally in a length-dependent fashion. These symptoms are symmetrical however sensory symptoms are more prominent than motor involvement (15). The further progression and down-stream effects of DSP symptoms can greatly affect the quality of life of individuals, both physically and mentally. DSP associated numbness can cause balance problems, leading to falls (15). People with severe DSP are at risk of ulcerations and lower-extremity amputations, with 15% developing ulcers during the course of their disease, and a high incidence of progressive lower-extremity amputations (15). Currently, research on the early diagnosis of DPN is limited, as is research on mechanisms to halt its progression of DPN. At present, there is no effective treatment available, except for tight control of blood glucose and pain management.

This might be a result of insufficient knowledge of the pathogenesis of DPN (16). Therefore, further research in the pathophysiology of DPN may provide much needed insight into the development of therapeutic measures.

Diabetic Nephropathy

DN or diabetic kidney disease (DKD) is another morbid complication of diabetes wherein persistent exposure to elevated blood glucose levels which leads to the damage and disruption of renal cellular architecture and microvasculature (17). Various complex pathways mediate these effects including metabolic, hemodynamic, intracellular and growth factors/cytokines (18). The resulting structural changes in the nephron ultimately lead to excessive urine albumin secretion, glomerular lesions and loss of glomerular filtration rate in diabetes (18). DN is a chronic and progressive disease, which as a result of the global diabetes epidemic, has become the most frequent cause of end-stage renal disease (ESRD) and the principal cause for requiring renal replacement transplants worldwide (19). At present 20-50% of people with diabetes are affected by DN and the increasing incidence will contribute to the increased prevalence of DN which will further expand its economic and health burden (20). Currently the management of DN relies on multifactorial intervention and targets the control of glycaemia, blood pressure and lipids in addition to lifestyle changes. Current treatments are reno-protective, anti-proteinuric and anti-hypertensive and have been shown to slow the progression of ESRD (21, 22) however these strategies still have limited clinical benefit and many individuals with diabetes will still go on to develop ESRD (17).

1.1.2 Macrovascular Complications of Diabetes

Macrovascular complications of diabetes arise due to atherosclerosis, which is characterised by the narrowing of endothelium arterial walls throughout the body, the damage of large blood vessels and the rupture of calcified cholesterol deposits causing thrombosis and clots

(23). Macrovascular complications includes cardiovascular events such as strokes and heart attacks as well as peripheral artery disease (PAD) (24). Diabetes increases the risk of cardiovascular disease (CVD), however the exact mechanisms on how diabetes increases the likelihood of atherosclerotic plaque formation has not been fully elucidated. Macrovascular diseases such as CVD are the leading cause of death for adults with either T1DM or T2DM, in which atherosclerotic disease is responsible for 42% of mortality in diabetes (25). PAD is present in approximately 29% of individuals with diabetes and may be undiagnosed due to confounders such as the lack of sensation from neuropathy (26, 27). Additionally individuals with PAD and diabetes are more likely to present with ulcers, increasing the risk for amputation of lower extremities (such as toes and feet), and are also more predisposed to cardiovascular events (24) (28). Diabetes is also correlated with a poor outcome and more disability after stroke (29). Among individuals admitted for acute stroke, diabetes was associated with a higher risk of death or functional dependency (29). Due to the fact that CVD accounts for the greatest component of health care expenditures in individuals with diabetes, and the fact that CVD is the leading cause of their morbidity and mortality, greater research is needed into the mechanisms to halt or prevent the progression of CVD and its fatal health outcomes (23) (30).

1.1.3 Other Complications of Diabetes

Periodontitis

Periodontitis is a chronic inflammatory disease characterised by the accumulation of dental plaque biofilm, within which microbial dysbiosis leads to a chronic, non-resolving and destructive inflammatory response (31). The resultant tissue destruction is caused by the hosts inflammatory response to the bacterial challenge presented within the biofilm (32). Periodontitis has been recognised as an additional complication of diabetes (33). A

bidirectional relationship exists between diabetes and periodontitis in which each disease negatively impacts the other (31). The comorbidity is linked to the common inflammatory pathophysiology which causes exacerbations of both diseases. The risk of periodontitis is increased up to 3 times in people with diabetes compared to those without (34). Similar to other diabetes complications the risk for periodontitis increases with poorer glycaemic control (35). The treatment of periodontitis requires professional interventions such as root surface debridement, oral hygiene instruction and patient education in addition to a life-long strategy involving regular professional monitoring and support (31). Multiple meta-analysis studies have shown that periodontal treatment alone can have beneficial effect on glycaemic control (36-38). Similarly anti-diabetic drugs play an indirect role in treating inflammation caused by diabetes, but also have a direct beneficial anti-inflammatory effect on periodontitis (39). Since the anti-inflammatory properties of anti-diabetic drugs have the potential to ameliorate periodontitis, future investigations that focus on the inflammation related connection between diabetes and periodontitis could illuminate the pathophysiology and lead to therapeutic targets (40).

Delayed Wound Healing

Diabetes causes delayed wound healing and impairs each phase of wound healing such as reduced blood supply due to vascular damage during haemostasis phase, impaired inflammation response, hindered endothelial proliferation and disrupted remodelling which consequently leads to a postponed, incomplete or uncoordinated healing process (41). These chronic and unresolving wounds have long-term negative effects on quality of life of individuals with diabetes and furthermore increasing morbidity and mortality and disrupt lifestyles as diabetic wounds can lead to leg or foot ulcers and amputations. Diabetes individuals have a 15-25% risk of developing diabetic foot ulcers of which 40-80% become severely infected which can extend to the bone and lead to osteomyelitis (42-44). Crucially,

the rate of recurrence of a foot ulcer is 65% at 3-5 years from the first episode and impaired wound healing in DM represents a major health care issue (45). It adds a significant economic burden since the costs for diabetic foot ulceration treatments are a further addition to the general costs of diabetes care. Delayed wound healing in diabetes involves a complex pathophysiology that includes vascular, neuropathic, immune and biochemical components. Whilst there are various treatments available they do not guarantee a rapid and definitive reparative process (41).

1.1.4 NAFLD and its Complications in Diabetes

Non-alcoholic fatty liver disease (NAFLD) is an umbrella term that encomposes a continuum of chronic liver conditions ranging from steatosis (fatty liver) to non-alchoholic steatohepatitis (NASH), which eventuates to fibrosis and cirrhosis (46). T2DM increases the risk for developing NAFLD and NASH and adds to the progression of NAFLD to its more severe forms including hepatocellular cancer (47). Globally, the prevalence of NAFLD in individuals with T2DM is approximated to be more than 55%, and NASH expected to occur in 37% of individuals with T2DM (48).

Whilst NAFLD is not a vascular condition but rather a disorder of lipid metabolism characterized by the accumulation of fat in the liver, it is recognised as a co-morbid condition with diabetes and has been linked to various complications of diabetes, including macrovascular, microvascular and other complications of diabetes (49). Studies in chronic kidney disease (CKD) have reported that NAFLD is associated with an increased risk of both prevalence and incidence of CKD in individuals with and without T2DM (50). Another study found that NAFLD was associated with a 43% increased risk of CKD, and this association was slightly increased when the analysis was restricted to the study cohort involving individuals with diabetes exclusively (51). Furthermore the presence of NAFLD may even be

associated with DN progression (52). Collectively, investigations concerning the association between NAFLD and DR have not yet reached a cohesive conclusion. For instance in a meta-analysis it was suggested that in China, Korea and Iran, T2DM individuals with NAFLD had a decreased risk of DR when compared with those without NAFLD, whereas in Italy and India, T2DM individuals with NAFLD had an increased risk for DR (53). Concerning macrovascualar complications, several studies have found the associations between cardiac dysfunction in individuals with T2DM and NAFLD, independently of CVD or traditional CVD risk factors (54). Due to a close relationship between NAFLD and micro- and macrovascular complications in T2DM, it has been suggested that the assessments of the degree of NAFLD could also be used as a screening tool to assess the degree of micro- and macrovascular complications in diabetes (55). NAFLD is a common inflammatory condition that co-exists with diabetes and its complications, this prompts the investigation of how the systemic low-grade inflammation characteristic of diabetes associates with NAFLD.

1.2 Chronic Low-grade Inflammation in Diabetes Complications

Inflammation plays a central role in the body homeostasis and protects the body from illness and disease. Chronic low-grade inflammation occurs when there is a failure to resolve an acute inflammatory response or there is repeated stimulation of the immune system, resulting in a long-lasting, low-grade inflammation that is damaging to tissues in comparison the protective acute inflammatory response. DM is characterised by a state of low-grade inflammation and uncontrolled inflammation is a critical factor in the progression of diabetes micro- and macrovascular complications leading to damage at affected organ sites (56).

Inflammatory processes are associated with both T1DM and T2DM, however there are distinct pathophysiology's between the two types, which suggests different causal mechanisms (57). In T2DM inflammatory processes are associated with development of

insulin resistance and impaired glucose tolerance (57). There is a possibility that obesity (a risk factor for T2DM) and the activation of adipose tissue in particular, may further enhance the release of inflammatory factors that underlie the development of insulin resistance (57). Insulin resistance and insulin deficiency give rise to a hyperglycaemic state that is a major risk factor for the development of diabetes complications as it acts destructively through various pathways including the aldose reductase pathway, advanced glycation end product pathway, reactive oxygen intermediate pathway and protein kinase-C pathway (57). These pathways induce oxidant and inflammatory mediators that can have damaging effects, at the systemic level and also locally in the tissues. The downstream effect of these pathways contribute to cellular dysfunction and damage associated with micro- and macrovascular complications including the activation of leukocytes and activation of cytokines (57).

Cytokines refer to a large group of small, secreted proteins released by cells. They include chemokines (cytokines with chemotactic function) and interleukins (IL-; cytokines made by one leukocyte to act on other leukocytes) (58). Chemokines are further classified as CXC-, CC-, C- and CX3C- chemokines according to the position of cysteine residues in their structure (59). Upon the binding of cytokines to their corresponding receptor, cytokines may have exert an autocrine action as either pro-inflammatory or anti-inflammatory by acting on the cells that secrete them, or surrounding cells by a paracrine action (58). Pro-inflammatory cytokines IL-6 and IL-8 and chemokines CCL1, CCL2, CCL4, CCL5, CCL11, CXCL8, CXCL10 and CX3CL1 have been found to be elevated in the serum of individuals with T2DM (60-62). Furthermore evidence suggests that IL-6 is a systemic marker of inflammation and is increased in and associated with complications such as DN (63) (57). Table 1.1 contains a list of cytokines and chemokines implicated in chronic inflammation conditions.

Table 1.1 Cytokines in chronic inflammation conditions.

Type of Cytokines	*Circulating Protein*	*Primary Cell Sources*	*Function in Chronic Inflammation*
Interleukins	**IL-1α**	Various	Promotes activation, co-stimulation, and secretion of cytokines and other acute phase proteins (64)
	IL-4	Th Cells	T and B cell proliferation (64)
	IL-6	Macrophages, Th Cells, endothelial cells, adipocytes	Synthesis of acute phase proteins and activation of immune cells
	IL-8	Macrophages, CD31+, T cells and endothelial cells	Angiogenesis and chemotaxis
	IL-10	Macrophages and DCs	Inhibition of Th1 inflammatory pathways in Th1 cells and macrophages (65)
	IL-12p70	Activated inflammatory cells including macrophages, neutrophils, microglia and DCs	Cell differentiation and activation (66)
	IL-15	Many cell types, but specifically monocytes, DCs, epithelial cells, bone marrow, stromal cells and fibroblasts	Activates NK cells, NKT cells, CD8+ cells (67)
Interferon	**INF-α2**	Plasmacytoid DCs, virally affected leukocytes	Modulates the anti-viral immune response (64)
Others	**TNF-α**	Many cell types	NFKβ pathway activation
Chemokines	**MDC**	Mature macrophages and monocyte-derived dendritic cells	Chemoattracted for CCR4 expressing cells including lymphocytes, monocytes, monocyte derived dendritic cells and NK cells (68)
	MCP-1	Macrophages and DCs, cardiac myocytes	Recruitment of leukocytes and monocytes (69)
	MCP-3	Endothelial cells, vascular smooth	Chemotactic factor for monocytes and neutrophils (70)

		muscle cells and myelomonocytic cells	
	IP-10	Immune and non-immune cells	Chemoattractant for various immune cells such as activated T cells, NK cells, DCs and macrophages (71)
	MIP-1α	Monocytes, T cells, B cells, neutrophils, DCs and NK cells	Inflammation, wound healing (72)
	MIP-1β		

The chronic low-grade inflammation in diabetes and its complications also involves the activation and dysregulation of leukocytes. Whilst the higher total white blood cell counts have been reported to be associated with T2DM occurrence (73), aberrant immune cell activation is described as a contributing mechanism to the development of the macrovascular complications in diabetes such as CVD (56). Additionally other studies have found that the dysregulation of leukocytes in diabetes included an imbalance between the ratio of M1/M2 macrophages, leading to an increased number of M1 pro-inflammatory macrophages compared to anti-inflammatory M2 macrophages (74). This imbalance in the phenotype of leukocytes may serve as a contributing factor to the prolonged damage of diabetes complications.

The precursor to the macrophage, the monocyte, has also been found changed in diabetes complications (75). Monocytes can be categorized into classical, intermediate and non-classical monocytes based on the intensity of CD16 expression (further details in the section 1.4.1). In a recent study, Min et al reported a decrease in the number of CD16+ monocytes in individuals with diabetes complications (75). In addition, the same research group has reported that another monocyte subset, the anti-inflammatory CD163+ monocyte, has also been found to be decreased in individuals with diabetes complications compared to those without complications (76). Furthermore, the soluble form of CD163 (sCD163) has been

identified as having potential predictive value for diabetes onset (77). This has prompted the current investigation to identify the role of CD163+ monocytes in diabetes complications.

1.3 Characterization and Structure of CD163

CD163 is a scavenger receptor exclusively expressed on monocytes and macrophage cells. It acts in an anti-inflammatory manner by internalising toxic free circulating haemoglobin.

1.3.1 Structure of CD163

CD163 belongs to class I of the scavenger receptor cysteine rich (SRCR) superfamily (78). Whilst there are many different classes of scavenger receptor (A-I), CD163 is uniquely characterized by the presence of one or several repeats of an ancient and highly conserved protein module, the type B SRCR domain (79, 80). The type B SRCR domain consists of 100-110 amino acid residues which are encoded by a single exon and contains 6 or 8 cysteine residues (78), resulting in the formation of three-four disulphide bonds which affect the folding and stability of the receptor.

CD163 is a type 1 transmembrane glycoprotein of 130kDa molecular weight known as M130 prior to its name change to CD163 (81). Structurally it consists of nine extracellular SRCR type B domains (SRCR1-9). Following the SRCR domains, a short proline-serine-threonine rich linker domain connects SRCR9 with a transmembrane domain and a short intracellular cytoplasmic tail. Of the nine SRCR domains, each contains four disulphide bridges, except for SRCR8 which lacks the C2-C7 bridge (82-84). The extracellular SRCR domains 2, 3, 7 and 9 contain consensus calcium binding sites that coordinate acidic amino acids electrostatic interactions with ligands (85, 86). SRCR domain 3 is as a crucial binding site for haemoglobin-haptoglobin (Hb-Hp) complexes (87, 88). Structurally, CD163 is post-translationally modified by glycosylation (84).

Several different CD163 mRNA variants, all arising from alternative splicing of a single CD163 gene have been identified (83, 89). Three different isoforms of CD163 exist, with differences pertaining to the intracellular tail component. The most abundant form has a tail of 49 amino acids, while the less abundant forms have 84 or 89 amino acids. The first 42 amino acids after the membrane spanning segments are in common for the three isoforms (84). Although the variants have differing cytoplasmic C-termini, all CD163 isoforms mediate endocytosis (79).

CD163 also exists as a soluble form (sCD163) and this soluble homogenous protein has been found containing more than 90% of the extracellular portion of nine SRCR domains (90), thus confirming that sCD163 is not a splice variant but rather a shedding product of the receptor from a site near the plasma membrane of monocytes and macrophages due to enzymatic cleavage (91).

1.3.2 Function of CD163

Scavenger receptors such as CD163, are a subclass of membrane bound pattern recognition receptors, which function to recognize and remove a diverse range of ligands including 'self-molecules' such as apoptotic cells, mineral laden debris of damaged proteins and also exogenous unmodified molecules or components such as microorganisms or foreign particles (92). As a scavenger receptor CD163 has been found to internalize and degrade tumour necrosis factor (TNF)- like weak inducer of apoptosis (TWEAK) which is a secreted cytokine belonging to the TNF superfamily (93). There are multiple interaction sites between TWEAK and CD163, including the Hb-Hp recognition site. Functional studies have demonstrated that the interaction of TWEAK with CD163 leads to neutralization of both molecules, suggesting that CD163 may function as a 'decoy' receptor for TWEAK (93). In addition, CD163 has been proposed to function as an erythroblast adhesion molecule, wherein CD163 promotes

the growth and survival of the erythroblast (94). CD163 has also been proposed to be an innate immune sensor for bacteria, as it binds to both gram-negative and gram-positive bacteria, subsequently promoting local immunity (95). The most widely known and most extensively studied established function of CD163 however is that of a scavenger receptor which endocytoses Hb-Hp complexes (84). Following this discovery in 2001, CD163 was also named the haemoglobin scavenger receptor (HbSR) (85). During pathological conditions of infections, trauma or autoimmune diseases, wherein the number of red blood cells undergoing lysis substantially increases, free Hb is released into the blood circulation. Free Hb must be removed from the circulation due to the oxidative and toxic properties of the iron-containing heme present in Hb (96). Free Hb is highly toxic due to its ability to rapidly destroy the nitric oxide deoxygenation region of proteins and to produce reactive oxygen species (ROS) leading to an oxidatively stressed environment (97). As depicted in Figure 1.2, upon release into the circulation, Hb binds to the plasma glycoprotein Hp with high affinity in a strong non-covalent interaction (98). The newly formed Hb-Hp complex interacts with high affinity via a newly exposed neo-epitope with the third SRCR domain of CD163 in a calcium and pH dependent manner (85), then the receptor-ligand complex is internalized to early endosomes (99). Subsequently, CD163 recycles to the plasma membrane (99) and the Hb-Hp is complex progresses to lysosomal degradation, the normally oxidative heme is converted there to overall anti-inflammatory molecules: CO, Fe^{2+} and bilirubin (100). Therefore this removal of free Hb from plasma prevents inexpedient oxidative stress caused by heme (101, 102). Furthermore, the binding of Hb-Hp complex to CD163 elicits a direct anti-inflammatory effect via the secretion of IL-10, which in turn up-regulates CD163 expression, thereby amplifying Hb-Hp scavenging via a positive feedback loop (84, 103).

Therefore, by binding to both endogenous and exogenous ligands, CD163 participates in the initiation and perpetuation of the inflammatory response exhibiting anti-inflammation functions.

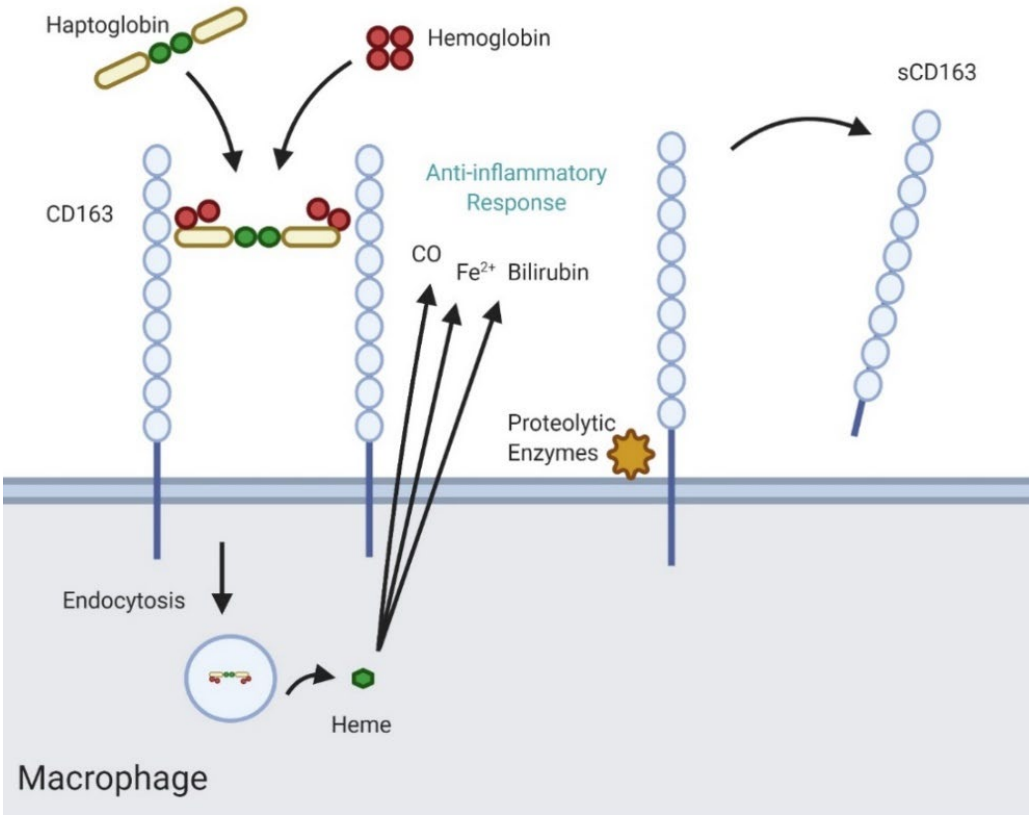


Figure 1.2. The structure and anti-inflammatory function of CD163, and the formation of sCD163. CD163 is characterized by nine extracellular SRCR type B domains and the SRCR domain 3 is the crucial binding site for Hb-Hp complexes. In the presence of pathological conditions such as trauma or infections, red blood cells are lytic and release highly toxic free Hb into the circulation. Hb complexes with haptoglobin in a high affinity manner, this complex is then endocytosed by CD163 expressing macrophages in which Hb is degraded and converted to anti-inflammatory molecules CO, Fe^{2+} and Bilirubin. This anti-inflammatory function of CD163 is compromised by proteolytic enzymes which cleave CD163 from the cell surface of macrophages, disabling the clearance of toxic Hb from circulation (84, 101). Created in BioRender.com

1.3.3 Expression of CD163

The expression of CD163 is exclusively limited to cells of the monocyte and macrophage lineage. It was initially reported that CD163 has a low to moderate protein expression in human peripheral blood monocytes (81, 82, 104). However in a study that utilized monoclonal antibodies to the N-terminal portion of CD163, more than 80% of the monocytes in human peripheral blood were identified as being CD163 positive, indicating that most, and conceivably all human peripheral blood monocytes do express CD163 (105). Of the three different subpopulations of monocytes, the highest expression of CD163 is found on intermediate monocyte subset and the lowest on the non-classical subset (106). The expression of CD163 also correlates positively with monocyte activation and differentiation. In a human blister model, tracking the monocyte phenotype from wound to resolution, Evans et al found a significant increase in the proportion of circulating $CD163^+$ cells in whole blood 40 hours after a blister was created, suggesting that the monocyte population had undergone differentiation in concordance with the blister developing (107). This supported previous findings that $CD163^+$ monocyte expression was upregulated during the healing phase of wounded and inflamed tissue (108) and also in acute or chronic inflammatory conditions (103, 109). Furthermore in an *in vitro* study, human monocytes were isolated and cultured under non-adherent conditions with human serum in the absence of additional growth factors and this stimulated the differentiation of monocytes into macrophages. Tippett and group found that the frequency of CD163 expressing monocyte-derived-macrophages (MDM) rapidly increased over their lifetime, indicating that the surface CD163 expression increases during maturation of monocytes to macrophages (109).

Mature macrophages in tissues also express CD163, examples are the Kupffer cells in the liver, red pulp macrophages in the spleen, cortical macrophages of the thymus, resident mature bone marrow macrophages, perivascular and meningeal macrophages of the central

nervous tissue, peritoneal and alveolar macrophages and scattered macrophages present in various other tissues (110). This indicates that the tissue macrophages which have increased expression of CD163 are more mature than the circulating monocytes where CD163 is lower. (111).

1.3.4 Regulation of CD163

The expression of CD163 has been found to be regulated by a variety of factors during monocyte differentiation and maturation, including cytokines and the process of proteolytic cleavage from the monocyte cell surface membrane. Additionally systemic inflammation has been shown to cause an elevation in the levels of circulating CD163+ monocytes (103). Furthermore the expression of CD163 in tissues has been found increased during the resolution of the acute inflammatory response (107, 108). The binding of Hb-Hp complexes to CD163, appeared to increase CD163 expression through IL-10 in a positive feedback mechanism both *in vivo* and *in vitro* (103) indicating an anti-inflammatory role for CD163. *In vitro* studies have shown that the differentiation of monocytes into macrophages strongly induces CD163 mRNA and protein expression (109). Likewise, the *in vitro* administration of macrophage stimulating factor (M-CSF), a growth factor that induces differentiation of monocytes into macrophages, up-regulates CD163 mRNA and protein expression (106). Glucocorticoids have proven to be a strong inducer of CD163 expression both *in vitro* and *in vivo* (76, 112). In a human study, healthy volunteers receiving a glucocorticoid injection increased $CD163^{+}$ monocyte population by 80% in 6 hours (113).

Anti-inflammatory mediators such as IL-10 also strongly up-regulate CD163 mRNA levels in both monocytes and macrophages (106, 112). In contrast, pro-inflammatory mediators such as LPS, interferon-gamma (IFN-ɣ) and TNF suppress the expression of CD163 both at mRNA and protein levels (106, 114). Endogenous pro-inflammatory cytokines such as IL-1α

and IL-1β and chemokines such as CXCL-8, also decrease CD163 expression (112). Interestingly, transforming growth factor beta (TGF-β) which is an anti-inflammatory mediator inhibits CD163 (115). Another growth factor, granulocyte macrophage colony stimulating factor (GM-CSF) also down-regulates CD163 protein and mRNA expression (106). Additionally, hypoxia can down-regulate CD163 mRNA levels (116). The activation of toll-like receptors (TLRs), TLR-2, TLR-4 and TLR-5 leads to rapid down-regulation of CD163 protein, however this is a result of ecto-domain shedding of CD163 from the cell surface (117). Similarly the reduction in expression caused by LPS is also due to this extracellular proteolytic cleavage (118). The extracellular shedding of CD163 from the plasma membrane results in increased formation of its soluble form, soluble CD163 (sCD163), which will be described in section 1.3.5.

The cleavage of CD163 receptor from the monocyte cell surface can be attributed to ADAM17, an enzyme belonging to the "a disintegrin and metalloproteinase (ADAM)" group of transmembrane proteases (119). ADAM17 has been shown to cleave and thus down-regulate the surface expression of CD163 (120). Similarly, matrix metalloproteinases (MMPs), another family of proteinases, have also been reported to be implicated in the cleavage of CD163. More specifically MMP-9 has been found to be correlated with sCD163, thus implicating MMP-9 as a key agent in the shedding mediated down-regulation of CD163 (94). However, the role of MMPs in the regulation of CD163 in diabetes and its complications has not yet been fully established.

Matrix Metalloproteinases

MMPs are a family of 23 proteinases specifically characterised by a zinc-binding motif. These endopeptidases are zinc-dependent and calcium-dependent and are involved in the breakdown and remodelling of extracellular matrix components (ECM) and the basement

membrane (121). MMPs participate in different biological and physiological processes that are regulated by hormones, growth factors and cytokines (122). Based on their sub-cellular distribution and specificity for particular components of the ECM, MMPs are divided into collagenases, gelatinases, stromelysins, matrilysins and membrane-type matrix metalloproteinases (MT-MMPs) (121). Collagenases include MMP-1, MMP-8, MMP-13 and MMP-18 which degrade interstitial collagens I, II and III as well as other ECM proteins (121). Gelatinases (MMP-2 and MMP-9) mainly digest gelatin but also other ECM proteins such as collagens and laminin. Gelatinases are involved in the cellular processes of angiogenesis and neurogenesis, which the action of these proteases may lead to cell death (121). Stromelysins (MMP-3, MMP-10 and MMP-11) degrade segments of the ECM and also involve in pro-MMP activation (121). Matrilysins (MMP-7 and MMP-26) process cell surface molecules and digest ECM components (121). MT-MMPs (MMP-14, -15, and -16 and -24) can degrade collagen and can activate some proteases and components of the cell surface (121).

MMPs are secreted as inactive pro-enzymes or bound to cell surface membranes, and the activity of MMPs can be inhibited by the tissue inhibitors of MMPs (TIMPs)(123). Traditionally it was thought that MMPs only function in tissue remodelling and maintenance, however recent studies have demonstrated that MMPs can regulate the release or activation of chemokines, cytokines, growth factors and other bioactive molecules thus participating in innate and adaptive immunity, inflammation and angiogenesis (124). In conditions of oxidative stress, or in the presence of inflammatory stimuli, MMPs have been reportedly involved in the proteolytic shedding of CD163 from the monocyte cell surface, resulting in the formation of sCD163, and therefore the indirect regulation of the CD163 receptor presence on the cell surface (76). MMP-9 has been shown to be strongly positively correlated with plasma sCD163 concentrations, supporting the notion that MMPs, especially MMP-9

are vital in the regulation of CD163 shedding (94). Correspondingly a recent study found that in all diabetes subjects, MMP-2 was increased compared to control subjects, and in diabetes complications MMP-3 was increased in plasma (76). In this same study sCD163 was found to be increased in individuals with diabetes complications compared to those without, suggesting that it was derived from the proteolytic cleavage of CD163 from the cell surface, however the specific MMPs implicated have not been identified, and should be further explored (76). As such MMPs are implicated to be key potential regulators of the circulating CD163+ monocyte, due to ecto-domain shedding leading to the formation of sCD163.

1.3.5 Soluble CD163

sCD163 is a homogenous protein that spans 94% of the extracellular domain of the CD163, and is the product of extracellular cleavage from a site near the cell membrane (91). sCD163 has been detected in the serum and plasma of healthy individuals, at average concentration of 1.9mg/L (91, 125). However drastic increases are commonly observed during various pathological conditions that affect the monocyte-macrophage lineage including rheumatoid arthritis (RA), asthma, multiple sclerosis (MS) or celiac disease (101). Due to its ubiquitous presence not only in serum but also in tissue fluids such as cerebrospinal, synovial, or ascitic fluid, sCD163 has been proposed to be a clinical biomarker for certain inflammatory conditions (91, 126). The enzymatic cleavage of CD163 from the plasma membrane, is responsible for the inverse relationship between the cell surface protein CD163 and sCD163 (127). Notably, the ratio of sCD163 to the cell surface CD163 might be a better prognosis marker in certain diseases than sCD163 alone (94). In a recent investigation, sCD163 was found to be increased in the plasma of individuals with diabetes complications and strongly correlated with worsening renal function in diabetes complications (76). However there has not yet been a single cohort study investigating sCD163 changes in various subtypes of

diabetes-related micro- and macrovascular complications, or in the co-morbid condition of NAFLD.

1.3.6 CD163 and sCD163 in Health and Disease

CD163 is a marker of monocyte/macrophage activation and its expression is upregulated in acute and chronic inflammatory conditions as well as in the healing phase of inflammation (111, 128). As such, the soluble form of CD163 could serve as a biomarker in inflammatory disease conditions as shown in Table 1.2. This is observed in conditions such as Gaucher'disease and other chronic inflammatory conditions such as rheumatoid arthritis, asthma and multiple sclerosis (94, 128, 129).

Table 1.2: The direction of change in the levels of sCD163 in inflammatory conditions.

	Disease Condition	**Change in sCD163 Level**
Acute inflammatory conditions	Hepatitis (130)	↑
	Bacteraemia & Sepsis (131)	↑
	Malaria (132)	↑
	Meningitis (133)	↑
	Tuberculosis (133)	↑
	Ebola Virus Diseases (133)	↑
	Gaucher's Disease (128)	↑
Chronic Inflammatory conditions	Rheumatoid Arthritis (123)	↑
	Asthma (124)	↑
	Multiple Sclerosis (94)	↑
Low-grade inflammatory conditions	Obesity (134)	↑
	Liver Disease (134)	↑

	Diabetes (134)	↑
	Atherosclerosis (134)	↑

Gaucher's disease is characterized by an excessive accumulation of macrophages in the spleen and liver. Moller's research group observed from Western Blot analysis of spleen tissue biopsies, that the CD163 protein level was 1.5 – 2 fold higher in intensity in with Gaucher's disease than in controls (128). It was also found that the sCD163 level was 2-5 times higher in the plasma of individuals with Gaucher's disease compared with healthy controls (128). Moller's group concluded that the sCD163 level correlates with disease activity and therefore serves as a diagnostic marker which is useful in monitoring disease course and response to treatment (128).

Rheumatoid Arthritis (RA) is a chronic inflammatory condition wherein, $CD163^+$ cells have been found to accumulate in the affected synovial membrane (129). It has also been observed that the concentration of sCD163 was increased in the joint fluid of affected subjects compared to the paired serum samples from non-affected subjects, indicating sCD163 increase was from a local source (129). Whilst serum sCD163 is moderately increased in RA people (123), no clear relationship between disease severity and the CD163 level has been observed (101).

In the chronic inflammatory condition of asthma, Zhi and group reported a significantly lower percentage of $CD163^+$ monocytes and noticeably higher sCD163 from sputum in individuals with asthma compared to healthy controls (124). Furthermore they reported that both sputum sCD163 and serum sCD163 were significantly higher in individuals with severe asthma compared to those with mild or moderate asthma. A previous study reported a strong inverse correlation between membrane bound CD163 expression and plasma sCD163 levels

(127). In addition, Fabriek et al also investigated the relationship between membrane bound CD163 on monocytes and the plasma level of sCD163 in MS people (94). They reported the sCD163 as significantly elevated, while the cell surface CD163 on monocytes being decreased in people with MS compared to healthy controls, and reflected patients with a long duration of disease.

sCD163 is also significantly increased in acute inflammatory conditions such as infectious hepatitis (135), bacteraemia and sepsis (136), malaria (137), meningitis, tuberculosis and more currently Ebola virus infections (133). In recent times, increased plasma levels of sCD163 have been linked to states of low-grade inflammation such as obesity, liver disease, diabetes and atherosclerosis (134).

1.3.7 CD163 in Obesity and Insulin Resistance

Obesity is characterized by an excess of adipose tissue and low-grade inflammation which promotes insulin resistance and increases the risk of development of T2DM and its concomitant complications (138). Obesity-related inflammation is characterized by an inflammatory state in the blood mononuclear cells, infiltration of macrophage into visceral fat depots and their subsequent activation, which was often accompanied by elevated levels of inflammatory markers including IL-6, TNF-α, adiponectin and MCP-1 (139-141). The presence of increased sCD163 as a reflection of macrophage activation has only begun to be investigated within the context of obesity and insulin resistance. A recent study by Feldberg's group, sought to examine whether the phenotype of adipose tissue macrophages were altered in an obese state (142). Using RT-PCR on mRNA extracted from abdominal subcutaneous adipose tissue (SAT) macrophages, they demonstrated that the gene expression of CD163 was significantly increased in SAT of obese individuals in comparison with lean subjects (142). This phenomenon was also observed by Sindhu et al (143). Shakeri-Manesch et al reported

increased CD163 mRNA in both visceral adipose tissue (VAT) macrophages and SAT macrophages in obese individuals compared to control subjects (144). Correspondingly, elevated CD163 protein expression has been reported in the adipose tissue of both diabetes and non-diabetes obese individuals, confirming that CD163 gene and protein expression are upregulated in people with obesity compared with lean controls (143). Fjeldborg also reported a positive correlation between adipose tissue CD163 mRNA and the serum level of sCD163 (142), indicating an association between local inflammation within the fat depots, low grade systemic inflammation and tissue remodelling by MMPs as reflected by the rise in sCD163.

Currently only two studies have reported on the effect of obesity and insulin resistance on the CD163 expression from peripheral blood mononuclear cells (PBMCs). Satoh's research group isolated PBMCs using magnetic assisted cell sorting and flow cytometry and reported that there was a significant decrease in the surface expression of CD163 derived from obese diabetes individuals compared to obese non-diabetes individuals (145). In another study, the CD163 mRNA expression in PBMCs was measured in individuals with metabolic syndrome (146). They reported decreased gene expression of CD163 on PBMCs from obese subjects compared with lean, and that an even greater decrease in CD163 occurred in obese diabetes individuals compared with their lean counterparts. Therefore, it appears well established that in conditions of obesity, insulin resistance and diabetes, the gene and surface expressions of CD163 in PBMCs were significantly lower compared to a lean condition (145, 146).

Numerous other studies have confirmed the association of sCD163 with obesity and insulin resistance. In a cross-sectional study of T2DM patients, Kawarabayashi's group found the monocyte surface expression of CD163 was inversely related to insulin resistance and serum sCD163 was positively correlated with insulin resistance (147). A significant number of papers have reported that elevated sCD163 levels were found in the sera of obese individuals

compared to normal weight controls and patients with T2DM (77, 90, 141, 147-151). Furthermore, a univariate analysis confirmed that sCD163 was significantly and independently associated with BMI, waist circumference, SAT mass and VAT mass (141). Whilst obesity reveals a rise in serum sCD163, Fjeldborg's group reported that weight loss resulted in a reduction of sCD163, further confirming the significant correlation of sCD163 with BMI, weight circumference and insulin resistance (152).

Significant changes in cellular CD163 and sCD163 have been implicated in obesity and insulin resistance which are known to be central in the development of T2DM. Obesity and insulin resistance are considered to be in a state of low-grade inflammation and this was supported by the increased presence of macrophages, especially $CD163^{+}$ macrophages, in the adipose tissue of such individuals (142, 143). Equal support for a state of low grade systemic inflammation is offered by the increased presence of sCD163 in the circulation of obese individuals (142). Whilst obesity and insulin resistance can precede the development of T2DM in humans, a role for the changes of CD163+ cells, protein expression and sCD163 levels in the subsequent complications of diabetes has not been explored in great depth.

1.3.8 CD163 in Diabetes Complications

As described above inflammation has a central role in the development and progression of many diabetes complications, the expression of CD163 on circulating monocytes, tissue macrophages and sCD163 level in various body fluids, have been investigated in various diabetes complications. However, this data has not been collated sufficiently to identify to clear trends for the involvement of CD163 in the progression of diabetes complications. CD163 expression on circulating monocytes was found decreased in individuals with DR compared to those with diabetes without any complications (76). CD163+ tissue macrophages, however, were found increased in DR compared to individuals with other

ocular disease and no diabetes (153, 154). This finding contrasted to the report of CD163+ tissue macrophages being decreased in atherosclerotic plaque of individuals with diabetes and CVD (155). Interestingly sCD163 level has been observed increased in the vitreous fluid, plasma, serum and CSF of individuals with DR, DN, DPN, CVD and PAD (76, 151, 153, 154, 156, 157), therefore indicating that sCD163 level is different between individuals with and without diabetes complications. Correspondingly, the level of sCD163 has been found to be different in individuals with NASH compared to those without (76, 158). It has been reported that sCD163 may be a good non-invasive marker for the progression of NASH, however this has yet to be confirmed in a cohort that included diabetes as a majority. The ability of sCD163 to gage the severity of disease has been investigated in the progression of NASH, but not in the progression of diabetes complications.

Whilst the number of CD163+ monocytes has been found to be decreased in individuals with diabetes complications compared to those without, the profile differences of CD163+ monocytes between those with and without complications, and how these differences may contribute to the progression of diabetes complications, have yet to be defined (76).

1.4 Cells of the Immune System in Diabetes Complications

The human immune system is made up from a large network of organs, cells, proteins, and chemicals to protect body from the invaders which comprises of two main parts, the innate immune system and adaptive immune system (159). The innate immune system is the body's initial response to foreign molecules and pathogens. It responds in a non-specific and rapid manner and includes granulocytes and antigen presenting cells (APCs) such as monocytes/macrophages and dendritic cells (DCs) (159). In the instance where the innate system cannot eliminate infectious organisms or cannot recognize pathogens, the T and B lymphocytes of the adaptive immune system take over. The adaptive immune system is

comprised of T and B lymphocytes which together coordinate a highly specific immune response (160). Due to being antigen-specific, the adaptive immune system acts in accordance with a slower response time (160).

1.4.1 Monocytes and Macrophages

Monocytes are a heterogenous population of innate immune cells that account for ~10% of nucleated blood cells (161). They circulate in the peripheral blood and have the ability to migrate to the inflammatory site, but also exhibit plasticity by being able to transform into tissue macrophages (162). Human monocytes play a key role in anti-microbial immunity through phagocytosis and cytokine production as well as regulating other cells of the innate and adaptive systems (163). Monocytes can be categorized into three phenotypically and functionally distinct types depending on differences in the expression of lipopolysaccharide receptor CD14 and low-affinity FCγ receptor CD16 as: classical ($CD14^{hi}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$) and non-classical ($CD14^{lo}CD16^{+}$) monocytes (164).

Classical monocytes account for 80-90% of all monocytes, whilst intermediate and non-classical monocytes account for the remaining 10-20 % (165). Classical monocytes are primed for innate sensing and are highly phagocytic. They are also known to be important scavenger cells (165). Intermediate monocytes are responsible for the proliferation and stimulation of T cells, as they express higher levels of surface markers involved in the interactions between antigen presentation cells and T cells (165). These cells are also known for great production of ROS, and a role in angiogenesis (166). Non-classical monocytes are highly motile in nature and survey the luminal side of the endothelium in a 'crawling' manner to recognize pathogens and dying cells and to repair the vasculature (164, 167, 168). This behaviour suggests that non-classical monocytes are involved in maintaining vascular integrity by slowly and constantly surveying the endothelium for signs of inflammation or

damage, clearing cell debris and potentially transmigrating rapidly (75). As such non-classical monocytes may play role in wound healing. In addition, genes associated with cytoskeleton mobility such as *Rho GTPase*, Ras homolog family member *RHOC* and *RHOF* are highly expressed by the non-classical subset (165). This suggested that the non-classical subset of monocytes is highly motile in behaviour. In addition, non-classical monocytes are also involved in antigen presentation and T cell stimulation (166, 169).

After migrating into the tissue, monocytes undergo differentiation into macrophages, exhibiting diverse functions such as: 1) phagocytosis, 2) presentation of antigens and activation of lymphocytes, 3) release and stimulation of cytokine secretion, 4) modulation of the immune response, and 5) regulation of tissue reorganization after the inflammation process is no longer active (159). Additionally, macrophages can polarize into 'M1' or 'M2' phenotypes, influencing their function within the tissues (162).

Multiple diseases have been reported associated with changes in circulating monocyte subsets as well as in tissue macrophages. Intermediate monocytes are elevated in bacterial sepsis, dengue fever, Crohn's disease, cardiovascular disease and rheumatoid arthritis, whereas non-classical monocytes are more prevalent in periodontitis and reduced in stroke (164, 170-176). A recent investigation has found that the number of CD16+ monocytes was decreased in diabetes complications (75). However which specific CD16+ subset, intermediate or non-classical monocytes, were decreased in diabetes complications has not yet been investigated. Additionally the phenotype of monocytes and macrophages have been found to be altered by the diabetic environment and in the presence of atherosclerosis to become pro-atherogenic (177), such that monocytes and macrophages are identified as key players in the progression of atherosclerosis. Monocytes have been also reported to have: 1) increased recruitment to lesions 2) increased inflammatory activation; 3) altered macrophage lipid accumulation and metabolism; 4) increased macrophage cell death; and 5) reduced efferocytosis (177-181).

These investigations of monocytes/macrophages involvement in atherosclerosis have been conducted in animal models of diabetes (177) which support the idea that increased monocytes in circulation can result in increased monocyte recruitment to the artery wall, however increased monocyte population has not been consistently observed in people with diabetes. As such this thesis endeavoured to investigate the difference in the expression of the circulating subsets of monocytes between those with and without diabetes complications.

Human and mouse models of diabetes have also reported that diabetes causes monocytes to make a more inflammatory phenotype. For instance isolated CD14+ peripheral blood monocytes from T1DM and T2DM individuals expressed and produced increased levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, when compared to monocytes isolated from control subjects (177). This demonstrates that diabetes affects monocyte phenotype such that the function of the cells is impacted in a potentially pathological manner, essentially contributing to the development and progression of diabetes complications.

1.4.2 Dendritic Cells

Dendritic cells (DCs) are a heterogenous group of leukocytes that originate from either lymphoid or myeloid precursors. The diverse group of DCs varies by their anatomical locality, cytokine production pattern and immunological responses (182). DCs include plasmacytoid DCs (pDCs) from lymphoid precursors, classical DCs (cDCs) from myeloid precursors, and monocyte-derived DCs that differentiate from peripheral blood monocytes in response to inflammation (182). DCs are found in every part of the body including the skin, peripheral blood, mucosal surfaces, interstitial tissues, lymphoid and non-lymphoid areas of the body (183). DCs are highly motile cells with a stellate morphology that express high levels of MHC molecules and the integrin CD11c. They are specialized to work as sentinel cells which can also phagocytose, process and professionally present a myriad of antigens

including micro-organisms released by dead cells, extracellular fluid, and apoptotic cells which when processed to present on MHC class I and class II molecules to naïve T cells in the form of peptides (182). By presenting peptides from various damage associated molecular patterns (DAMPS) on the surface of MHC molecules to T-cells, DCs thus act as a crucial link between the innate and adaptive immune response (184). Furthermore, DCs prime immature T cells and further activate and generate memory T cells as well as inducing the activation of B cells (182). Dendritic cells also play a role in immune tolerance by maintaining immune homeostasis through continually presenting tissue-derived self-antigens to CD4+ and CD8+ T cells, therefore contributing to tolerance to self-antigens. As such DCs are viewed as the centre of the immune system as they serve a mediating role between innate and adaptive immune systems (182). Furthermore, DCs can secrete growth factors and cytokines that modulate ongoing immune responses (185).

Studies have shown that the numbers of DCs are reduced in both T1DM and T2DM (184). Seifarth et al have found that the numbers of both myeloid and plasmacytoid DCs are decreased in individuals with T2DM with poor metabolic control compared to health controls. It is suggested that the decrease in DCs could incline diabetes individuals as susceptible to opportunistic infections (184, 186). Another study has shown that the hyperglycaemic medium and sera derived from T2DM individuals prevented the maturation of monocytes into effective DCs and their activation *in vitro* (184, 187). Furthermore, some studies suggested that the function of DCs in diabetes complications was impacted. For instance, in a study of men with diabetes and chronic atherosclerotic complications, it was found that the number of circulating DCs were reduced as well as their capacity to produce pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. However this study was limited as it only focused on the plasmacytoid subset of DCs but not the myeloid DCs (188). In the diabetes complication, DN, DCs have been found to be significantly increased in the

interstitium of both experimental and human kidney diseases, and therefore DCs may be implicated in the pathological progression of DN (189). As such we endeavoured to phenotypically profile the function and activation markers of circulating DCs in diabetes complications.

1.4.3 Granulocytes

Apart from monocytes and DCs, granulocytes are another innate immune cell which are predominantly part of the early or acute phase of the innate immune response. These cells function to identify, ingest and destroy microbial pathogens through various mechanisms including 1) receptors; 2) oxidative mechanisms; 3) enzymes such as lysozyme, collagenase and elastase (159). Granulocytes can be further categorized into neutrophils, eosinophils, basophils and mast cells. Neutrophils are the most abundant and effective cells during the inflammation and phagocytosis process. They have stored granules containing enzymes that can lyse microbial pathogens (159). Eosinophils are less abundant than neutrophils, and are specifically localized to the respiratory, gastrointestinal and urinary tract. These cells conduct their effector function by degranulation and release of histamine, cationic proteins, major basic protein, sulfatases, and chemotactic factors such as leukotrienes and prostaglandins (190). Finally, basophils and mast cells are the lowest proportion of granulocytes in circulation, as they are located mainly in the mucosa. They differ from other granulocytes as the granules contain heparin, serotonin and histamine. They are mainly involved in allergic and viral processes (159).

Recent studies have found that neutrophil behaviour and function are altered by hyperglycaemia associated with diabetes, such that hyperglycaemia causes aberrant activation of neutrophils resulting in increased production in reactive oxidation species which may play a pathological role in the progression of complications (191). The activation of neutrophils by

diabetic hyperglycaemia elicits an inflammatory immune response through oxidative bursts and release of DAMPS. A recent cross-sectional study found that a higher neutrophil-to-leukocyte ratio (NLR) was associated with an increased prevalence of DN and DR in individuals with diabetes (192). As such we endeavoured to characterize neutrophils, to identify functional and activation markers that may be implicated in diabetes complications.

1.4.4 T Cells

The adaptive immune response involves T cell-mediated immunity which is an adaptive process of developing antigen specific T lymphocytes to eliminate viral, bacterial or parasitic infections or malignant cells (193). T lymphocytes are produced in bone marrow and then migrate to the thymus through the bloodstream, where they mature. Peripheral T cells include a myriad of subsets including naïve T cells, which to respond to new antigens, memory T cells which derive from pervious antigen activation and maintain long-term immunity, and regulatory T (Treg) cells which regulate the immune response (194).

Upon antigen presentation by APCs such as DCs, the naïve T cells encounter the antigen and produce cytokines, proliferate and differentiate into the effector cells that migrate to diverse sites to promote pathogen clearance (160). Activated T cells have a relatively short lifespan, however a proportion of them remain as memory T cells from several months to several decades (194). CD4+ and CD8+ T cells account for the majority of T lymphocytes. Following activation and differentiation into distinct effector subsets CD4+ T cells are involved in mediating the immune response through the secretion of specific cytokines (195). Furthermore CD4+ T cells execute numerous functions such as the activation of innate immune cells, B lymphocytes, CD8+ T cells as well as non-immune cells, and also the suppression of the immune response (195). CD4+ T cells encompass various subsets including classical T-helper 1 (Th1), Th2, Th17, follicular helper T cell (Tfh) and induced T-

regulatory cells (iTreg) (195). The differentiation of CD4+ T cells into the above mentioned subsets depends on the complex network of specific cytokine signalling and transcription factors (195). CD4+ helper T cells also play a critical role in diabetes complications. Activated T lymphocytes and inflammatory cytokines have been found increased in the kidneys in individuals with T2DM (196). Furthermore Th17 is a pro-inflammatory subset secreting IL-17, and has been found increased in diabetic nephropathy (197).

Similarly recent investigations have found CD8+ T cells to be implicated in diabetes complications. CD8+ recognize antigens presented on the MHC class I molecule on APCs and are predominantly cytotoxic. They use various mechanisms to kill their targets. A study in individuals with T2DM and PAD reported that CD8+ T cells are increased in the ischemic tissues of individuals with T2DM and PAD and therefore be involved in the pathogenesis of PAD in T2DM (198). However, the role that CD8+ T cells play in the pathogenesis of diabetes complications has not been fully established. Another study has found that high glucose conditions alter the phenotype of CD8+ T cells such that CD8+ T cells are recruited into the tissues of DPN, and further mediate cytotoxicity towards Schwann cells therefore playing an important role in the development of DPN (199).

Tregs which constitute 5-20% of total CD4+ T cells have also been found to be altered by diabetes. Tregs function to control adaptive immune responses by suppressing T cells, NK cells, B cells and DCs. They are crucial in the maintenance of peripheral tolerance and down-modulate the amplitude of the immune response as well as prevent autoimmune diseases (193, 200). In a systematic review investigating changes in Tregs in individuals with T2DM, it was found that individuals with T2DM had decreased percentage of peripheral CD4+CD25+Foxp3+Tregs, and this was further reduced in individuals with complications (201). Therefore Tregs, in conjunction with CD4+ T helper cells and CD8+ cytotoxic cells

are influenced by the condition of diabetes and may contribute to the pathogenesis of diabetes complications.

1.4.5 B Cells

B lymphocytes represent 5-15% of circulating blood lymphocytes and are derived from the bone marrow (202). They are a population of cells that express clonally diverse cell surface immunoglobulin receptors which recognize specific antigenic epitopes (160).

B cells are involved in humoral immunity, wherein once activated by T helper cells they have the ability to proliferate and transform into plasmacytes and rapidly produce large amounts of antibodies (203). Additionally, B cells are also involved in antigen presentation, allowing them to interact with cells involved in cell-mediated immunity and produce cytokines (203).

Studies have found that the adaptive B cell response is impaired in individuals with obese and diabetes, which may contribute to increased systemic inflammation, when compared to individuals without diabetes (204). This indicates that diabetes induces functional changes in B cells that may play a pathological role and may contribute to the development of diabetes complications.

1.4.6 Natural Killer Cells

Natural Killer (NK) cells are members of the innate lymphoid cell family and comprise approximately 10% of all peripheral blood lymphocytes. They are characterized in humans by the expression of the CD56 marker and the absence of CD3 marker. NK cells have the ability to kill virus-infected or tumour cells by the degranulation of their cytotoxic vesicles; additionally NK cells can also release pro-inflammatory cytokines (205). Traditionally NK cells were only identified as killer cells, however recent technological advancements have enabled NK cells to be re-characterized as important mediators and influencers of adaptive immune responses, therefore highlight their immunoregulatory functions (206). Human

mature NK cells are usually divided into two subsets based on the surface expression of CD56 and CD16 as: $CD56^{bright}$ $CD16^{-}$ cells, which their function is to produce cytokines and are more abundance in secondary lymphoid tissues; $CD56^{dim}$ $CD16^{+}$ cells, which are involved in stronger natural cytotoxicity, and are predominantly present in peripheral blood, accounting for 90% of all NK cells (207). The expression of CD56 indicates the degree of activation (208). Recent studies have indicated that diabetes affects NK cells by reducing their function. For instance, a recent animal study reported that NK cells isolated from mice with T1DM or T2DM had reduced toxicity against cancer cells, and furthermore the reduced toxicity was correlated to the duration of diabetes. This study also showed that in diabetic mouse models, expression of activation markers on NK cells was reduced (207). Another study reported that in individuals with long-duration T2DM, NK cells demonstrated reduced functional abilities, such as reduced degranulation (209). This could possibly explain why individuals with T2DM are highly susceptible to infection and additionally may have increased incidence of some tumours. Despite these recent developments characterizing NK cells in diabetes, the functional differences and role of NK cells in the progression of diabetes complications is still ambiguous.

1.5 Thesis Hypothesis and Aims

The background provided above in the literature review, provides evidence that supports the central hypothesis of this thesis: that alterations in the circulating CD163+ monocytes and sCD163 level are impacted in diabetes complications and secondly that functional differences in CD163+ monocytes contribute to the development of diabetes complications and sCD163 can serve as both a biomarker for certain complication of diabetes and a marker for disease progression.

In summary, the aims of this thesis were to:

1. Systematically analyse human studies published up until January 31, 2022, to assess CD163 expression, including cell surface CD163 and sCD163, in diabetes complications and to determine whether CD163 can be implicated as a potential biomarker or mediator in the progression of diabetes complications from analysis of the consolidated data.
2. Determine whether the level of sCD163 is different in those with diabetes and various complications including NAFLD and whether sCD163 has clinical utility as a biomarker to assess complication status and severity in a diabetes cohort.
3. Deeply characterize the circulating CD163+ monocytes in individuals with diabetes complications compared to those without at both gene and protein levels by RNA sequencing and mass cytometry.
4. Investigate whether there are phenotypic alterations in circulating immune cells across individuals with and without diabetes complications including monocytes, DCs, granulocytes, T cells, B cells and NK cells as well as their subsets.
5. Investigate the potential pathways responsible for the regulation of circulating CD163+ monocytes in diabetes complications including cytokines, chemokines and MMPs.

This study will offer a comprehensive understanding of the involvement of CD163+ monocytes in diabetes complications, potentially providing valuable insights for targeted treatments or biomarker to prevent or treat complications in diabetes in future.

Chapter 2: Materials and Methods

As described in Chapter 1, 4 out of 5 aims in this thesis were investigated the changes of CD163 in diabetes complications. The focus was on identifying differences in circulating CD163 monocytes and sCD163, immunophenotype and transcriptome between individuals with and without diabetes complications, and to determine the regulation of CD163 in diabetes complications.

This chapter of the thesis is intended to supplement the methodology described in the manuscripts including Chapters 3, 4 and 5 as the word limitations of published manuscripts. The full methodology utilised in Chapters 6 and 7 is described in detail in this chapter. The relationships between the major studies in this thesis is depicted in Figure 2.1.

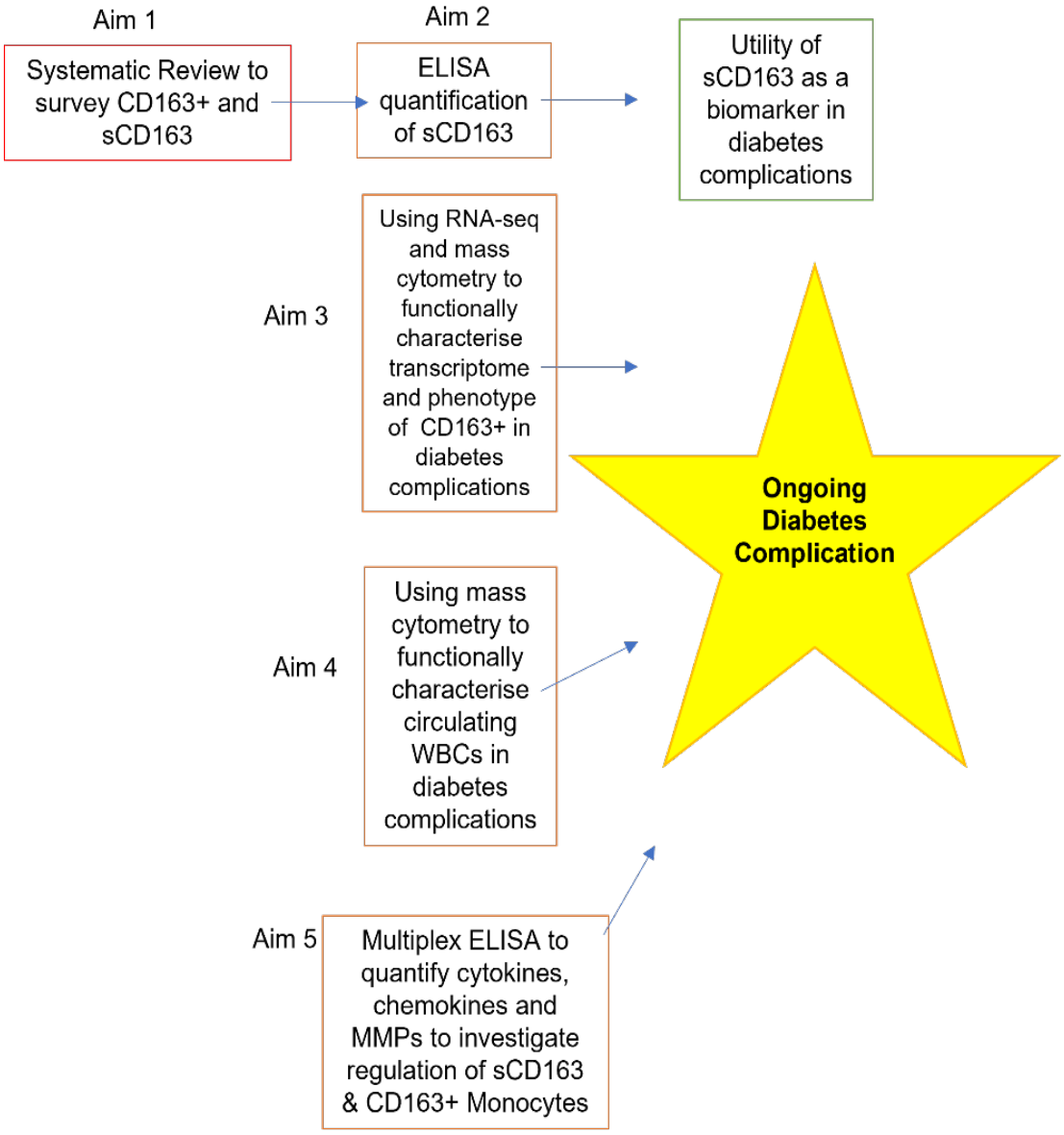


Figure 2.1. Diagram representation of the aims investigated in this thesis. Aim 1 involved a systematic review; aim 2, ELISA to quantify sCD163; aim 3, to characterise the transcriptome and phenotype of CD163+ monocytes, aim 4, to characterise the phenotype of other white blood cells (WBCs) and aim 5, to quantify circulating proteins in individuals with and without diabetes complications.

2.1 Methods for Systematic Review

The first study is a systematic review which aimed to examine human studies on the expression of CD163 at both gene and protein levels from various sources including tissues and peripheral blood in diabetes complications. Furthermore, it also aimed to investigate whether CD163 may be implicated in the disease progression. The curated keyword search for published articles up until January 31, 2022, was conducted following the guidance of an

experienced librarian from the University of Sydney. Following the search, the PRISMA workflow was utilised to distinguish included and excluded studies according to a pre-set criteria between 2 independent reviewers. Details on the complete methodology have been published as a peer-reviewed manuscript during this PhD candidature and has been included in Chapter 3.

Siwan E, Twigg SM, Min D. Alterations of CD163 expression in the complications of diabetes: A systematic review. Journal of Diabetes and its Complications. 2022 Feb 9:108150.

2.2 Experimental Procedures

The subsequent investigations in this thesis aimed to determine the plasma sCD163 levels by ELISA assay, immunophenotype the circulating CD163+ monocytes and other immune cells by mass cytometry, and profile the transcriptome of circulating CD163+ monocytes by RNA-sequencing. In addition, the potential regulators of CD163+ monocytes including circulating chemokines, cytokines and MMPs were investigated by multiplex ELISA assays.

2.2.1 Study Participants

Two different study cohorts have been utilised across the investigations in Chapter 4-7. The participants used in Chapter 4 "*Circulating soluble CD163 as a potential biomarker of diabetes complications*" were derived from the DASH Study previously investigated by Williams and colleagues (210). These participants had either T1DM or T2DM, were evaluated in accordance with a full diabetes complications assessment; as well as liver steatosis presence measured by ultrasound, and liver stiffness measurement (LSM) measured by transient elastography (TE). Since this cohort included individuals with liver steatosis, the exclusion criteria were alcohol consumption exceeding 140g per week and/or 40g per day, current hepatitis B or C infection or autoimmune or metabolic liver disease. The full detailed inclusion and exclusion criteria have been described by Williams and colleagues (210). The

non-alcoholic fatty liver disease fibrosis score (NFS) was calculated to classify participants as either low, indeterminate or high risk for clinically significant liver fibrosis. All participants were provided with the written informed consent prior to undertaking any study procedures. All procedures described were approved by the relevant New South Wales Health Human Research Ethics Committees (HRECs; Protocol NO X11-0105 & HREC/11/RPAH/144) and the University of Sydney HREC and were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

For the investigations pertaining to CD163+ monocyte phenotype, CD163+ cell RNA profile and CD163 regulation as well as immunophenotyping of circulating immune cells, the respective cohorts have been described in Chapters 5-7. The number of participants used in each chapter has been described in Supplementary Table 2.1.These investigations used cohorts derived from an original cohort that included adults who had diabetes for a duration of greater than 10 years, and were recruited from the Diabetes Centre, Royal Prince Alfred Hospital. The inclusion and exclusion criteria for this cohort has been previously described in our earlier publication (75). The participants were sorted according to the presence or absence of diabetes complications; complication status was determined based on the presence of retinopathy confirmed by fundal examination and/or photography. Diabetic nephropathy was confirmed by abnormal serum creatinine and urinary albumin-creatinine ratio >2.5mg/mmol for men and >3.5 mg/mmol for women. The presence of macrovascular disease was determined by any relevant symptom of vascular disease or if the participant had a reported history of abnormal investigation of or a prior macrovascular event. This study was approved by the Human Ethics Review Committee of Sydney Local Health District (HREC Protocol No X14-0074 (Prev X08-0255) & HREC/08/RPAH/431) and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. All participants gave the written informed consent.

2.2.2 Blood Sample Processing

For the DASH study used in Chapter 4: fasting blood samples were obtained from all participants and cell free plasma was stored at -80˚C for ELISA analysis later.

For the investigations pertaining to CD163+ monocyte profile and immunophenotyping, fresh venous fresh whole blood was collected into the EDTA collection tubes. CD163+ monocytes were isolated from the fresh collected blood. In addition, 0.5mL blood was aliquoted into a cryotube with 0.7mL Smart Tube Proteomic Stabilizer (Smart Tube Inc., California, USA) and incubated at room temperature for 10 minutes before being stored at -80 ˚C for immunophenotyping later. The remaining blood was centrifuged following which cell free plasma was aliquoted into 1.5mL cryotubes and stored in a -80˚C freezer.

2.3 Laboratory Measurement

2.3.1 ELISA Assay

The concentration of plasma sCD163 in individuals with and without diabetes complications was quantified by the Human Soluble CD163 ELISA kit (Aviscera Bioscience, CA, USA). The cohort for this investigation also included individuals with liver steatosis and has been described in section 2.2.1. Plasma samples were thawed for 30 minutes at 4˚C then were diluted 1:200 using the provided dilution buffer included in the ELISA kit. All reagents were reconstituted as per the manufacturer's protocol, all reagents including standards, positive control, detection antibody concentration and wash buffer were reconstituted. 100µL of dilution buffer was added to the blank wells of a 96-well ELISA plate, then the serial diluted standards, positive controls and samples were added to designated wells in duplicate respectively. The plate was sealed and incubated for 2 hours on a microplate shaker at room temperature. After that the plate was washed with 300 µL of wash buffer and aspirated three times. 100µL of detection antibody was added to each well, and the plate was sealed and

incubated for 2 hours. Next, 100µl of Streptavidin-HRP conjugate was added to each well and the plate was then incubated for 1 hour on a microplate shaker at room temperature, and washed three times according to the manufacturers' instructions. Following the addition of 100µl of substrate solution to each well and the plates were incubated with shaking for 3-7 minutes. Finally, the addition of 100µl of stop solution and the optical density of each well was measured using the TECAN® Infinite M1000Pro Plate Reader (Palm Springs, USA) set at 450nm. A log-log standard curve was generated with a coefficient of variation of <20%.

2.3.2 CyTOF

Mass cytometry, also known as cytometry by Time-Of-Flight (CyTOF) is used to identify and determine the quantity of specific CD markers on single cells. This high-dimensional, high throughput technology enables immunophenotyping including the analysis of immune cell function and activation (211). In CyTOF, white blood cells are incubated with antibodies tagged with specific and unique non-radioactive heavy metal isotopes. The *Helios* machine (Fluidigm, California, USA) then nebulizes and processes the cell mixture as single-cell suspensions. The single cells are ionised, then separated by mass-to-charge ratio, and can be distinguished by the unique metal isotope associated with each antibody. The amount of antibody bound can be detected in the mass spectrometer for each cell and therefore will correspond to the expression levels of the target antigen (211). Flow cytometry was the traditional method to immunophenotype immune cells, however this was limited by the overlapping of the limited emission spectra of fluorescence-tagged antibodies. CyTOF employs metal isotopes to mitigate signal overlap, enabling more accurate and precise analysis (212). Utilising CyTOF we were able to design a panel of 37 different CD markers that identified and quantified circulating immune cells and their phenotypes. In addition, the panel also included the markers to characterise CD163+ monocytes. The completed panel of CyTOF markers is listed in Table 2.1.

CyTOF was used for the quantification and characterisation of CD163+ monocytes, as presented in Chapter 5. The methods used in data analysis of CD163+ monocytes are presented in Chapter 5. Additionally, the specific methods and data analysis for the quantification of other immune cells and their subsets are presented in Chapter 6.

Table 2.1 Antibodies used for mass cytometry in this study

Metal		Company	Target	Marker purpose
^{106}Pd	Palladium	Biolegend	CD45	Lineage/Barcode
^{108}Pd	Palladium	Biolegend	CD45	Lineage/Barcode
^{143}Nd	Neodymium	BD	CD45RA	Activation
^{147}Sm	Samarium	Biolegend	CD45RO	Activation
^{113}In	Indium	Biolegend	CD61	Lineage/platelet
^{167}Er	Erbium	Abcam	CD66	Lineage
^{160}Gd	Gadolinium	BD	CD14	Lineage
^{148}Nd	Neodymium	Biolegend	CD16	Lineage
^{170}Er	Erbium	Biolegend	CD3	Lineage
^{168}Er	Erbium	Biolegend	CD4	Lineage
^{145}Nd	Neodymium	Biolegend	CD8a	Lineage
^{142Nd}	Neodymium	Biolegend	CD19	Lineage
^{176}Yb	Ytterbium	Miltenyi	CD56	Lineage
^{174}Yb	Ytterbium	Biolegend	HLA-DR	Lineage
^{169}Tm	Thulium	Biolegend	CD25	Activation
^{165}Ho	Holmium	Biolegend	CD127	Activation
^{171}Yb	Ytterbium	Biolegend	CD282(TLR-2)	Activation
^{149}Sm	Samarium	BD	CD284(TLR-4)	Activation
^{172}Yb	Ytterbium	BD	CD38	Activation
^{151}Eu	Europium	Biolegend	CD192(CCR2)	Chemokine receptor
^{144}Nd	Neodymium	Bio-Rad	CD195(CCR5)	Chemokine receptor
^{164}Dy	Dysprosium	Biolegend	CX3CR1	Chemokine receptor

^{163}Dy	Dysprosium	Miltenyi	CXCR3	Chemokine receptor
^{209}Bi	Bismuth	Biolegend	CD11b	Adhesion
^{115}In	Indium	Biolegend	CD11c	Lineage and adhesion
^{155}Gd	Gadolinium	BD	CD31	Adhesion
^{152}Sm	Samarium	Biolegend	CD36	Phagocytosis and clearance
^{153}Eu	Europium	Biolegend	CD68	Phagocytosis and clearance
^{175}Lu	Lutetium	Biolegend	CD39	Immune regulation
^{173}Yb	Ytterbium	Biolegend	CD73	Immune regulation
^{162}Dy	Dysprosium	BD	CD80	Immune regulation
^{156}Gd	Gadolinium	BD	CD86	Immune regulation
^{139}La	Lanthan	Biolegend	CD206	Immune regulation
^{159}Tb	Terbium	R&D	ADIPOR1	Metabolic function
^{166}Er	Erbium	Abcam	ADIPOR2	Metabolic function
^{141}Pr	Praseodymium	Biolegend	CD15	Activation
^{158}Gd	Gadolinium	Biolegend	VEGFR2	Adhesion

Due to the fact that data acquisition in the *Helios* is time-consuming, a barcoding technique was incorporated into the protocol. The use of barcodes allows for multiple samples to be processed and acquired as a single sample, and later during data analysis, the paired samples (one from diabetes with complications and another from without complications) can be separated and analysed individually.

2.3.2.1 Thaw and Red Blood Cell Lysis Procedure for Fixed Blood Samples

A previously established method was utilised to stain white blood cells. Whole blood samples were previously stored with proteomic stabilizer (Smart tube Inc., California USA) in a -80˚C freezer. Blood samples were then transferred from the -80˚C freezer into a 4 ˚C fridge, where they thawed for 45 minutes. Following which the samples was mixed and incubated for 1 minute with 1mL of Thaw-Lyse Buffer 1 (TLB1) (Smart Tube Inc., California, USA). The solution was then filtered through a 70µm cell strainer (Falcon®), mixed and incubated with 10 mL TLB1 at room temperature for 10 minutes. After that the solution was then centrifuged at 600 x *g*, 4˚C for 5 minutes, the supernatants were poured off and pellets resuspended in TLB1 with incubation for 10 minutes before centrifugation again. The cell solution was then washed twice with TLB2 (Smart Tube Inc., California, USA), and finally the pellet resuspended in 1mL FACS buffer (0.5% BSA, 0.02% NaN_3, 2mM EDTA in PBS), and stored on ice.

2.3.2.2 Antibody Staining for Mass Cytometry

Each individual sample, after lysis of red blood cells, was counted with trypan blue, and then up to 2.0 x 10^6 cells were transferred into a falcon tube. Samples were then centrifuged for 8 minutes at 500 x g at room temperature. The cell pellet was resuspended in FACS solution with heparin (100µL heparin in 600µL FACS) and incubated for 20 minutes at room temperature. After two washing cycles in FACS buffer, the samples were barcoded using ^{106}Pd-CD45 and ^{108}Pd-CD45 and incubated on ice for 30 minutes. After three FACS buffer washing cycles, each barcoded sample was combined with an alternatively barcoded sample, and paired samples were stained with cell surface antibodies listed in Table 2.1 and incubated on ice for 30 minutes. Following three final washes in FACS buffer, DNA intercalator

(1:2000 Iridium intercalator in 1mL Perm Buffer and 1mL 4% paraformaldehyde) was added to samples, incubated at room temperature for 20 minutes, followed by 12 hours at 4˚C.

After the 12-hours incubation, the samples were washed once with FACS buffer and once with ultrapure water before being resuspended in Cell Acquisition Solution (Fluidigm, California, USA). Subsequently cells were counted on a haemocytometer using trypan blue and resuspended cells at 1 x 10^6 cells/mL in ratio 1:10 EQ calibration beads (Fluidigm, California, USA) and ultrapure water. Samples were then filtered into a FACS tube and all events were acquired in the time-of flight mass cytometer, *Helios*.

2.3.2.3 Optimizing Antibody Titration for Fixed Whole Blood

A trial experiment was initially conducted in order to determine the optimal concentration of antibodies to be used in the final experiment. A donated blood sample was collected in an EDTA tube, and cryopreserved with proteomic stabiliser, following thawing and red blood cell lysis, the remaining white blood cells were stained with serial dilutions of the full panel of cell surface antibodies at concentrations of 8µg/mL, 4µg/mL, 2µg/mL, 1µg/mL and 0.5µg/mL. A control sample containing gating antibodies at well-known titrated concentrations previously was included. As described above, following the overnight incubation with DNA intercalator, samples were prepared with EQ calibration beads and filtered before acquiring 100,000 events on the *Helios* Mass Cytometer. The results of this titration, pertaining to the optimal concentration of antibodies have been presented in Chapter 6, Table 6.1.

In instances where the antibody did not exhibit clear separation, FMO (fluorescence minus one) controls, shown in Supplementary Figure 2.1 as an example, were included. In addition, the antibody was gated within a population known to be negative for this respective antibody,

facilitating a more accurate determination of the gating boundaries as shown in Supplementary Figure 2. 2.

2.3.2.4 Mass Cytometry Data Analysis

Following data acquisition, Flow Jo version 10.7.1 (FlowJo LLC., Oregon, USA) was used to analyse the data. A manual gating strategy was developed and utilised for the quantification of each major leukocyte subtypes including monocytes, dendritic cells, granulocytes, T-cells, B-cells and NK cells, under which various activation and function CD markers were quantified by 'frequency of parent' and classified as subsets. Figure 2.2 depicts a representative manual gating strategy for the identification of monocytes (CD14+HLADR+). Normalisation beads were excluded, then single cells were gated on event length against DNA. The paired D^{+Comps} and D^{-Comps} individuals' samples were gated for the expression of ^{106}Pd-CD45 and ^{108}Pd-CD45, thus de-barcoded and resolved for individual samples. The remaining acquired events were manually gated by selecting positive and negative cells for the leukocyte population of choice as well as excluding other cell subtypes, in order to attain the desired leukocyte subtype. For the selection of CD14+ monocytes, the exclusion of other cell populations was necessary due to co-expression of CD14+ previously found with CD66+ granulocytes and CD3+ T cells (213, 214), and also in the current dataset (Supplementary Figure 3) .Thus individual and customised manual gating strategies were established to identify all leukocyte subtypes including dendritic cells, granulocytes, T-cells, B-cells, NK cells, and monocytes including classical, intermediate and non-classical monocytes. Various activation and function markers were gated as a subset of each leukocyte subtype, and these cells were quantified as a 'frequency of parent". Gated populations were then compared for cell population frequency between the experimental groups.

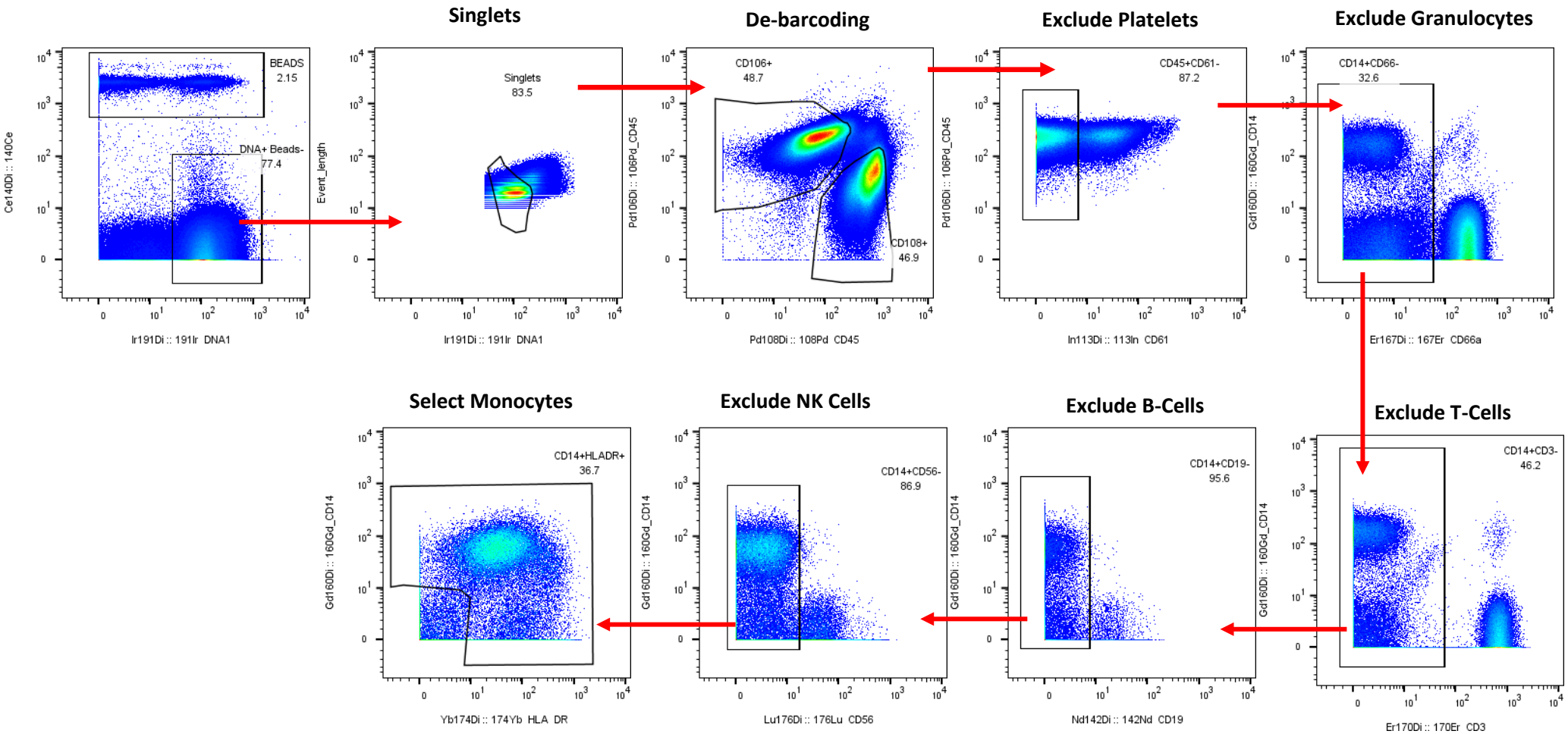


Figure 2.2: A flow diagram of the manual gating strategy used to identify monocytes (CD14+HLADR+). DNA+ events were selected, and thus normalisation beads were excluded, singlets were then gated. Total cells were separated into individual patient samples by de-barcoding. Platelets (CD61+) were excluded and then the leukocyte population of investigation (monocytes) was positively gated with other leukocyte subtypes excluded in a negative selection process.

2.3.3 RNA-sequencing

RNA-sequencing is a modern high-throughput technique that provides insight into the transcriptome of cells, by quantifying gene expression. The data generated by RNA-sequencing is most frequently used to analyse differential gene expression between two different cohort groups (215). For this study, we have sought to examine the differentially expressed genes in CD163+ cells between individuals with and without diabetes complications. The workflow involves RNA extraction, mRNA enrichment or ribosomal RNA depletion, cDNA synthesis and preparation of a sequencing library, which is then sequenced at a depth of 50 million reads per sample on a high-throughput platform Illumina. Following data acquisition computational methods and platforms are utilised to process the raw data including the removal of adaptors, aligning the reads to a reference human transcriptome, quantifying the reads and finally the filtering and normalizing between samples. The detailed methods are presented in Chapter 5.

2.3.3.1 Isolation of CD163+ Cells

Human CD163+ monocytes were isolated from fresh blood by fluorescence activated cell sorting (FACS) FACS Aria (Becton Dickinson, San Jose, CA, USA) using fluorescent-conjugated monoclonal antibodies to CD45-PerCP-Cy5.5 (Becton Dickinson, San Jose, CA, USA), CD14-PE-Cy7 (Becton Dickinson, San Jose, CA, USA), and CD163-APC (R&D Systems, Minneapolis, MN, USA). The dye diamidino-2-phenylindole (DAPI) (Invitrogen, Waltham, MA, USA) was used to allow identification of dead cells. The CD163+ cells were stored in RPMI media after sorting with a purity of 95%.

2.3.3.2 RNA Extraction and Sequencing

A subset of samples including six individuals with and six without complications was selected for RNA profile analysis which was selected from the samples with the highest

number of isolated CD163+ cells. The QIAGEN RNeasy Micro Kit (QIAGEN, Germany) was utilised to extract total RNA from isolated CD163+ cells according to the manufacturer's protocols, and the concentration of extracted RNA was determined using NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). At this point, the extracted RNA was sent to the Australian Genome Research Facility (AGRF), wherein quality and purity of the extracted RNA was assessed by electropherogram analysis using Agilent Bioanalyzer RNA Nano Chip Bioanalyzer (Agilent Technologies, USA). Quality RNA was then converted into sequencing ready libraries using Illumina Stranded Total RNA Prep, following which the Illumina Ribo-Zero Plus rRNA Depletion Kit was used to remove abundant RNA by enzymatic depletion. RNA from each of the total 12 samples were normalised at 25ng, and then progressed using the Illumina bcl2fastq 2.20.0.422 pipeline to generate the sequence data by NovaSeq 6000 System (Illumina, San Diego, CA, USA). Sequence libraries were prepared at 150bp paired end sequencing, with a depth of 50 million reads. The data generated met the quality standards, and the resultant FASTQ files were saved and used for data analysis later.

2.3.4 Multiplex Immunoassay

In order to determine the potential regulatory effect of cytokines, chemokines and MMPs on circulating CD163+ monocytes and sCD163; multiplex immunoassay was used to determine the concentration of cytokines, chemokines and MMPs. The MILLIPLEX Multi-Analyte Profiling (MAP) Human Cytokine/Chemokine Kit and MMP kit (Merk, Germany) were employed to quantify the circulating levels of proteins as listed in Table 2.2.

Table 2.2 Cytokines, chemokines and matrix metalloproteinases (MMPs) included in the study panel.

Group of Circulating Protein	Circulating Protein
Cytokine	**IFN-α2**
	IL-1α
	IL-4
	IL-6
	IL-8
	IL-10
	IL-12p70
	IL-15
	TNF-α
Chemokine	**MDC**
	MCP-1
	MCP-3
	IP-10
	MIP-1α
	MIP-1ß
Growth Factor	**VEGFα**
Matrix Metalloproteinases	**MMP-1**
	MMP-2
	MMP-3
	MMP-7
	MMP-9
	MMP-10
	MMP-12
	MMP-13

All reagents used were purchased from Merk (Millipore, Massachusetts, USA). The MAGPIX® analyser with xPONENT® software analyser was calibrated and verified prior to the commencement of the immunoassay. Plasma samples were collected and stored as described in section 2.2.2; they were then thawed for 30 minutes at room temperature, vortexed for 10 seconds and centrifuged for 5 minutes at 1500 x *g* to remove particulates, prior to use in the assay. In accordance with the manufacturer's protocol the working

standards, control solutions, matrix solutions, beads and wash buffers were prepared. The preparation of the plate involved the 96-well plate to be washed with 200µL of wash buffer including agitation on a plate shaker for 10 minutes at room temperature. Subsequently the wash buffer was thoroughly decanted and 25µL of working standards to added to the designated wells, 25µL of assay buffer added the sample wells, 25µL serum matrix solution was added to the standard and control wells and finally 25µL of plasma was added to the sample wells. Following vertexing for 30 seconds, 25µL of the premixed beads was added to each well. The plate was sealed using a plastic plate sealer, wrapped in foil, and placed on plate sharker with agitation for incubation for 16 hours at 4°C.

The plate was then attached onto a hand-held magnetic washer for 60 seconds, and then the wells were thoroughly decanted. The plate was removed from the magnetic washer, wells washed by adding 200µL wash buffer and then agitated on the plate shaker for 30 seconds at 500rpm. This washing process was repeated twice.

Next, 25µL of detection antibodies were added to each well and the plate was incubated for 1 hour on the plate shaker at room temperature, followed by 25µL of Streptavidin-Phycoerythrin. The plate was then sealed, wrapped in foil and agitated on the plate shaker for 30 minutes at room temperature. Proceeding three rounds of washing, 150µL Drive Fluid (Luminex, Texas, USA) was added to each well and the plate was incubated for 5 minutes at room temperature, before being run on the MAGPIX® analyser.

For each protein analyte, the xPONENT® 3.1 software automatically generated a range of standard curves including 4. Plog, 5. Plog or 'best-fit' which were the manufacturer's recommended procedure. The quality of each standard curve was analysed using a 4 point criteria which involved i) inter-assay %CV <15%; ii) R^2 close to 1; iii) recovery 70-130%;

iv) sigmoid shaped curve. The standard curve that satisfied each of these criteria was utilised as the curve from which the plasma concentration of the analytes was derived.

2.4 Statistical Analysis

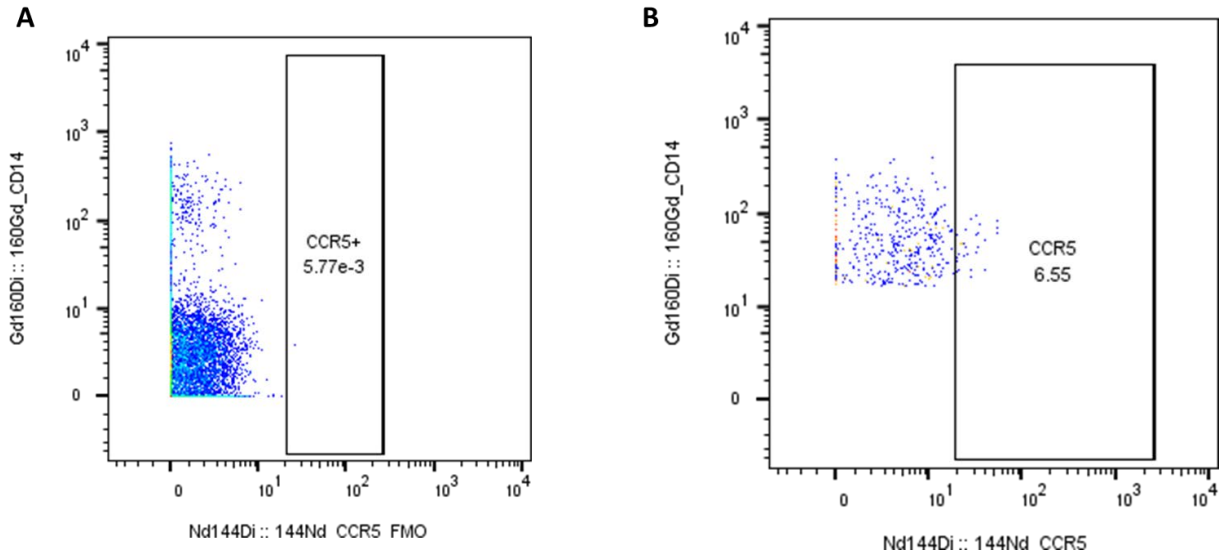
The GraphPad Prism 9 statistical package (GraphPad Software, San Diego, CA, USA) was utilised to statistically analyse all the data generated. All continuous data were examined for normality and presented as mean ± standard deviation (SD) for parametric data, median and IQR or proportion for non-parametric data as indicated. Grouped data were compared by either Welch's t-test for parametric data, or Mann-Whitney test for non-parametric data or Fisher's exact test to identify differences between groups. Significance was accepted at $p<0.05$, and Bonferroni correction was used to determine the adjusted p value. According to the normality test, the non-linear regression Spearman's correlation coefficient was used to verify the significance of correlations between the proportion of CD163+ cells and circulating proteins, or the sCD163 level and circulating proteins.

Supplementary Material

Supplementary Table 2.1. Cohort size of groups: diabetes cohort without and with traditional complications; in each of three investigations; including RNA-sequencing (Chapter 5), Immunophenotyping (Chapter 6), and Multiplex ELISA (Chapter 7).

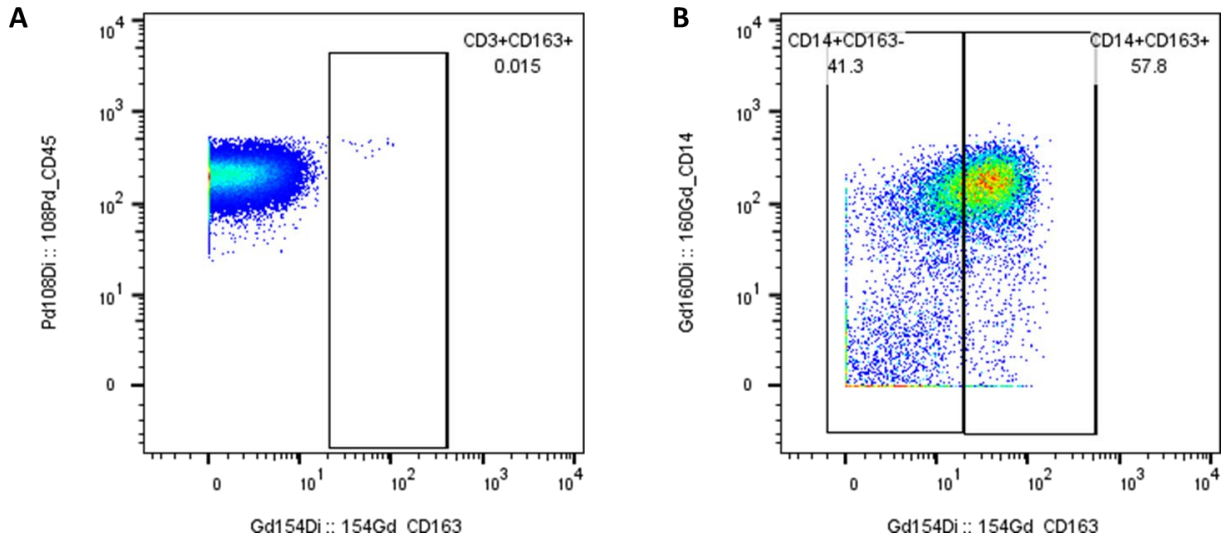
Chapter	Diabetes without traditional complications (n)	Diabetes with traditional complications (n)
5	6	6
6	13	8
7	12	6

Supplementary Figure 2.1.



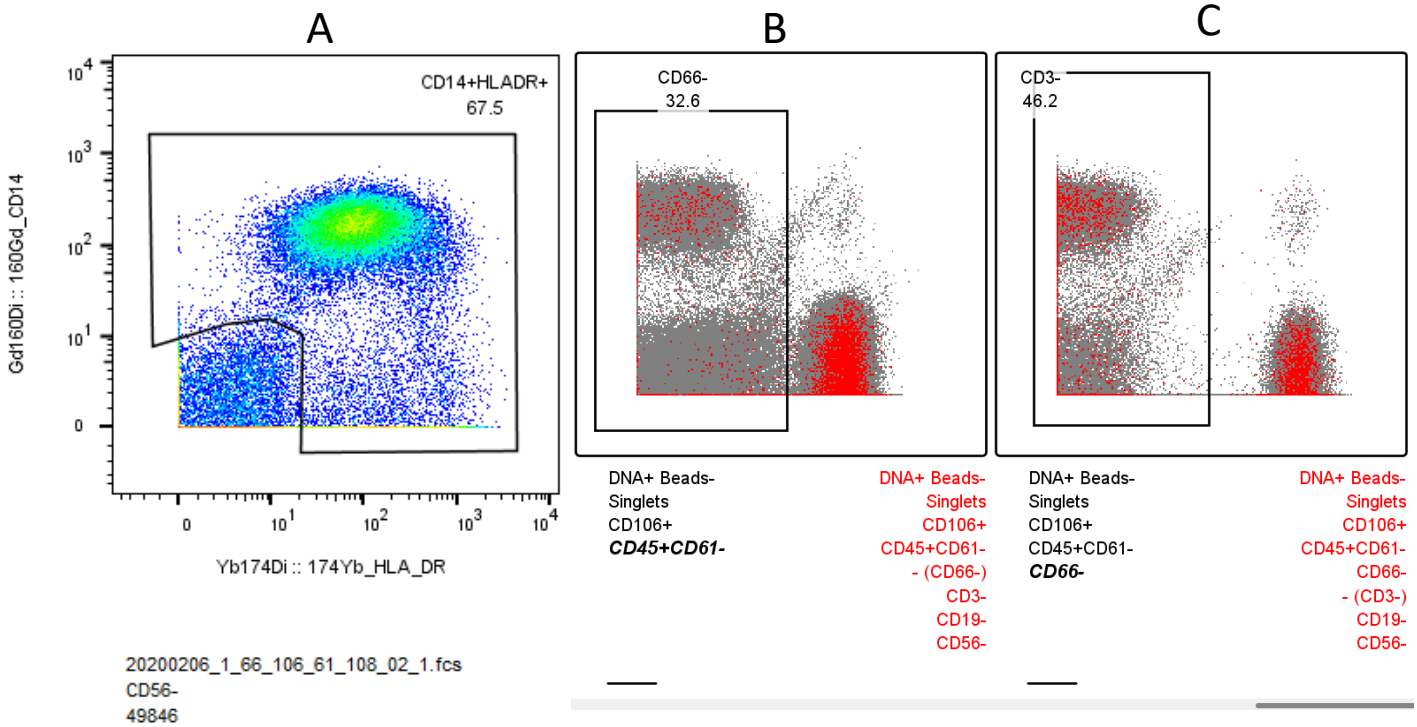
Supplementary Figure 2.1: Gate determination using FMO for CCR5. A and B are representative images depicting the gating of CD14+CCR5+ population by using an antibody panel without CCR5 (A) and with CCR5 (B).

Supplementary Figure 2. 2.



Supplementary Figure 2.2: Gate determination in a negative population. A) depicts the CD163+ gate being determined from a CD3+ population, which is known to negative for CD163 staining. B) depicts the CD163+ gate being applied to CD14+ monocytes, thus selecting for CD14+CD163+ monocytes.

Supplementary Figure 2.3.



Supplementary Figure 2. 3: Back-gating of CD14+ Monocytes: Flow-go back-gating diagram of CD14+ monocytes (A); Back-gating CD14+ cells on CD66 (B). The ungated CD66+ cells contain CD14+, indicated by red dots. Back-gating CD14+ cells on CD3 (C) and the ungated CD3+ cells contain CD14+, indicated by red dots.

Chapter 3: Alterations of CD163 Expression in The Complications of Diabetes: A Systematic Review

Presented as a manuscript.

This chapter presents the findings from a systematic review of human studies investigating CD163 levels in diabetes complications. The review was conducted with the initial guidance from a USYD librarian and in accordance with the PRISMA workflow. The levels of CD163 in tissues and peripheral blood at gene and protein levels including cell surface and sCD163 were collated and surveyed in order to identify trends in diabetes complications, and to assess if CD163 can be implicated in the progression of diabetes complications. Whilst this investigation has already been published by the *Journal of Diabetes and its Complications*; all permissions have been granted to include it in this thesis.

This chapter is presented as the published peer-reviewed manuscript:

> **Siwan E**, Twigg SM, Min D. Alterations of CD163 expression in the complications of diabetes: A systematic review. Journal of Diabetes and its Complications. 2022 Feb 9; 36 (4):108150.

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Alterations of CD163 expression in the complications of diabetes: A systematic review

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ABSTRACT

Aims: Diabetes mellitus is a state of chronic low-grade inflammation. Scavenger receptor CD163, expressed on monocyte/macrophage cells with anti-inflammatory functions, has been observed in diabetes complications. This review aimed to systematically survey human studies published until 31st January 2022 for CD163 expression, in particular diabetes complications and additionally to investigate whether CD163 may be implicated as a biomarker of, and mediator in, the progression of diabetes complications.

Methods: A systematic literature search undertaken in Scopus, Embase and Medline established 79 papers of relevance. Data extraction and assessment followed the PRISMA workflow.

Results: Based on specific criteria, 11 studies totalling 821 participants were included in this review. CD163 was quantified in various forms including soluble, cell surface, and mRNA measures. This review found that soluble CD163 was upregulated in diabetes complications in various local body fluids and systemically in plasma or serum and therefore implicated in the progression of those complications. CD163+ cells and mRNA were variably expressed across diabetes complications.

Conclusions: CD163 was altered in series of diabetes complications and the circulating sCD163 has potential utility as an inflammation biomarker. The variable expression of CD163 on cell surfaces and its mRNA across different diabetes complications warrants further systematic investigation.

1. Introduction

1.1. Current state of diabetes complications

In present times, diabetes has proven to be one of the most common non-communicable diseases globally.[1] The increasing prevalence of diabetes foreshadows the rise of diabetes complications which themselves contribute to much of the premature mortality and morbidity of the disease.[2] This reality reflects a need for new and sustained investigations concerning the development and management of diabetes complications.

A large proportion of individuals with diabetes will develop long-term vascular complications. Microvascular complications affect intricately vascularised organs such as the nervous system, retina and kidneys and can be clinically diagnosed as diabetic polyneuropathy (DPN), diabetic retinopathy (DR) and diabetic nephropathy (DN).[3] Macrovascular complications characterise disorders of the large blood vessels such as diabetic cardiovascular disease (CVD) and peripheral artery disease (PAD). Another diabetes-related complication which pathologically has tissue inflammation present is the multifactorial diabetes-related foot ulceration (DFU).[3]

Organ complications of diabetes tend to be progressive in nature and can extend to a state of irreparable damage. DN is a leading cause of end stage renal disease (ESRD). DR is a common cause of working age blindness, and DFU is the major risk factor for amputations.[1] Current therapies are typically ineffective in reversing or preventing further

Abbreviations: CAD, coronary artery disease; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CSF, cerebrospinal fluid; CVD, cardiovascular disease; DFU, diabetic foot ulcer; DN, diabetic nephropathy; DPN, diabetic polyneuropathy; DR, diabetic retinopathy; ESRD, end stage renal disease; FVMs, fibrovascular membranes; GC, glucocorticoids; Hb-Hp, hemoglobin-haptoglobin; IBD, inflammatory bowel disease; IFN-γ, interferon gamma; IL, interleukin; ROS, reactive oxygen species; PAD, peripheral artery disease; sCD163, soluble CD163; TNF, tumour necrosis factor.

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damage at affected organ sites, and are only predictably effective in slowing disease progression.[2] A therapeutic target that can halt the progression of vascular complications is yet to be determined and requires greater attention. Studies in immunological and inflammatory involvement in the progression of vascular complications could reveal a therapeutic target to help prevent diabetes complications progression at a tissue level or more holistically.

Chronic low grade systemic inflammation is phenotypic of diabetes; additionally, local inflammation features at affected organ sites as complications progress alongside the developing pathophysiology, which can promote further tissue injury.[4,5] The function and nature of the inflammatory milieu present at affected organ sites is undefined, especially which immune pathways in diabetes complications are essentially protective rather than markers and/or pathological.[3]

1.2. Scavenger receptor CD163

As an anti-inflammatory haptoglobin hemoglobin scavenger receptor, CD163 is a marker of monocyte and macrophage activation, and it therefore may act as a proxy indicator for the degree of inflammation present.[6] Functionally, this receptor is most well described to endocytose free circulating toxic hemoglobin (Hb) via hemoglobin-haptoglobin (Hb-Hp) complexes[7] (Fig. 1). This clearance of free circulating Hb is a highly beneficial anti-inflammatory function as it counteracts toxic Hb which otherwise can produce reactive oxygen species (ROS) creating an oxidative stress to environment.[8]

The membrane bound form of CD163 is exclusively limited to cells of the monocyte and macrophage lineage and its expression is upregulated by anti-inflammatory mediators such as interleukin (IL)-10, IL-6 and glucocorticoids (GC).[9–11] A positive feedback loop created by the binding of Hb-Hp complexes to the CD163 receptor also results in enhanced expression.

Pro-inflammatory mediators such as tumour necrosis factor (TNF), interferon gamma (IFN-γ), and IL-4 downregulate the expression of CD163, and increased shedding of the extracellular domain of CD163 from the plasma membrane results in decreased membrane bound CD163 and increased formation of its soluble form, soluble CD163 (sCD163).[12] Recent investigations have found that various acute and chronic inflammatory conditions cause dysregulation in the expression of CD163.[12,13]

1.3. CD163 and sCD163 in inflammatory conditions

CD163+ macrophages have been found to accumulate locally at sites of chronic inflammation such as in rheumatoid arthritis and this was accompanied by a greater concentration of sCD163 found in local synovial joint fluid.[13] In the chronic inflammatory condition of asthma, CD163+ macrophages were significantly reduced, and sCD163 significantly increased, in sputum samples.[14] The systemic expression of CD163+ monocytes was reported to be downregulated in individuals with multiple sclerosis, especially with a long duration of disease. This was partnered with a significant elevation of sCD163 in the plasma.[15] In recent times, increased plasma levels of sCD163 has been linked to states of low-grade inflammation such as obesity, liver disease, diabetes and atherosclerosis.[16–19] Correspondingly in the chronic inflammatory condition of diabetes, in a novel investigation, our research group reported that circulatory CD163+ monocytes are significantly decreased and plasma sCD163 is increased in individuals with diabetes complications compared to those without complications.[20] However, the presence of CD163+ monocytes/macrophages or sCD163 in microvascular or macrovascular diabetes complications has not been systematically profiled, analysed for trends, or reviewed.

1.4. Aim of systematic review

Due to the progressive nature of many diabetes complications, there is a greater need for understanding their respective pathophysiology. Since CD163 is a marker of macrophage activation and sCD163 is reflective of local and systemic inflammation, there will likely be benefit in profiling the expression of CD163 and sCD163 across individual diabetes complications.

As an expansion from our previous original article findings, this

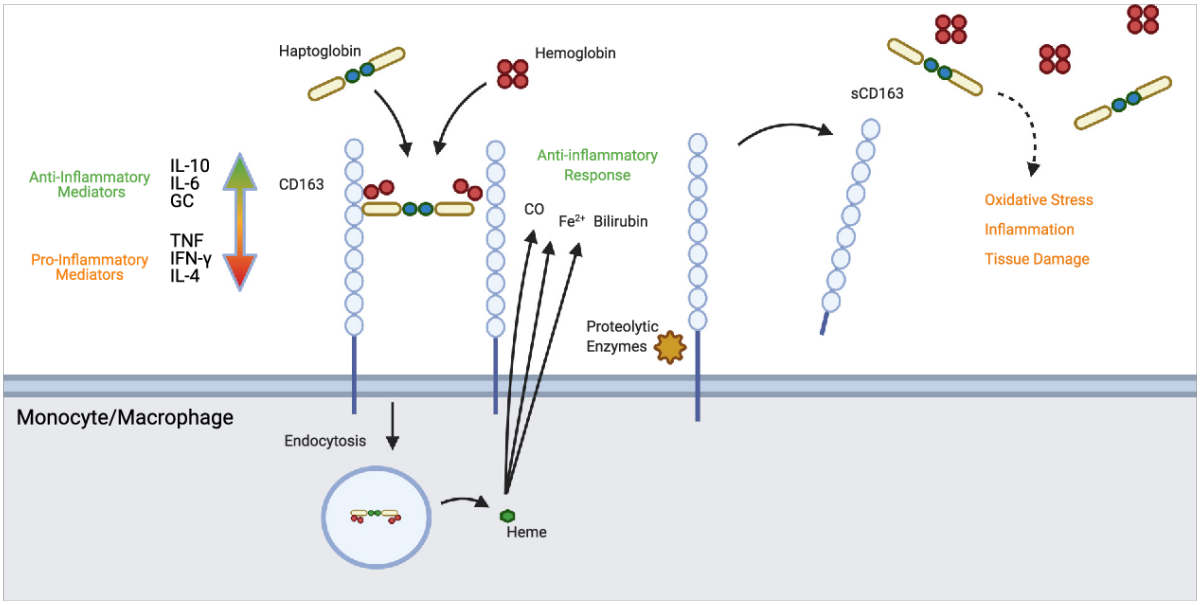

Fig. 1. The structure, anti-inflammatory function of CD163, and the formation of sCD163.
In the presence of pathological conditions such as trauma or infections, lytic red blood cells release highly toxic free hemoglobin (Hb) into the circulation. Hb complexes with haptoglobin (Hp) in a high affinity manner, and this complex is then endocytosed by CD163 on macrophages in which Hb is degraded and converted to anti-inflammatory molecules CO, Fe^{2+} and Bilirubin. This anti-inflammatory function of CD163 is compromised by proteolytic enzymes which cleave CD163 from the surface of macrophages, disabling the clearance of toxic Hb from the circulation.[7,45] Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

current systematic review aimed in diabetes complications to survey human studies and report the empirical data on the expression of CD163 pertaining to tissues and peripheral blood at gene and protein levels including cell surface CD163 and sCD163. Additionally, it also aimed to investigate whether CD163 may be implicated in affecting the progression of diabetes complications. The resultant data from this systematic review thus aimed to establish if CD163 may be implicated as a biomarker of, and mediator in, the progression of diabetes complications.

2. Materials and methods

2.1. Search protocol and study eligibility

A systematic search was conducted to identify published studies investigating CD163+ cells, protein, mRNA and sCD63 in various diabetes complications (DR, DN, DPN, CVD, PAD, DFU) in humans between 1946 and January 31st, 2022. A protocol for key search terms (Appendix: Supplementary Table 1), inclusion and exclusion criteria were established as determined by the authors. The search was performed in Scopus and Ovid Database which included platforms of: MEDLINE, EMBASE and AMED. Articles were limited to English language and full text availability. To ensure that all the most relevant studies and key articles were found, no publication parameters were set other than requiring human, not preclinical, studies.

In performing literature searches "CD163", "sCD163" and its alternate names "hemoglobin-haptoglobin scavenger receptor" were searched alongside varying terms for diabetes, as "diabetes mellitus", 'T1DM' or 'T2DM'. The complications were searched as 'diabetic nephropathy' and 'retinopathy' and the alternate search terms such as 'diabetic kidney disease' and 'diabetic eye disease' were also included. 'Peripheral neuropathy' was also used as a search term synonymously with 'nerve damage'. Similarly, 'diabetic foot disease' was searched with terms 'diabetic wound' or 'diabetic ulcer' or 'diabetic foot disease' or 'diabetes-related foot disease'. The synonyms for diabetes complications were searched by the term 'OR' and then combined by 'AND' to the synonyms for CD163.

Research studies found through the literature search were exported to Endnote, which was then used to organise and maintain the articles found. Following the literature search, this systematic review was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.[21] The titles and abstracts of the exported studies were screened, and duplicates were removed from the search results. If the article was deemed suitable, the full text was assessed. Eligibility of the papers was assessed by reading the abstracts and method sections of the publications. Studies were considered 'not eligible' if any of the following were realised: i) the study group had disease conditions other than a traditionally recognised diabetes-related complication; ii) if isolated primary cells were stimulated with any reagents prior to CD163 quantification; iii) if the study was an animal study; or iv) if the authors of the research paper in question did not respond to an essential request for study details of the specified finding. Studies were deemed eligible if any of the following were realised: i) CD163 was measured in soluble form in any body fluid; ii) CD163 protein or mRNA was measured in any tissue organ; or iii) membrane bound CD163 was measured in unstimulated cells. Following the first selection round, the studies were selected for a full-text screen, and these second round selected studies were reviewed for inclusion by two reviewers. The number of studies included in the review is indicated by 'n'.

2.2. Data extraction

The included studies were analysed for details regarding study type and control groups, duration of diabetes and/or complications, form of CD163 measured (membrane bound, soluble, protein or mRNA), the tissues or body fluids investigated, whether the diabetes complications were graded by severity, and whether there was a reported association between CD163 expression and disease progression. The level of expression (increase, decrease or no difference) of CD163 was reported utilising the significance threshold used in individual studies.

2.3. Risk of bias assessment

The study design of each included study was determined. The Newcastle-Ottawa Scale (NOS) was used to assess the risk of bias and quality of each included study, with information provided from the paper or by the authors from individual studies.[22] The prospective study in the series was assessed using a 9 point scale based on criteria of cohort selection, comparability of cohorts regarding design or analysis and adequacy of study outcome measures.[22] The case-cohort studies were examined using the same criteria, however, they were assessed by a 10-point scale. A score of 6 and above was considered low risk of bias.[16]

3. Results

3.1. Search results

Results of the systematic search and article inclusion process are presented in Fig. 2. A total of 93 papers between 1946 and 31st January 2022, were identified, 14 of which were duplicates and removed. The remaining 79 studies were screened based on title and abstract, and of this 51 were excluded based on the depicted reasons. The full text of the remaining 28 studies were reviewed and 17 studies were excluded based on the exclusion criteria. Finally, 11 human studies met the inclusion criteria and qualified for further qualitative assessment.

Overall, 11 papers with 821 participants were included in this review, and comprised: 5 case-cohort studies,[20,23–26] 1 case-cohort investigation within a cross-sectional study,[27] 1 case cohort within a case control study,[28] 1 case controlled study,[29] 2 retrospective cohort studies using autopsy samples[30,31], and 1 prospective case cohort.[32]

The included studies reported on diabetes complications were found to be distributed as: DR (n = 3), DN (n = 3), CVD (n = 2), DPN (n = 1), PAD (n = 1), and DFU (n = 1). Across the reports CD163 expression was examined and quantified in various forms including soluble form, cell surface or at the gene expression level; and compared to: healthy controls, non-diabetes related disease conditions at the same organ site as a diabetes complication, or diabetes without any complications or diabetes with more severe complication. The change in CD163 expression in diabetes complications compared with the aforementioned control groups, is reported in Table 1. All studies were assessed as being of low risk of bias: the NOS assessment tool[22] used for study quality, and subsequent results reflecting moderate to top high study quality, are provided within the Appendix (Supplementary Tables 2 & 3).

3.2. Diabetic retinopathy

Three studies investigated CD163 expression in DR. Soluble CD163 was measured in vitreous fluid (n = 2), serum (n = 1) or plasma (n = 1).[20,23,24] It was found to be 8 ($p < 0.001$) and 10 fold ($p < 0.0001$) significantly increased in the local ocular vitreous fluid of patients with proliferative diabetic retinopathy (PDR) compared to patients with non-diabetes related ocular disease conditions from two separate studies.[23,24] Systemically, sCD163 was also found to be significantly increased by 1.5 fold ($p < 0.05$) in plasma of DR patients compared to diabetes individuals without any complications,[20] however in serum, sCD163 was not found to be significantly different between PDR patients and those with non-diabetic ocular disease.[23] When patients with PDR were categorised into those with or without fibrovascular membranes (FVMs), it was found that vitreous sCD163 was significantly elevated in patients with FVMs (72.45 ± 9.88 ng/mL) compared to patients without FVMs (34.22 ± 5.61 ng/mL)($p = 0.014$), indicating that sCD163 was increasingly present as DR worsens in progression.[23]

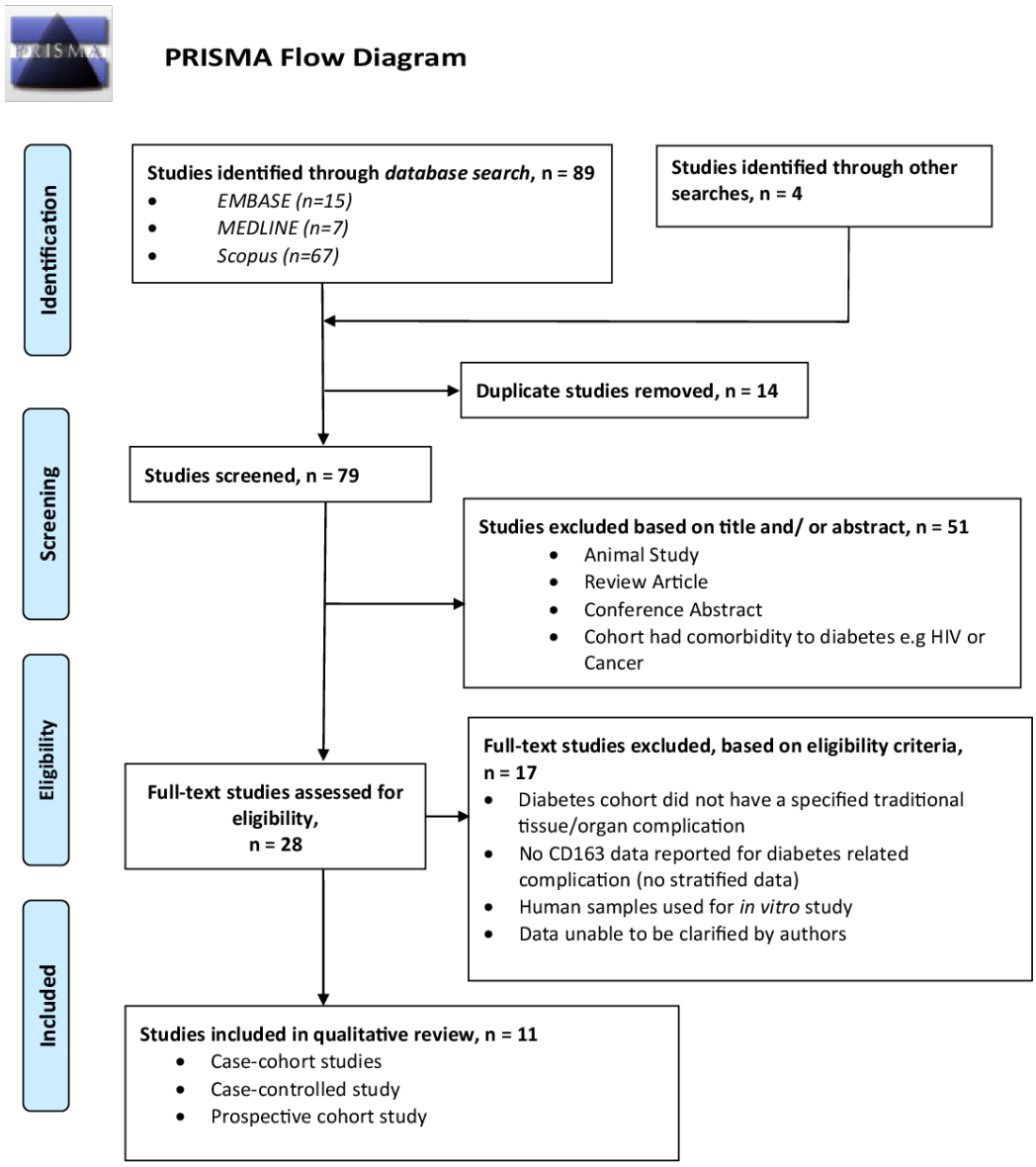

Fig. 2. Literature search PRISMA consort diagram depicting the summary of evidence search and seleciton.
The process of literature search for the profiling of CD163 in various forms (soluble, cell surface, protein and mRNA) in human studies of microvascular, macrovascular and other diabetes complications, is shown. Of the 93 studies identified, 14 duplicates were excluded and 51 excluded based on title and abstract. Subsequently, 28 studies were reviewed in full, by two authors. Finally 11 studies met the inclusion criteria and qualified for further analysis.

Furthermore, macrophage expression of CD163 was quantified by immunohistochemistry staining in FVMs from the surface of the neuroretina of patients with PDR and found to be significantly increased ($p < 0.001$) compared to those patients without diabetes but with other ocular conditions.[23] Additionally, El-Asrar et al. further characterised PDR FVMs into two categories according to severity of disease progression. Active neovascularisation in FVMs indicated greater disease severity, whereas inactive neovascularisation indicated a lesser progressed PDR. CD163+ macrophages were significantly elevated by 10 fold ($p < 0.001$) in the FVMs of patients with active neovascularisation compared to those with inactive neovascularisation. This indicates that CD163+ tissue macrophages are increasingly present as DR progresses in severity.[24] Whilst CD163+ cells in local tissue were increased in DR compared to non-diabetes individuals, CD163+ cell numbers in the peripheral blood measured by flow cytometry were found to be significantly reduced by 52.2 fold ($p < 0.05$) in patients with DR compared to individuals with diabetes who did not have any complicaitions.[20] In addition, Min et al. further reported no significant difference in blood monocyte CD163 mRNA expression, which indicated that the increase in plasma sCD163 and decrease in circulating CD163+ monocytes may potentially be due to increasing cleavage of CD163 from monocyte cell surfaces.[20]

3.3. Diabetic nephropathy

Three studies investigated CD163 expression in DN in the forms of sCD163 (n = 1), CD163+ cells (n = 1) and at the mRNA level (n = 1). In Samuelsson's prospective study, plasma sCD163 was measured in a diabetes cohort at the point of diagnosis.[32] After a period of 10 years, individuals with diabetes were assessed for the presence of nephropathy. It was found that sCD163 was trending higher at onset of diabetes in individuals who later developed DN (346 ng/mL), compared to those who did not (287 ng/mL)($p = 0.058$).[32] This indicates that plasma sCD163 has potential clinical utility as an early biomarker of DN,

Table 1
CD163 expression across various diabetes complications.

Organ and study number	Soluble	CD163+ Cells	mRNA	CD163 expression in worsening complication?
Diabetic retinopathy (n = 3)	Vitreous fluid↑[c] (n = 2)[23,24] Serum↔[c] (n = 1)[23] Plasma↑[b] (n = 1)[20]	Epiretinal FVMs blood vessels↑[c] (n = 2)[23,24] Blood↓[b] (n = 1)[20]	Blood↔[b] (n = 1)[20]	↑Vitreous fluid[d] (n = 2)[23,24] ↑Cell surface[d] (n = 1)[24]
Diabetic nephropathy (n = 3)	Plasma↑[b] (trend, n = 1)[32]	NR	Glomerular↑[c] (n = 1)[25]	↑ CD163+ cells with nephropathy progression[31]
Diabetic polyneuropathy (n = 1)[29]	CSF↑[b] (trend, n = 1) Serum↑[b] (trend, n = 1)	NR	NR	CSF sCD163 correlated with neuropathy progression
Cardiovascular disease (n = 2)	Serum↑[b] (n = 1)[27]	Plaque↓[c] (n = 1)[30]	Plaque↓[c] (n = 1)[30]	N/A
Peripheral artery disease (n = 1)[28]	Serum↑[d] (n = 1)	NR	NR	↑Serum[d] (n = 1)
Diabetic foot ulcer (n = 1)[26]	NR	Skin↑[a], ↔[b] (n = 1)	NR	N/A

The number of studies indicated as '**n**'. Direction of change is indicated by ↑ (statistically significant increase), ↓ (statistically significant decrease), ↔ (no significant difference).
NR = not reported.
[a] Indicated compared with healthy controls;
[b] Indicated compared with diabetes but without any complications;
[c] Indicated compared with disease conditions without diabetes;
[d] Indicated compared with exacerbated diabetes complication.

however whether the systemic level of sCD163 rises or remains elevated with progressive DN has not yet been investigated. At a kidney level, immunohistochemical analysis of CD163+ cells in glomeruli in DN demonstrated a positive correlation with progressive DN, and glomerular CD163 mRNA was found to be 5-fold significantly higher in individuals with DN compared to individuals with other nephrotic conditions but without diabetes.[25,31]

3.4. Diabetic polyneuropathy

One study investigated sCD163 in the cerebrospinal fluid (CSF) and serum from individuals with DPN.[29] The level of sCD163 in both CSF and serum was significantly higher in individuals with diabetes (CSF: 107 μg/L; serum: 2305 μg/L) compared to healthy controls (CSF: 84 μg/L; serum: 1420 μg/L) (both $p < 0.01$), however the level of sCD163 was only trending to being increased in individuals with DPN when compared to those with diabetes but without DPN who had other neuropathological conditions [CSF: 131 (86–173) vs 101 (70–190) μg/L, $p = 0.08$; serum: 3725 (920–7060) vs 2220 (1130–4780) μg/L, $p = 0.06$].[29] A neuropathy rank score was calculated for all participants with diabetes according to peripheral nerve function with the rank scores for neuropathy symptom score, neuropathy impairment score, nerve conduction study rank and vibration perception threshold. There was a trend towards a positive correlation between CSF sCD163 and neuropathy impairment score ($p = 0.06$), indicating that CSF sCD163 was near-correlated with neuropathy progression. No studies were reported on CD163+ cells and CD163 expression at protein or mRNA levels in DPN.

3.5. Cardiovascular disease/coronary artery disease (CAD)

Two studies investigated CD163 expression from individuals with diabetes and cardiovascular disease.[27,30] Serum sCD163 was significantly increased in those with diabetes and CAD [1.4 (0.9–3.8) μg/ mL] compared to those without CAD [1.1 (0.4–2.0) μg/mL]($p = 0.006$).[27] Contrastingly in a tissue level study as a retrospective autopsy study of atherosclerotic plaques, CD163+ cells and mRNA were found to be significantly decreased 1.8 fold ($p < 0.0001$) and 75% ($p = 0.002$) respectively in those with diabetes and CAD compared to individuals with CAD alone.[30] No studies or investigations have examined the relationship between CD163 or its expression and the progression of CAD.

3.6. Peripheral artery disease (PAD)

Only one study was found that investigated sCD163 in the serum of those with diabetes and PAD, at Fontaine stage I or II.[28] Stratified data provided by the authors confirmed that serum sCD163 was 1.2-fold significantly increased ($p = 0.002$) in those with diabetes and PAD II compared to PAD I, suggesting that sCD163 may be involved in PAD progression in individuals with diabetes. However, no further investigation to date has reported upon the local tissue or systematic circulation of CD163 expression in people with diabetes and PAD.

3.7. Diabetic foot ulcer

One study measured CD163+ cells by immunohistochemical staining in non-ulcerated skin biopsies from the forearm of people with diabetes with or without foot ulcer and also healthy controls.[26] Compared to healthy controls, CD163+ cells were significantly increased in individuals with DFU by 1.8 fold; ($p = 0.006$) and also in individuals with diabetes but without DFU [3.7 vs 7.0; $p = 0.002$]. But there was no significant difference in the number of CD163+ cells in non-ulcerated forearm skin biopsies between diabetes individuals with DFU and without DFU,[26] which suggests the change in CD163 may be due to diabetes conditions rather than ulceration. However, as the biopsies were from a non-ulcerated site, further studies are required to investigate CD163 regulation in the ulcer site and bed itself.

4. Discussion

4.1. Potential for sCD163 as a biomarker in diabetes complications

In microvascular complications of DR and DPN, sCD163 examined locally in the vitreous fluid and CSF was found to increase aligned with disease progression in severity in individuals with DR or DPN compared to control groups including in individuals with either non-diabetes related diseases, or in diabetes alone with no complications, or in healthy individuals without diabetes.[23,24,29] Although sCD163 expression has not been quantified in DN from an organ specific fluid such as urine, recent studies in other kidney diseases such as lupus nephritis and renal vasculitis have described that sCD163 was shed from the cell surface in glomeruli directly into urine.[25,33,34] The sCD163 was therefore quantifiable in urine, and additionally it corroborated with the presence and degree of inflammation.[25,33,34] These studies indicate that

urinary sCD163 is an attractive potential biomarker of nephritic inflammation suggesting it can potentially be utilised in individuals with DN for future studies.

Systemically, sCD163 has been examined in either serum or plasma of all microvascular complications including DR, DN and DPN.[20,23,29,32] Three of four of these investigations reported sCD163 to be significantly increased in individuals with diabetes complications when compared to those with diabetes but without any complications.[20,29,32] However, no significant difference was found when those with DR were compared to individuals with non-diabetes related diseases such as non-diabetes based ocular disease.[23]

In macrovascular complications, sCD163 in CAD and PAD was found to be significantly elevated in individuals with complications compared to individuals with diabetes but without complications, or individuals with a less severe graded PAD complication.[27,28]

At present, no studies have examined sCD163 in the wound fluid of people with DFU; investigations in this setting could provide biomarker measures for DFU healing and could potentially be used to predict and monitor wound healing progress of DFU.

Preceding these more recent studies addressing sCD163 measures, there are other inflammatory markers that have earlier been found to be associated with diabetes complications, such as IL-6 and C-reactive protein (CRP). IL-6 is released from neutrophils, macrophages and many tissue resident cells during acute and chronic inflammation which has been reported to be associated with DR, DN, and DPN.[35–37] Our previous study has also found that the circulating sCD163 level was associated with the IL-6 level in diabetes with DR.[20] CRP is produced by the liver as a common clinical pathological test to indicate acute phase infection and inflammation which has also been reported as a potential biomarker to determine the severity of DR.[38] Both IL-6 and CRP are secreted in large amounts and are unanimously regarded as inflammatory biomarkers.[39] Contrastingly sCD163 is specifically reflective of the activation status of monocytes and macrophages which may provide extra information of the underlying pathological conditions of diabetes complications. Thus, while there is a biological rationale that sCD163 may have particular clinical utility in predicting diabetes complications over other established markers, such data has yet to be reported in well-defined longitudinal cohorts.

4.2. Variable CD163 expression in diabetes complications

CD163 expression has been examined in diabetes complications as: CD163+ labelled cells, or at mRNA levels.[23,24,26,30,31] CD163+ tissue macrophages and their mRNA level in local tissues were found to be increased with disease progression in microvascular complications of DR[23,24] and DN[25] but only when compared with people who did not have diabetes suggesting these changes in CD163 expression may be mainly due the impact of diabetes. Whilst it was not the focus of the current sytematic review in traditional diabetes complications, a similar finding in chronic periodontitis was supported where CD163 protein was increased in subgingival plaque of people with diabetes and chronic periodontitis compared to those without diabetes and chronic periodontitis, and the mRNA was decreased.[40] CD163+ cells were also found to be increased in the non-ulcerated forearm skin of people with diabetes compared to healthy controls but no difference was observed between people with diabetes with and without DFU[26] further suggesting that diabetes presence itself may affect CD163 expression,[25] although, it must be noted that this investigation utilised non-ulcerated forearm skin tissue and therefore did not reflect CD163 expression in the ulcerated foot tissue in DFU. Thus, future studies in ulcerated skin are required to determine the extent of local inflammation, and whether CD163 in local tissues may be associated with worsening progress.

Increased CD163 expression in local tissues has been reported in other chronic inflammatory conditions such as inflammatory bowel disease (IBD); wherein increased CD163 expression was more pronounced at sites of active inflammation.[41] Additionally, CD163+ macrophage accumulation in alveoli in chronic obstructive pulmonary disease (COPD) was correlated with disease severity and may be involved in the pathogenesis of COPD.[42] There was also a report that CD163+ cells were deeply infiltrated surrounding sarcoidal granulomas.[43] These recent investigations have revealed an increased local expression of CD163 in chronic inflammatory diseases which is similar to this systematic review which has demonstrated CD163 expression in local tissues in diabetes complications.

Interestingly, CD163+ cells and mRNA was decreased in atherosclerotic plaque in people with diabetes with CAD[20,30] which is the reverse trend compared to increased CD163 expression in tissues in DR, DN and DFU. Decreased CD163 expression in CAD in diabetes has been attributed to the Hp 2–2 genotype which leads to Hp 2–2/Hb complexes being cleared more slowly than other Hp genotypes, resulting in increased accumulation of toxic Hb in the plasma and the tissues and prolonged inflammation.[44] However, the difference of CD163 expression in tissues in DR, DN and DFU indicates that other mechanisms are involved.

CD163+ monocytes in diabetes complications has only been investigated in DR and found to be decreased in individuals with DR compared to those with diabetes and no complication,[20] indicating that the loss of circulating CD163+ monocytes maybe exacerbated with diabetes complications.[20] Decreased CD163 cell surface expression may also be attributed to increased proteolytic shedding as determined by increased sCD163.[20] Considering this finding of decreased systemic CD163+ monocytes and other studies reporting increased CD163+ cells in tissues; it is possible that reduced CD163+ monocytes in the circulation maybe also partly due to increased infiltration into the tissues. The study on colonic tissue samples of IBD patients confirmed that CD163+ cells accumulated mostly around and inside blood vessels, suggesting that CD163+ cells were recruited into the tissue from the systemic circulation.[41] Dynamic studies of labelled CD163+ cells could in future be undertaken in people with diabetes with and without particular diabetes complications to examine tissue uptake and systemic clearance of CD163+ cells.

This review has revealed varied CD163 expression in local tissues and systemically in diabetes. Further investigations are necessary concerning the effect of diabetes on CD163 expression, the infiltrating ability of CD163+ monocytes and the function of CD163 macrophages in tissues. It can then be determined whether CD163 has clinical potential as a therapeutic target in managing the development of or progression of diabetes complications.

4.3. Limitations

4.3.1. Limitations of study design

This systematic literature search found that 11 papers had been published investigating the expression of various forms of CD163 in individuals with diabetes complications. All the investigations conducted were observational in nature. The observational study design only measured CD163 expression at one timepoint in various studies and control groups. This limitation did not enable CD163 or sCD163 to be implicated in a causal nature in the development and progression of diabetes complications in the same individual longitudinally. In the future, prospective investigations would enable CD163 or sCD163 to be measured in the same individuals across various timepoints and assist in consolidating the nature of CD163 or sCD163 as disease progresses. Additionally, there are different control groups used across each study. Individuals with a particular diabetes complication are compared to either i) healthy controls; ii) individuals with diabetes and no complications; iii) individuals with an advanced form of diabetes complication of interest; or iv) individuals with a non-diabetes related complication. As a result of the small number of included studies in this review, a meta-analysis was not suitable given the differing study designs and various control groups.

4.3.2. Limitations of sCD163 as a biomarker for diabetes complications

sCD163 is increased in acute, chronic and low-grade inflammatory conditions however there are various factors also affecting its concentration. For instance sCD163 has been shown to increase significantly with lifestyle and health related factors such as chronological age and body weight.[6] It is not clear whether fasting or prandial states may significantly affect sCD163 appearance and clearance from the circulation thus influencing sCD163 concentration. In addition, there is a lack of information for an 'established normal range' for sCD163. It is also uncertain whether a systemic reflection of macrophage activation by sCD163 measure will correspond with the inflammatory status of tissue specific diabetes complications.

Although the studies included in this review are limited in type and nature, the current albeit limited sample size, indicates a clear relationship between increased levels of sCD163 and the progression of complications. Further investigations are required to study the function of CD163+ cells and sCD163 to establish how this immune cell receptor may be regulated by diabetes and contribute to chronic inflammation in diabetes, and whether it may be a therapeutic target to alleviate progressive disease, especially in certain organ complications.

5. Conclusions & future directions

In the long-term, most individuals with diabetes will develop defined diabetes-related complications which are variably progressive in severity and driven by chronic inflammation. Scavenger receptor CD163, expressed on monocytes and macrophages, has been implicated in various inflammatory conditions and has been found to be altered in many diabetes complications which has been systematically profiled in this review.

This systematic review revealed that soluble CD163 in blood and local body fluids was upregulated and implicated in paralleling, and in some cases, foreshadowing, the progression of complications suggesting its potential utility as an inflammation biomarker for diabetes complications. However, currently there is a lack of studies defining the normal baseline range of sCD163 in humans and biomarker validations such as the correlation of serial sCD163 concentration to disease clinical endpoints. Future investigations should be performed to enable a more robust judgment on the strength of sCD163 as a biomarker of disease progression. This would preferably involve well curated, preferably large cohorts of patients with their diabetes specific complications systematically documented from diabetes diagnosis, then at timed follow-up intervals, on each occasion in a standardised manner measuring sCD163, as well as other measures such ultra-sensitive CRP and IL-6. Determining the expression of CD163 on cell surfaces and mRNA levels across different diabetes complications in particular tissues would complement such blood measures, recognising the challenge in obtaining serial tissue samples in study cohorts.

This review suggests that sCD163 holds promise as a propective marker in series of diabetes complications, as does tissue mRNA expression and protein measures in tissues affected by diabetes complications. Additionally, mechanistically studying the potential function of CD163+ macrophages and relationships with the pathophysiology of diabetes complications, and whether targeting of CD163+ cells could suppresses chronic inflammation and progression of diabetes complications warrants further systematic investigation in appropriately designed studies.

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CRediT authorship contribution statement

ES, DM and ST conceptualized the review; ES and DM screened articles; ES extracted data and wrote the original draft; DM and ST revised and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdiacomp.2022.108150.

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Supplementary Table 1: Key search words and variants used in database searches in Embase, Medline and Scopus

Diabetes	CD163	Complications	Retinopathy	Nephropathy	Cardiovascular Disease	Neuropathy	Diabetic Foot Ulcer
Diabetes Mellitus	CD163	Microvascular	Diabetic retinopathy	Diabetic nephropathy	Coronary artery disease	Peripheral Neuropath*	Foot disease
T1DM		Macrovascular	Diabetic eye disease	Diabetic renal disease	Atherosclerosis	Neuropath*	Ulcer
T2DM		Diabet* complication	Diabetic macular* oedema	Diabetic renal nephrosis	Cardiovascular disease	Autonomic neuropath*	Wound
Diabetes				Diabetic kidney disease	Heart disease	Nerve	Chronic
Diabet*				Diabetic renal failure	Diabetic cardiomyopathy	Focal Neuropathy	Diabet* Foot
					Angiopath*	Proximal Neuropath*	Healing
					Diabetic peripheral angiopathy*		

Database	**Total retrieved**
Embase	15
Medline	7
Scopus	67
Manual Search	4
Total	**93**
Duplicates & Exclusions	82
Final Included Total	**11**

Supplementary Table 2: Study quality assessment using the NOS method[22] for the various publications identified and their respective study type.

Study	Study Design	Selection*	Comparability†	Outcome‡	Total Score§
Min et al	Case Cohort	4	2	4	10
Kobayashi et al	Case Cohort	4	2	3	9
Abu El-Asrar et al	Case Cohort	4	2	3	9
O'Reilly	Case Cohort	4	2	3	9
Eleftheriadou et al$^{\phi}$	Case Cohort	4	2	3	9
Sporrer et al	Case Cohort within cross-secitonal	4	2	0	6
Mrak et al$^{\phi}$ (emailed for details)	Case Cohort within case-control	3	2	4	9
Kallestrup et al	Case Controlled	4	2	3	9
Levy et al$^{\phi}$	Retrospective case cohort	4	2	3	9
Klessens et al	Retrospective case cohort	3	2	4	9

*Maximum 4 points awarded for adequate definition of cases, representativeness of cases, selection of controls, and definition of controls.

†Maximum 2 points awarded for comparability of cases and controls on the design or analysis.

‡ Maximum 3 points awarded for ascertainment of exposure, consistency of ascertainment of exposure for cases and controls, and non-response rate.

§ A maximum of 10 points could be awarded.

$^{\phi}$ Author contacted for additional details.

Supplementary Table 3: NOS study quality assessment[22] for prospective case cohort study.

Study	Study Design	Selection*	Comparability†	Outcome‡	Total Score§
Samuelsson et al	Prospective case cohort	4	2	3	9

*Maximum 4 points awarded for cohort representativeness, selection of non-exposed cohort, exposure assessment, and demonstration outcome not present at baseline.

†Maximum 2 points awarded for comparability of cases and controls on the design or analysis.

‡ Maximum 3 points awarded for follow-up length, adequacy of follow-up, and outcome assessment.

§ A maximum of 9 points could be awarded.

Chapter 4: Circulating Soluble CD163 as a Potential Biomarker of Diabetes Complications

Presented as a manuscript.

This chapter presents the results from an immunoassay investigation of plasma sCD163 in individuals with and without diabetes complications including non-alcoholic fatty liver disease (NAFLD). Other studies indicated that plasma sCD163 is increased in diabetes complications, implicated in the progression of those complications and has potential utility as an inflammation biomarker. However, this was observed over multiple diverse cohorts, with non-uniform disease backgrounds. Considering the studies from Chapter 3, we endeavoured to investigate plasma sCD163 level in a single cohort of individuals with diabetes and with or without various subtypes of micro- and macrovascular diabetes complications as well as NAFLD; and furthermore to investigate whether sCD163 had clinical utility to indicate disease severity in diabetes complications. A unique feature of our sCD163 study is that liver assessments for steatosis and fibrosis were performed for all participants, which distinguishes it from other studies where the presence of NASH fibrosis among participants was not determined. This manuscript has already been published by the *Journal of Diabetes and its Complications*, all permissions have been granted to include it in this thesis.

This chapter is presented as the published peer-reviewed manuscript:

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Circulating soluble CD163 as a potential biomarker of diabetes complications

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ABSTRACT

Aims: To investigate whether soluble CD163 (sCD163) is altered in those with diabetes and various subtypes of complications and non-alcoholic fatty liver disease (NAFLD), and whether it can assess disease complications and severity in people with diabetes.

Methods: Adults with diabetes ($n = 101$) were recruited and assessed for the presence of any complications (D^{+Comps}). Liver steatosis presence was determined by ultrasound and liver stiffness measurement (LSM) by transient elastography. Liver pathology other than non-alcoholic steatohepatitis (NASH) was excluded. Plasma sCD163 was measured by ELISA.

Results: sCD163 was higher in D^{+Comps} ($n = 59$) compared to D^{-comps} ($n = 42$) in those with microvascular complications ($n = 56$; 1.3-fold), including a 1.4-fold increase in chronic kidney disease (CKD) ($n = 42$). sCD163 correlated positively with HbA_{1c} and urinary albumin-creatinine ratio and negatively with HDL-c in D^{+Comps}. sCD163 was increased 1.7-fold in those with advanced NASH fibrosis (LSM $\geq$ 10.3 kPa, $n = 19$) compared to those without (LSM $<$ 10.3 kPa, $n = 80$). The AUC-ROC-curve was 0.64 for sCD163 to detect CKD and 0.74 to detect advanced NASH fibrosis.

Conclusions: In this study, the elevated circulating sCD163 occurred in people with diabetes who had microvascular complications or advanced NASH fibrosis, suggesting sCD163 may have clinical utility as a biomarker in certain diabetes complications and disease severity in NAFLD.

1. Introduction

Diabetes is a global health concern due to its commonality and the progressive nature of its concomitant complications. Microvascular complications of diabetes such as diabetic eye disease (DED), chronic kidney disease (CKD), diabetes-related polyneuropathy (DPN), diabetes-related foot ulcers (DRFUs) and macrovascular conditions such as coronary artery disease (CAD), cerebrovascular disease (CRD) and peripheral vascular disease (PVD), significantly contribute to the associated mortality and morbidity.[1] The increasing prevalence of diabetes combined with progressive nature of diabetes-related microvascular and macrovascular complications, advocates the need for a biomarker to assist in the management, or even prevention of, worsening prognosis.

We have recently identified that the anti-inflammatory CD163+ monocytes are significantly decreased in individuals with diabetes-related complications compared to those without complications.[2] Scavenger receptor CD163 is exclusively expressed on monocyte and macrophage cell surfaces, indicating the activation of these myeloid cells. The anti-inflammatory functions of CD163+ cells involve the clearance of free hemoglobin from the circulation via the haptoglobin-

Abbreviations: CKD, chronic kidney disease; CAD, coronary artery disease; CRD, cerebrovascular disease; DED, diabetic eye disease; DPN, diabetic polyneuropathy; DR, diabetic retinopathy; DRFUs, diabetes-related foot ulcers; LSM, liver stiffness measurement; MVD, macrovascular disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NFS, non-alcoholic fatty liver disease fibrosis score; TE, transient elastography; UACR, urinary albumin-creatinine ratio.

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hemoglobin complexes, resulting in reduced oxidative stress and inflammation. Proteolytic enzymes cleave CD163 from the cell surface, resulting in a soluble form, sCD163. The level of circulating sCD163 is considered to be a good proxy indicator of monocyte/macrophage activation and therefore a systemic marker of inflammation.[3]

A recent systematic review including our own research, found that circulating sCD163 was significantly increased in DED, DPN, CAD and PVD.[2,4–7] However according to our knowledge, there has not yet been an investigation of sCD163 in various subtypes of diabetes-related complications in a single study in which technical and study design factors are controlled, and where severity of the complication was considered.

Plasma sCD163 has also reported to be increased in individuals with non-alcoholic steatohepatitis (NASH), where a minority (<25 % of the individuals) had diabetes.[8] Non-alcoholic fatty liver disease (NAFLD) encompasses a range of conditions caused by the accumulation of fat in the liver (steatosis), which results in progressive liver inflammation causing NASH and its fibrosis. Although not a traditional vascular complication of diabetes, NAFLD is highly prevalent at 55 % or more amongst people with type 2 diabetes and is globally prevalent at ~37 % of adults.[9] Kazankov and co-authors report sCD163 to be a good non-invasive predictor for advanced liver fibrosis,[8] however, this has not been reported in a diabetes cohort.

Therefore, this investigation aimed to determine whether circulating sCD163 is different in those with diabetes and various subtypes of complications and whether it may have utility as a clinical biomarker to assess the disease status and severity of NAFLD in a diabetes cohort.

2. Subjects, materials and methods

2.1. Study subjects

Across a 14-month period, individuals aged 18 years and above were randomly recruited from the Diabetes Centre, Royal Prince Alfred Hospital. The exclusion criteria were: alcohol consumption exceeding 140 g/week and/or 40 g/day, current hepatitis B or C infection or autoimmune or metabolic liver disease and/or known malignancy or clinically significant heart failure; as previously described by Williams and colleagues.[10] All individuals underwent a detailed clinical history and examination.[10] Subsequently 101 adults with both type 1 or type 2 diabetes had a full diabetes complications assessment, and data was collected following a standardized protocol, as described previously.[10,11]

Traditional diabetes complications (D^{+Comps}) were classified as any microvascular complication including: DED, CKD, DPN, DRFUs or macrovascular disease (MVD). DED was defined by the presence of any retinopathy ($n = 19$). CKD was determined by eGFR ≤ 60 mL/min/1.73 m^2 and/or urinary albumin-creatinine ratio (UACR) of >3.5 mg/mmol in women and >2.5 mg/mmol in men ($n = 42$). A reporting of abnormal monofilament testing in either the right or left foot was used to determine the presence of DPN ($n = 20$). DRFUs were established by the event of a previous or current foot ulcer; and or amputation ($n = 9$). The presence of MVD was defined as any known presence of coronary, cerebrovascular or peripheral vascular disease (CVD; CRD; and/or PAD) ($n = 23$).

For NAFLD, liver steatosis presence was determined by ultrasound, and liver stiffness measurement (LSM), reflecting liver fibrosis severity, was determined by transient elastography (TE). The LSM cut-off for advanced liver fibrosis was set high at ≥10.3 kPa, as previously described.[10,12] Non-alcoholic fatty liver disease fibrosis score (NFS) was calculated for all subjects as previously described.[13] Individuals were classified as low, indeterminate, or high risk for clinically significant liver fibrosis if their NFS score was <−1.455, ≥−1.455 and ≤0.675, or >0.675, respectively.[13]

Fasting blood samples were obtained from all participants and cell free plasma was stored at −80 °C for later analysis.

All participants provided written informed consent prior to undertaking any study procedures. All procedures described were approved by the relevant New South Wales Health Human Research Ethics Committees (HRECs) and the University of Sydney HREC and were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

2.2. Measurement of sCD163

Plasma sCD163 was measured using the human soluble CD163 ELISA kit according to the manufacturer's protocols (Aviscera Bioscience, CA, USA). The samples were measured in duplicate, with a dilution factor of 1:200 to enable samples to be within the working range of the assay. Resulting data was analyzed using a log-log standard curve with a co-efficient of variation of ≤20 %.

2.3. Statistical analysis

Data analysis was performed using GraphPad Prism 9 statistical package. Continuous data were checked for normality and presented as mean ± standard deviation (SD) for parametric data, median and IQR or proportion for non-parametric data as indicated. Grouped data were compared by either Welch's *t*-test for parametric data, or Mann-Whitney test for non-parametric data or Fisher's exact test to identify differences between groups. According to the normality test, the non-linear regression Spearman's correlation coefficient was used to verify the significance of correlations between various clinical characteristics and the sCD163 level. IBM® SPSS® Statistics was used for ANCOVA analysis to adjust for the effect of pre-specified factors clinically relevant to microvascular complications, CKD or advanced NASH fibrosis, and to adjust for CKD and advanced NASH fibrosis. Furthermore, the sensitivities and specificities were analyzed by ROC curve, and the AUC and optimal cut-off values were determined. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. The expression of sCD163 in traditional diabetes complications

Clinical characteristics of individuals with (D^{+Comps}, $n = 59$) or without (D^{-Comps}, $n = 42$) any traditional diabetes complications are presented in Table 1. The majority of participants in this study had type 2 diabetes, approximately 95 % in each group. The D^{+Comps} group was older with longer duration of diabetes (both $p < 0.01$), slightly higher HbA_{1c} ($p < 0.05$), lower HDL-c ($p < 0.05$), higher UACR ($p < 0.0001$) and lower eGFR ($p < 0.01$) compared to D^{-Comps}. Additionally, 46 % of those in the D^{+Comps} group were using insulin to manage their blood glucose level compared to 24 % in the D^{-Comps} group. The BMI, total cholesterol and triglycerides were not significantly different between the two groups, however the LDL-c was trending lower in D^{+Comps} ($p = 0.05$) (Table 1). NAFLD was present at 91 % in the D^{+Comps} and 95 % in the D^{-Comps} group.

The level of circulating sCD163 was 1.3-fold higher in D^{+Comps} [282.7 (168.9–412.2) ng/mL] compared to D^{-Comps} [224.9 (152.1–322.9) ng/mL], but this was not statistically significant ($p = 0.08$) (Table 1). In D^{+Comps}, sCD163 positively correlated with HbA_{1c} ($r = 0.30$; 95%CI [0.04–0.52]; $p < 0.05$) and triglycerides ($r = 0.47$; 95%CI [0.23–0.65]; $p < 0.05$), negatively with HDL-c ($r = -0.26$; 95%CI [−0.48 to −0.003]; $p < 0.05$) and was trending with UACR ($r = 0.25$; 95%CI [−0.01–0.48]; $p = 0.05$). In D^{-Comps}, sCD163 was positively correlated with LDL-c ($r = 0.39$; 95%CI [0.09–0.63]; $p < 0.05$) (Table A.1). The level of sCD163 was not different between treatment with or without insulin in D^{+Comps} or D^{-Comps} (data not shown).

3.1.1. The expression of sCD163 in subtypes of traditional diabetes complications

When individuals in the D^{+Comps} group were further stratified

Table 1
Clinical characteristics and the level of sCD163 of individuals with or without any traditional diabetes complications.

	D^{+Comps} ($n = 59$)	D^{-Comps} ($n = 42$)	p value
Age (years)	64.6 (58.1–72.0)	60.2 (54.0–65.9)	$p < 0.01$
Men/women (n)	43/16	20/22	$p < 0.05$
Duration of diabetes (years)	12.0 (7.0–15.0)	8.0 (2.7–12.2)	$p < 0.01$
Type 2 diabetes/total cohort (%)	56/59 (95 %)	40/42 (95 %)	*ns*
Insulin use/total cohort (%)	27/59 (46 %)	10/42 (24 %)	$p < 0.05$
BMI (kg/m^2)	31.9 ± 7.3	29.8 ± 5.8	*ns*
HbA_{1c} (mmol/mol)	60 (49–70)	54 (45–62)	*ns*
HbA_{1c} (%NGSP units)	7.8 ± 1.2	7.3 ± 1.4	$p < 0.05$
Total cholesterol (mmol/L)	4.0 (3.3–4.4)	4.0 (3.5–4.8)	*ns*
HDL-c (mmol/L)	1.1 (0.9–1.3)	1.3 (1.1–1.4)	$p < 0.05$
LDL-c (mmol/L)	1.8 (1.5–2.3)	2.1 (1.7–2.9)	$p = 0.05$
Triglycerides (mmol/L)	1.7 (1.0–2.7)	1.5 (1.1–1.9)	*ns*
UACR (mg/mmol)	4.0 (2.0–15.0)	1.0 (1.0–2.0)	$p < 0.0001$
eGFR mL/min/1.73 m^2	86.0 (67.7–94.7)	92.4 (77.1–105.4)	$p < 0.01$
sCD163 (ng/mL)	282.7 (168.9–412.2)	224.9 (152.1–322.9)	$p = 0.08$

Results are expressed as mean ± SD or median with interquartile range (IQR) or proportion. Significance was accepted at $p < 0.05$ and no significance indicated by *ns*. BMI: body mass index; HbA_{1c}: glycated hemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio; eGFR: estimated glomerular filtration rate.

according to various complication subtypes, sCD163 was 1.3-fold significantly increased in those with any diabetes-related microvascular complication (combined DED, CKD, DPN or DRFUs; $n = 56$) compared to those without any microvascular complication ($n = 45$, $p < 0.05$; Table 2). More specifically, this increase was found in diabetes with CKD, in which sCD163 was 1.4-fold significantly increased compared to individuals with diabetes but without CKD ($p < 0.05$; Table 2). In contrast, the sCD163 level was not significantly different in diabetes with and without macrovascular complications.

The clinical characteristics of individuals with and without diabetes-related microvascular complications are presented in Table A.2. Those with microvascular complications were older with longer duration of diabetes, lower HDL-c and LDL-c, higher UACR and lower eGFR (all $p < 0.05$). Furthermore, sCD163 was correlated with HbA_{1c} ($r = 0.31$; 95% CI [0.04–0.53]; $p < 0.05$) and triglycerides ($r = 0.40$; 95%CI [0.15–0.60]; $p < 0.05$) in individuals with diabetes-related microvascular complications and LDL-c ($r = 0.30$; 95%CI [0.001–0.55]; $p < 0.05$) in individuals with diabetes but without microvascular complications (Table A.3). After adjusting covariates, age and duration of diabetes independently, the difference of sCD163 was no longer met significantly between individuals with or without microvascular complications (adjusted for age: $R^2 = -0.11$; for duration of diabetes: $R^2 = -0.03$; both $p > 0.05$), however there was no correlation found between sCD163 and age or duration of diabetes in either diabetes with or without microvascular complications (Table A.3).

Similarly, individuals with CKD were older with longer duration of diabetes, had higher HbA_{1c} and triglycerides, and lower HDL-c compared to individuals with diabetes without CKD (Table A.4). In addition, it is not surprising that the 6-fold elevated UACR ($p < 0.0001$) has been found with individuals with diabetes and CKD compared to those without. Circulating sCD163 was not significantly correlated with any clinical feature in this CKD cohort, however in those without CKD, sCD163 was positively correlated with total cholesterol ($r = 0.29$; 95%CI [0.03–0.51]; $p < 0.05$) and LDL-c ($r = 0.29$; 95%CI [0.03–0.52]; $p < 0.05$) (Table A.5). After adjusting for covariates of chronological age, and duration of diabetes, the level of sCD163 was no longer significantly different in individuals with or without CKD (adjusted for age: $R^2 = 0.01$; for duration of diabetes: $R^2 = 0.01$; both $p > 0.05$), but there was no correlation found between sCD163 and age or duration of diabetes in either diabetes with or without CKD (Table A.5).

The AUC of ROC curve for sCD163 to detect CKD was 0.64 ($p = 0.01$) (Fig. 1A). A sCD163 cut-off of 296.1 ng/mL was found to have only 55 % sensitivity and 69 % specificity to discriminate CKD from other complications of diabetes.

When individuals with CKD were stratified by their stage of CKD by eGFR according to Stage 1: >90; Stage 2: 60–89; Stage 3/4: 29–45, sCD163 was not significantly different between CKD Stage 1 [286.5 (174.6–451.1) ng/mL; $n = 16$], Stage 2 [305.9 (196.1–484.4) ng/mL; $n = 16$] and Stage 3/4 [346.4 (163.3–446.7) ng/mL; $n = 10$]. Furthermore, sCD163 was not correlated with eGFR in individuals with CKD ($r = 0.01$; 95%CI [−0.30–0.32]; $p > 0.05$).

Table 2
The level of circulating sCD163 in individuals with any traditional complications compared to those without complications.

sCD163 ng/mL	Diabetes with any traditional complications	Diabetes without traditional complications	Fold change	p value
Microvascular	290.6 (172.4–403.9) ($n = 56$)	222.6 (148.6–324.3) ($n = 45$)	1.3	$p < 0.05$
DED	215.2 (134.2–360.3) ($n = 19$)	238.9 (163.1–382.1) ($n = 82$)	0.9	*ns*
CKD	316.4 (186.6–467.1) ($n = 42$)	223.7 (146.2–327.2) ($n = 59$)	1.4	$p < 0.05$
DPN	309.0 (211.5–368.4) ($n = 20$)	229.5 (157.1–364.0) ($n = 81$)	1.3	*ns*
DRFUs	304.8 (195.2–407.7) ($n = 9$)	231.5 (158.3–362.3) ($n = 92$)	1.3	*ns*
Macrovascular	282.7 (158.9–478.5) ($n = 23$)	234.5 (161.0–358.2) ($n = 78$)	1.2	*ns*

Results are expressed as median with interquartile range (IQR). Significance was accepted at $p < 0.05$ and no significance indicated by *ns*. DED: diabetic eye disease; CKD: chronic kidney disease; DPN (diabetic polyneuropathy), DRFUs (diabetes-related foot ulcers).

3.2. Levels of sCD163 in those with NAFLD in diabetes

The level of sCD163 was not significantly different between individuals with diabetes with or without liver steatosis at 262.5 (163.4–379.2) ng/mL; $n = 91$ and 179.8 (144.8–295.5) ng/mL; $n = 10$, respectively. Individuals with diabetes were stratified by LSM score with LSM < 10.3 kPa ($n = 80$) indicating no advanced liver fibrosis and LSM ≥ 10.3 kPa ($n = 19$) indicating advanced liver fibrosis[10,12]; 100 % of individuals with advanced NASH fibrosis in this study had type 2 diabetes and a longer duration of diabetes ($p < 0.05$), higher UACR ($p < 0.01$), trending elevated BMI ($p = 0.06$), HbA_{1c} ($p = 0.07$), total cholesterol and triglycerides (both $p = 0.08$). Additionally, 53 % of individuals with advanced NASH fibrosis were using insulin treatment compared to 32 % in those with no advanced NASH fibrosis, however this was not significantly different (Table 3).

The expression of sCD163 was increased 1.7-fold in individuals with diabetes with advanced NASH fibrosis [368.9 (262.5–563.9) ng/mL] compared to those without advanced NASH fibrosis [222.9 (151.4–327.8) ng/mL], $p < 0.001$ (Table 3). Furthermore, the sCD163 level was correlated with LSM score ($r = 0.22$; $p < 0.05$) (Fig. A.1). However, the significance was only observed in individuals with diabetes with advanced NASH fibrosis ($r = 0.55$; $p < 0.05$), but not in those without advanced NASH fibrosis ($r = -0.04$; $p > 0.05$). In individuals with advanced NASH fibrosis, sCD163 was significantly correlated with age ($r = -0.52$; 95%CI [−0.79 to −0.09]; $p < 0.05$), HDL-c ($r = -0.49$;

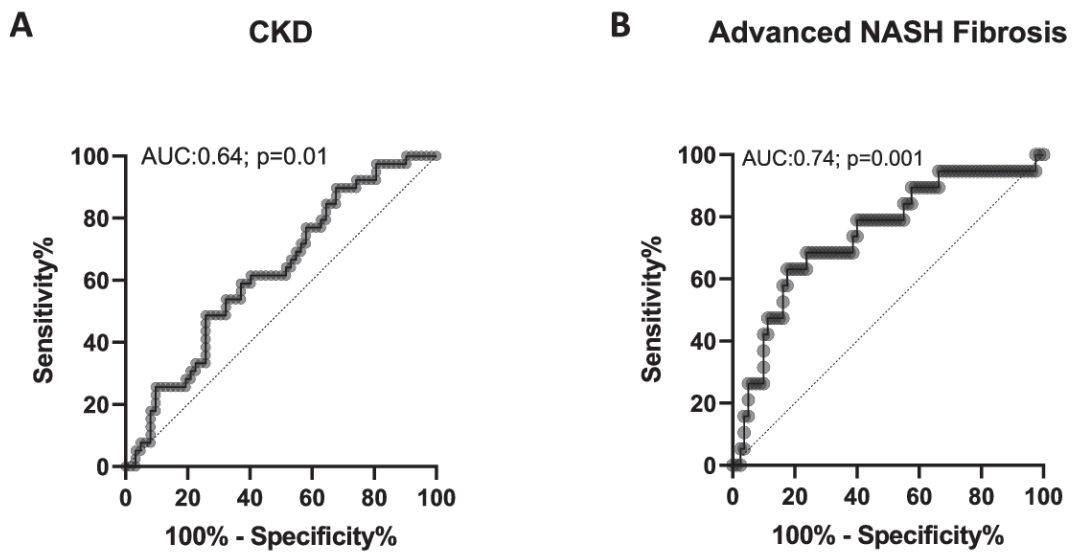

Fig. 1. ROC analysis of plasma sCD163 to detect CKD (A) and advanced NASH fibrosis (B) in individuals with diabetes. Significance at $p < 0.05$. The area under of the ROC curve was labelled, and the dashed line indicates the reference line.

Table 3
Clinical characteristics and the level of sCD163 of individuals with and without severe NASH fibrosis indicated by LSM score.

	LSM < 10.3 kPa ($n = 80$)	LSM ≥ 10.3 kPa ($n = 19$)	p value
Age (years)	62.1 ± 9.9	62.9 ± 11.0	*ns*
Male/women (n)	45/35	16/3	$p < 0.05$
Duration of diabetes (years)	9.5 (3.3–14.0)	14.0 (8.0–20.0)	$p < 0.05$
Type 2 diabetes/total cohort (%)	75/80 (94 %)	19/19 (100 %)	*ns*
Insulin use/total cohort (%)	26/80 (32 %)	10/19 (53 %)	*ns*
BMI (kg/m^2)	30.1 ± 6.3	33.0 ± 5.7	$p = 0.06$
HbA_{1c} (mmol/mol)	57 (48–65)	64 (49–70)	$p = 0.07$
HbA_{1c} (%NGSP units)	7.4 (6.5–8.1)	8.0 (6.6–8.6)	$p = 0.07$
Total cholesterol (mmol/L)	3.9 (3.4–4.6)	4.2 (3.9–4.6)	$p = 0.08$
HDL-c (mmol/L)	1.2 (1.0–1.4)	1.1 (0.9–1.3)	*ns*
LDL-c (mmol/L)	1.9 (1.5–2.5)	1.9 (1.6–2.3)	*ns*
Triglycerides (mmol/L)	1.5 (1.1–2.0)	2.1 (1.0–3.5)	$p = 0.08$
UACR (mg/mmol)	2.0 (1.0–4.0)	5.0 (2.0–27.0)	$p < 0.01$
eGFR mL/min/1.73m^2	88.1 ± 21.4	82.8 ± 22.5	*ns*
sCD163 (ng/mL)	222.9 (151.4–327.8)	368.9 (262.5–563.9)	$p <$ 0.001

Results are expressed as mean ± SD, median with interquartile range (IQR) or proportion (n). Significance was accepted at $p < 0.05$ and no significance indicated by *ns*. BMI: body mass index; HbA_{1c}: glycated hemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio; eGFR: estimated glomerular filtration rate.

95%CI [−0.77 to −0.05]; $p < 0.05$), and triglycerides ($r = 0.50$; 95%CI [−0.04–0.78]; $p < 0.05$) and trending to being correlated with duration of diabetes ($r = -0.42$; 95%CI [−0.73–0.04]; $p = 0.07$) (Table A.6). After adjusting covariate, duration of diabetes, sCD163 still remained significantly increased in individuals with diabetes with advanced NASH fibrosis compared to those without advanced NASH fibrosis ($R^2 = 0.48$; $p < 0.05$).

The AUC of ROC curve for sCD163 to detect advanced NASH fibrosis was 0.74 ($p = 0.001$). A sCD163 cut-off of 185.7 ng/mL was found to have 89 % sensitivity and 41 % specificity to identify advanced NASH fibrosis in diabetes (Fig. 1B).

3.2.1. *Clinical utility of sCD163 to assess the risk of advanced NASH fibrosis in diabetes*

In this study, there were $n = 59$ classified as 'indeterminant risk' for NASH fibrosis by NFS calculation. In the 'indeterminate risk' group, 46 participants (78 %) had an LSM score of <10.3 kPa, and 13 participants (22 %) had an LSM score ≥ 10.3 kPa indicating advanced liver fibrosis (Table 4).

A cut-off level of sCD163 at 185.7 ng/mL determined from ROC curve analysis, was applied to the 'indeterminate risk' group wherein <185.7 ng/mL indicated no advanced NASH fibrosis and ≥185.7 ng/mL indicated advanced NASH fibrosis. The results are illustrated in Table 4 in which 23 individuals (39 %) presented with sCD163 <185.7 ng/mL and 36 (61 %) greater than the cut-off. Within the 46 individuals who did not have advanced NASH fibrosis (<10.3 kPa), using the sCD163 cut-off demonstrated 46 % similarity to detect no advanced NASH fibrosis. Within the 13 individuals who had advanced NASH fibrosis according to LSM (≥10.3 kPa), the sCD163 cut-off demonstrated 84 % similarity to detect advanced NASH fibrosis, with a positive predictive value (PPV) of 89 % and negative predictive value (NPV) of 42 %.

3.3. *Soluble CD163 as a singular independent biomarker in CKD and advanced NASH fibrosis*

In individuals with diabetes and CKD, 32 % also had concomitant advanced NASH fibrosis, whereas in those with diabetes without CKD, 6 % had concomitant advanced NASH fibrosis. When adjusting for the presence of advanced NASH fibrosis, sCD163 was no longer significantly different in individuals with diabetes with and without CKD ($R^2 = 0.01$,

Table 4
The utility of applying a blood sCD163 cut-off to the 'indeterminate risk' group as calculated by NAFLD Fibrosis Score (NFS).

Median liver stiffness measurement (LSM)	Indeterminate risk group, $n = 59$	Indeterminate risk group subdivided by sCD163 measurement	
		sCD163 < 185.7 ng/mL 23/59 (39 %)	sCD163 ≥ 185.7 ng/mL 36/59 (61 %)
<10.3 (kPa), number (%)	46/59 (78 %)	21/46 (46 %)	25/46 (54 %)
≥10.3 (kPa), number (%)	13/59 (22 %)	2/13 (15 %)	11/13 (84 %)

$p > 0.05$).

Similarly in individuals with diabetes and advanced NASH fibrosis, 68 % also had CKD, and in individuals without advanced NASH fibrosis, 32 % concomitantly had CKD. After adjusting for the presence of CKD, sCD163 was no longer significantly different between individuals with and without advanced NASH fibrosis ($R^2 = 0.028$, $p > 0.05$). This suggests that the phenotype of advanced NASH fibrosis and CKD are both associated with circulating sCD163 level in this study cohort.

4. Discussion

Globally, both diabetes and its consequential microvascular and macrovascular complications, are increasing in prevalence. The progressive nature of these complications warrants the need for enhanced management.[1] Although NASH fibrosis is not a vascular complication traditionally associated with diabetes, it is highly prevalent in individuals with diabetes. In addition, type 2 diabetes is an independent risk factor for NASH fibrosis.[9,14,15] Currently liver biopsy is the gold-standard to diagnose NASH fibrosis severity, however the risk with this invasive procedure warrants the need for a non-invasive method.[16] In this study, we have investigated the utility of sCD163, a marker of monocyte/macrophage activation, to assess disease severity in traditional diabetes complications as well as advanced NASH fibrosis in a diabetes cohort with majority as type 2 diabetes. Our investigation demonstrated that circulating sCD163 was increased in individuals with diabetes-related microvascular complications, especially CKD compared to those without; but it was not implicated with the severity of CKD. In contrast, circulating sCD163 was increased in individuals with diabetes with advanced NASH fibrosis, and it also correlated with progressing fibrosis severity, suggesting sCD163 may have utility as a biomarker of certain diabetes complications and disease severity in NASH fibrosis.

In individuals with any traditional diabetes complications, plasma sCD163 exhibited a trend of increase in those with complications compared to those without. The significant increase appeared in diabetes-related microvascular complications and more specifically in the diabetes cohort with CKD. After adjusting for covariates, age and duration of diabetes, sCD163 was no longer significantly different between the cohort with and without microvascular complications or with and without CKD, suggesting that the relationship between sCD163 and the occurrence of microvascular complications or CKD may be affected by age and duration of diabetes. In an earlier prospective study, it was reported that plasma sCD163 was trending higher at onset of diabetes in individuals who later developed CKD, compared to those who did not, indicating that sCD163 has potential as an early biomarker of CKD.[17] This phenomenon is fortified in this study, such that in those with diabetes, sCD163 was increased in CKD compared to individuals without. However, the level of sCD163 was not found to be linked to the severity of CKD in this study. The level of sCD163 was moderately correlated with UACR suggesting that sCD163 has some potential in its ability to detect CKD, and further investigations are needed to bring clarity to the changes of sCD163 in CKD progression.

Within DED, DPN, and diabetes-related macrovascular complications, we found that sCD163 was not significantly different in individuals with those complications compared to without. In contrast to our finding, previous studies including from our own group have reported a significant increase in sCD163 in DED,[2] a trending increase in DPN,[5,18,19] increase in CAD and peripheral artery disease (PAD) with disease progression compared to individuals with diabetes without these complications.[6,7] However, in these earlier studies it is unclear whether the study cohort also had NASH. As sCD163 changes with NASH fibrosis, future investigations will benefit from larger cohort studies with NASH assessment.

In DRFUs, novel data from this investigation demonstrated that plasma sCD163 was not significantly different when compared to individuals with diabetes without DRFUs. Similarly, one study reported tissue expression of CD163+ cells was unaltered in individuals with DRFUs compared to individuals with diabetes without DRFUs[20]; and our previous study reported circulating %CD163+ monocytes did not change according to duration of wound healing or healing status in individuals with diabetes with DRFUs.[18] Contrastingly in that same study from our unpublished data, plasma sCD163 was not affected by duration of wound healing and healing status (data not shown), indicating that CD163 may not be implicated in DRFUs and its healing status.

In this investigation, we found that plasma sCD163 was significantly elevated in individuals with diabetes and advanced NASH fibrosis compared to those without advanced fibrosis according to LSM score, and it correlated well with LSM score in the advanced NASH fibrosis cohort. This finding parallels those of Kazankov and colleagues, who also reported plasma sCD163 to be correlated with NASH fibrosis, confirmed by liver biopsy histological staining, but in a non-diabetes focused study as the majority (75 %) of the study cohort lacked diabetes.[7,8] Our current work combined with Kazankov's study suggest that sCD163 may be a good biomarker to detect advanced NASH fibrosis in the cohort either with or without diabetes.

It is notable that NAFLD especially the more severe form of NASH with fibrosis, is an increasingly recognized form of diabetes-associated complication in type 2 diabetes.[10,14,15] Ongoing challenges relate to screening for NASH fibrosis in people with diabetes; recent recommendations have suggested a clinical risk algorithm such as NFS, followed by an informative blood test or test panel in the 'indeterminant risk' group, then confirmed by non-invasive transient elastography.[21,22] It is in the 'indeterminant risk' group rather than the low or high risk group after use of the clinical algorithm where a blood test may be most useful. In our previous study, Williams and colleagues found that lower levels of circulating fibroblast activation protein demonstrated negative predictive value for clinically significant NASH fibrosis in the 'indeterminant risk' group.[10] In this study, we have found that sCD163 has reasonable assessment value for current liver fibrosis in the 'indeterminant risk' group as applying a plasma sCD163 cut-off of 185.7 ng/mL can sensitively identify 84 % who may have advanced NASH fibrosis, suggesting that it holds promise as a tool to aid risk stratification of NASH fibrosis in type 2 diabetes. To our knowledge, this is the first study to present a clinical application of plasma sCD163 to be used as a biomarker to screen for advanced NASH fibrosis in diabetes. Therefore, with the aim of detecting advanced liver fibrosis earlier, it would be highly beneficial to use this easy to perform and inexpensive substitute biomarker to screen individuals in the 'indeterminate risk' group. A multicenter clinical trial is needed to develop and validate it as a useful clinical biomarker.

Based on the study design, there are some limitations with this cross-sectional observation study. Firstly, this is a single center study where >90 % had NAFLD in this study cohort which may not reflect the general or some other diabetes populations. Secondly, the study number was relatively small when the complications group was further divided into sub-groups based on the complication subtypes and nearly all participants had more than one traditional diabetes complication mixed with NAFLD, thus this does not allow for independent assessment of sCD163 in singular disease conditions. For example, after adjusting for the presence of advanced NASH fibrosis in CKD individuals, and inversely adjusting for the presence of CKD in individuals with advanced NASH fibrosis, we found that sCD163 was no longer significantly increased in either disease state. Future studies focusing on individuals with specifically either CKD or NAFLD will be beneficial. However, this also highlights that it is biologically and clinically inevitable that individuals with diabetes may present with multiple co-morbid traditional complications and NASH. Therefore, this phenomenon may reflect realistic clinic conditions. In addition, this study was limited by the inability to assess the stage of liver fibrosis using biopsy, for ethical reasons as invasive tissue sampling was not approved in the study. Finally, the effect of medication or other possible inflammatory diseases with this study cohort may also impact on plasma sCD163 levels. Even so, this study has revealed that circulating sCD163 level is altered in a diabetes

cohort with CKD and advanced NASH fibrosis. Future investigations would likely benefit from standardized reference ranges of sCD163 in healthy individuals, so to enable cross-study comparisons.

In conclusion, this study found that plasma sCD163 is altered with diabetes-related complications, particularly CKD and advanced NASH fibrosis. Soluble CD163 may serve as a biomarker in assessment the presence of more advanced NASH fibrosis in people with diabetes, and to help differentiate risk of advanced NASH fibrosis in those categorized as 'indeterminate risk' based on clinical algorithm scores alone. Future studies should test this hypothesis in larger cohorts and prospective series in people with diabetes.

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CRediT authorship contribution statement

ES performed the experiment, data analysis and wrote the first draft of the manuscript. SP assisted with data analysis. KW acquired the clinical samples and clinical data. MM, TW and JW assisted with recruitment. ST contributed to study cohort design, interpretation of the results, and revision of the manuscript. DM contributed to study experiment design, interpretation of the results and writing and revision of the manuscript. All authors contributed to review/edit and approved the final version for submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available due to restrictions in the ethical permission.

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Supplementary Material for Review

Figure A. 1

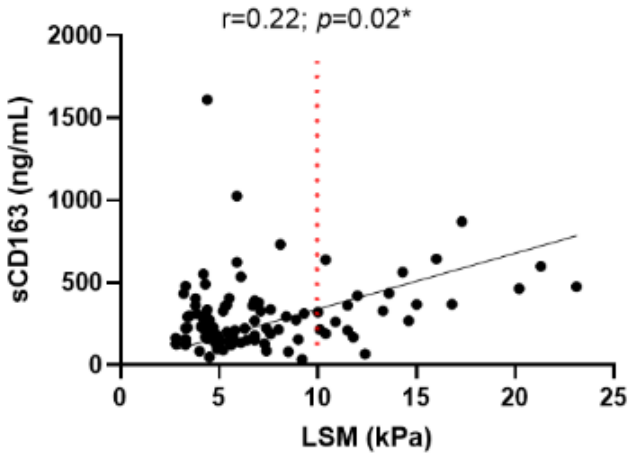

Figure A. 1

Scatter plot displaying correlation of sCD163 with liver stiffness measurement (LSM) score in the whole diabetes cohort (n=99). Spearman's rank non-linear correlation analysis indicated a significant relationship between plasma sCD163 and LSM score, with r correlation coefficient and p-value provided within plot. The red dashed line indicates LSM 10.3 kPa, from which the correlation with sCD163 is strengthened.

Table A. 1: The relationship between sCD163 and clinical features in individuals with or without any traditional complications.

sCD163 vs.	D^{+Comps} (*n*=59)	D^{-Comps} (*n*=42)
Age	0.07 (-0.20 - 0.33) *ns*	(-0.31 - 0.32) *ns*
Diabetes Duration	0.07 (-0.20 - 0.33) *ns*	-0.21 (-0.49 - 0.11) *ns*
BMI	0.01(-0.08 - 0.43) *ns*	0.11 (-0.21 - 0.41) *ns*
HbA_{1c} (NGSP units)	0.30 (0.04 - 0.52) *p*<0.05	-0.19 (-0.47 - 0.13) *ns*
Total Cholesterol	0.08 (-0.19 - 0.34) *ns*	0.28 (-0.03 - 0.55) p=0.06
HDL-c	-0.26 (-0.48 - -0.003) *p*<0.05	-0.13 (-0.42 - 0.19) *ns*
LDL-c	-0.08 (-0.35 - 0.19) *ns*	0.39 (0.09 - 0.63) *p*<0.05
Triglycerides	0.47 (0.23 - 0.65) p<0.05	-0.08 (-0.39 - 0.23) *ns*
UACR	0.25 (-0.01 - 0.48) *p=0.05*	-0.05 (-0.36 - 0.27) *ns*

Results are expressed as *r* (95%CI). Significance was accepted when p<0.05 and no significance indicated by *ns.* BMI: body mass index; HbA_{1c}: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio; eGFR: estimated glomerular filtration rate.

Table A. 2: Clinical characteristics of individuals with or without diabetes-related microvascular complications.

	With microvascular complications (*n*=56)	**Without microvascular complications (*n*=45)**	***p* value**
Age (years)	65.3 (57.8 -72.0)	59.9 (54.1- 65.7)	*p*<0.01
Men/Women (*n*)	42/14	21/24	*p*<0.01
Duration of diabetes (years)	12.5 (7.3 - 16.5)	8.0 (2.5 -12.0)	*p*<0.01
T2DM/total cohort (%)	54/56 (96%)	42/45 (93%)	*ns*
Insulin use/total cohort (%)	25/56 (45%)	12/45 (27%)	*ns*
BMI (kg/m^2)	30.6 (26.9 - 34.9)	28.8 (26.9 - 34.2)	*ns*
HbA_{1c} (mmol/mol)	61 ± 13	57 ± 14	ns
HbA_{1c} (%NGSP units)	7.0 (6.2 - 8.2)	7.1 (6.4 - 8.0)	*p*=0.06
Total Cholesterol (mmol/L)	3.9 (3.3 - 4.3)	4.1 (3.5 - 4.9)	*p=0.06*
HDL-c (mmol/L)	1.1 (0.9 -1.3)	1.3 (1.1 -1.4)	*p<0.05*
LDL-c (mmol/L)	1.8 (1.5 -2.3)	2.2 (1.7 -2.9)	*p*<0.01
Triglycerides (mmol/L)	1.7 (1.0-2.7)	1.5 (1.1-1.9)	*ns*
UACR (mg/mmol)	4.0 (2.0 - 15.8)	1.0 (1.0 - 2.0)	*p*<0.0001
eGFR mL/min/1.73m^2	87.0 (67.7 - 94.7)	92.1 (76.1 - 106.2)	*p*<0.01

Results are expressed as median with interquartile range (IQR) or proportion (*n*). Significance was accepted when $p<0.05$ and no significance indicated by *ns.* BMI: body mass index; HbA_{1c}: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio; eGFR: estimated glomerular filtration rate.

Table A. 3: The relationship between sCD163 and clinical features in individuals with or without diabetes-related microvascular complications.

sCD163 vs	With microvascular complications (*n*=56)	Without microvascular complications (*n*=45)
Age	0.06 (-0.22 - 0.32) *ns*	0.01 (-0.29 - 0.31) *ns*
Diabetes Duration	0.05 (-0.22 - 0.32) *ns*	-0.17 (-0.44 - 0.14) *ns*
BMI	0.23 (-0.04 - 0.47) *ns*	0.05 (-0.24 - 0.36) ns
HbA_{1c} (NGSP units)	0.31 (0.04 - 0.53) p<*0.05*	-0.14 (-0.42 - 0.17) *ns*
Total Cholesterol	0.12 (-0.15 - 0.38) *ns*	0.24 (-0.06 - 0.51) ns
HDL-c	-0.17 (-0.42 - 0.11) *ns*	-0.19 (-0.47 - 0.11) *ns*
LDL-c	-0.04 (-0.31- 0.24) *ns*	0.30 (0.001 - 0.55) *p*<0.05
Triglycerides	0.40 (0.15 - 0.60) p<0.05	-0.6 (-0.25 - 0.36) *ns*
UACR	0.24 (-0.04 - 0.48) *ns*	-0.04 (-0.34 - 0.27) ns

Results are expressed as *r* (95%CI). Significance was accepted when $p<0.05$ and no significance indicated by *ns*. BMI: body mass index; HbA_{1c}: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio.

Table A. 4: Clinical characteristics of individuals with diabetes with or without CKD.

	With CKD (*n*=42)	Without CKD (*n*=59)	*p* value
Age (years)	64.9 ± 11.0	60.5 ± 8.8	*p*<0.05
Men/Women (*n*)	30/12	33/26	*ns*
Duration of diabetes (years)	11.5 (7.7 - 15.7)	8.0 (3.0 - 14.0)	*p*<0.05
T2DM/total cohort (%)	40/42 (95%)	56/59 (95%)	*ns*
Insulin use/total cohort (%)	18/42 (43%)	19/49 (39%)	*ns*
BMI (kg/m^2)	30.9 (27.2 - 35.1)	29.5 (26.5 - 33.2)	*ns*
HbA_{1c} (mmol/mol)	62 (50 – 74)	55 (48 -64)	*p<0.05*
HbA_{1c} (%NGSP units)	7.8 (6.7 - 8.9)	7.2 (6.5 - 8.0)	*p*<0.05
Total Cholesterol (mmol/L)	4.1 (3.4 - 4.3)	3.9 (3.5 - 4.7)	*p*=0.7
HDL-c (mmol/L)	1.1 (0.9 -1.3)	1.2 (1.1 - 1.4)	*p*<0.05
LDL-c (mmol/L)	1.8 (1.5 - 2.3)	1.9 (1.5 - 2.7)	*ns*
Triglycerides (mmol/L)	2.0 (1.3-2.7)	1.5 (1.0-1.8)	*p<0.05*
UACR (mg/mmol)	6.5 (4.0 - 27.2)	1.0 (1.0 - 2.0)	*p*<0.0001
eGFR mL/min/1.73m^2	85.8 (59.5 - 94.3)	91.1 (77.3 - 100.5)	*p*<0.01

Results are expressed as median with interquartile range (IQR) or proportion (*n*). Significance was accepted when $p<0.05$, and no significance indicated as *ns.* BMI: body mass index; HbA_{1c}: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio; eGFR: estimated glomerular filtration rate.

Table A. 5: The relationship between sCD163 and clinical features in individuals with diabetes with or without CKD.

sCD163 vs	With CKD (*n*=42)	Without CKD (*n*=59)
Age	0.01 (-0.31 - 0.32) *ns*	0.09 (-0.18 - 0.34) *ns*
Diabetes Duration	-0.03 (-0.34 - 0.28) *ns*	-0.07 (-0.33 - 0.20) ns
BMI	0.06 (-0.25 - 0.37) *ns*	0.18 (-0.09 - 0.42) *ns*
HbA_{1c} (NGSP units)	0.25 (-0.06 - 0.52) *ns*	-0.05 (-0.31 - 0.21) ns
Total Cholesterol	-0.08 (-0.39 - 0.23) *ns*	0.29 (0.03 - 0.51) *p*<0.05
HDL-c	-0.25 (-0.53 - 0.06) *ns*	-0.12 (-0.37 - 0.15) *ns*
LDL-c	-0.24 (-0.52 - 0.08) *ns*	0.29 (0.03 - 0.52) *p<0.05*
Triglycerides	0.29 (-0.23 - 0.55) *ns*	0.15 (-0.12 - 0.40) *ns*
UACR	0.13 (-0.19 - 0.43) *ns*	0.01 (-0.26 - 0.27) *ns*

Results are expressed as *r* (95%CI). Significance was accepted when $p<0.05$ and no significance by *ns.* BMI: body mass index; HbA_{1c}: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio.

Table A. 6. The relationship between sCD163 and clinical features in individuals with diabetes with or without advanced liver fibrosis according to LSM score.

sCD163 vs	LSM<10.3 kPa (*n*=80) r (95%CI)	LSM≥10.3kPa (*n*=19) r (95%CI)
Age	0.20 (-0.02 - 0.41) *p=0.06*	-0.52 (-0.79 - -0.09) p<0.05
Diabetes Duration	-0.001 (-0.23 - 0.22) *ns*	-0.42 (-0.73 - 0.04) *p=0.07*
BMI	0.08 (-0.15 - 0.30) *ns*	0.20 (-0.28 - 0.60) *ns*
HbA_{1c} (NGSP units)	0.05 (-0.18 - 0.27) *ns*	0.24 (-0.24 - 0.62) *ns*
Total Cholesterol	0.14 (-0.09 - 0.36) *ns*	-0.07 (-0.51 - 0.39) *ns*
HDL-c	-0.11 (-0.33 - 0.12) *ns*	-0.49 (-0.77 - -0.05) p<0.05
LDL-c	0.13 (-0.10 - 0.34) *ns*	-0.19 (-0.62 - 0.34) *ns*
Triglycerides	0.17 (-0.05 - 0.39) *ns*	0.50 (0.04 - 0.78) p<0.05
UACR	0.14 (-0.09 - 0.35) *ns*	0.12 (-0.36 - 0.56) *ns*

Results are expressed as *r* (95%CI). Significance was accepted when $p<0.05$ and no significance indicated by *ns.* BMI: body mass index; HbA_{1c}: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; UACR: urinary albumin-creatinine ratio.

Chapter 5: Deep Profiling Revealed Distinct CD163+ Monocytes in Diabetes-related Complications.

Presented as a manuscript which is in the process for submission.

This chapter presents the findings of an in-depth characterization of the CD163+ monocyte in diabetes complications. Drawing from the survey of CD163+ cells in diabetes complications presented in the systematic review of Chapter 3, a previous investigation reported circulating CD163+ monocytes as decreased in individuals with diabetes complications. This finding therefore prompted the inquiry into the function of the CD163+ monocytes, especially the role they play in the unresolving nature of diabetes complications. This chapter therefore aimed to investigate functional characterisation of the CD163+ monocytes in individuals with and without diabetes complications. Mass cytometry was utilised to immunophenotype, and RNA-sequencing used to profile the RNA of CD163+ monocytes. This investigation found that in diabetes complications the CD163+ monocytes may be apoptotic, have reduced capacity to migrate into tissues, have reduced functional abilities and are involved in dysregulated activation of the adaptive immune system. This unique and altered phenotype of the CD163+ monocytes in diabetes complications, suggests that CD163+ monocytes may be involved in the progression of diabetes complications.

Deep profiling revealed distinct CD163+ monocytes in diabetes-related complications

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Abstract: CD163, a scavenger receptor with anti-inflammatory function expressed exclusively on monocytes/macrophages, is dysregulated in diabetes complications. This study aimed to deeply characterize circulating CD163+ monocytes in the presence or absence of diabetes-related complications.

RNA-sequencing and mass cytometry were conducted in CD163+ monocytes in adults with long-duration diabetes, with (D^{+Comps}) or without complications (D^{-Comps}). A total of 10,868 differentially expressed genes were identified in D^{+Comps} compared to D^{-Comps} including 885 up-regulated and 190 down-regulated genes (fold-change≥1.5). In D^{+Comps}, 'regulation of centrosome cycle' genes were 6.7-fold enriched. *MIR27A, MIR3648-1* and *MIR23A* the most up-regulated (>6-fold) and *CD200R1* the most down-regulated (-3.8-fold) genes were detected in D^{+Comps} from the list of 75 'Genes of Interest'. CD163+ monocytes in D^{+Comps} had a low proportion of recruitment markers CCR5, CD11b, CD11c, CD31, and immune regulation markers CD39, CD86. A gene-protein network identified *TLR4* and CD11b as 'hub-nodes'. In conclusion, this study reports novel insights into CD163+ monocytes dysregulated in diabetes-related complications. Enriched centrosome cycle genes and up-regulated *miRNAs* implicated in apoptosis, along with down-regulated monocyte activation, recruitment and immune regulation suggest functionally distinct CD163+ monocytes in diabetes complications. Further investigation is needed to confirm their role as markers and in pathogenesis of diabetes tissue pathology.

Article Highlights

- This study addressed the profile of the anti-inflammatory CD163+ monocytes in people with longstanding diabetes and with diabetes complications.
- In individuals with long duration of diabetes and complications, the CD163+ monocytes presented with significantly enriched centrosome cycle genes and up-regulated miRNAs indicating apoptosis, along with down-regulated monocyte activation, recruitment, and immune regulation, compared the CD163+ monocytes in those without complications.
- This deep characterization of CD163+ monocytes suggests decreased anti-inflammatory and altered inflammatory activities which may contribute to the pathogenesis of diabetes-related complications.

Keywords

- Diabetes-related complications
- Anti-inflammation
- Monocyte
- CD163
- RNA-seq
- Mass cytometry

In modern times diabetes has increased in prevalence at pandemic proportions and has become a major health concern due to the progressive nature of its concomitant complications, which contribute to the mortality, morbidity and cost associated with the disease (1). Chronic low-grade systemic inflammation is a feature of diabetes, and persistent inflammation will induce a further influx of inflammatory cells, specifically monocytes into the tissue which contributes to the pathophysiology of complications in diabetes (2). Recently, our group reported that diabetes complications status co-segregated with monocyte phenotype, such that the circulating percentage of CD163+ monocytes was significantly decreased in individuals with complications compared to those without any complications (3). CD163, the haemoglobin (Hb)- haptoglobin (Hp) scavenger receptor, is involved in the endocytosis of free circulating toxic Hb via Hb-Hp complexes. The clearance of this Hb is a highly beneficial anti-oxidative to protect the tissues from oxidative damage caused by uncontrolled Fe2+. As such CD163+ monocytes have an anti-inflammatory function, as they reduce the production of reactive oxygen species which create oxidative stress to the local environment, leading to organ damage (4, 5). Therefore, CD163+ monocytes act as scavengers and in this manner, they resolve systemic or local inflammation.

Recent investigations have found that tissue expression of CD163 may differ between individuals with and without diabetes complications. For instance in individuals with diabetes and cardiovascular disease, CD163+ cells are decreased in the atherosclerotic plaques of these individuals compared to those with cardiovascular disease but without diabetes (6). Similarly, in renal complications, CD163+ positive cells in glomeruli were reported to be positively correlated with progression of diabetic nephropathy (7). These discordant reported tissue profiles for CD163+ cells show that dysregulation of CD163+ macrophages is associated

with diabetes complications; however a detailed profile of circulating CD163+ monocytes, the precursor of macrophages, is unclear. Therefore, we aimed to deeply characterize circulating CD163+ monocytes in individuals with long-duration of diabetes and complications compared to those without complications. We aimed to utilize RNA-sequencing (RNA-seq) to identify the RNA profile of the CD163+ monocyte; and mass cytometry for characteristic phenotyping to discover potential underlying mechanisms whereby CD163+ monocytes regulation leads to diabetes complications.

Research Design and Methods

Study Subjects

Adults with diabetes duration of greater than 10 years, either with (D^{+Comps}; n=6) or without any microvascular or macrovascular complications (D^{-Comps}; n=6) were recruited from the Diabetes Centre, Royal Prince Alfred Hospital. Complication status has been assessed as described previously (8). Briefly, individuals were included based on the presence of retinopathy, which was confirmed by fundal examination and/or photography. The absence of diabetic nephropathy was confirmed by normal serum creatinine and urinary albumin-creatinine ratio <2.5mg/mmol for men and <3.5 mg/mmol for women. Participants were considered to have macrovascular disease if they had any relevant symptoms of vascular disease or had a reported history of abnormal investigation or prior macrovascular event. This study has been approved by the Human Ethics Review Committee of Sydney Local Health District and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. All participants gave their written informed consent (8).

RNA Sequencing of CD163+ Monocytes

Isolation of CD163+ cells

CD163+ monocytes were isolated from freshly collected blood by fluorescence activated cell sorting (FACS) FACSAria (Becton Dickinson, San Jose, CA, USA) using antibodies CD45-PerCP-Cy5.5 (Becton Dickinson, San Jose, CA, USA), CD14-PE-Cy7 (Becton Dickinson, San Jose, CA, USA), and CD163-APC (R&D Systems, Minneapolis, MN, USA). The dead cells were excluded by the dye diamidino-2-phenylindole (DAPI) (Invitrogen, Waltham, MA, USA) staining. The purity of the isolated CD163+ cells approached 95% as shown in Supplementary Figure 1.

RNA extraction and sequencing

Total RNA was extracted from the isolated CD163+ monocytes by RNeasy Micro Kit (QIAGEN, Germany) according to the manufacturer's protocols. The quality and purity of RNA were determined by optical density on NanoDrop 2000 (Thermo Fisher Scientific Inc., USA) and Agilent Bioanalyzer RNA Nano Chip Bioanalyzer (Agilent Technologies, USA). Samples were excluded if the RNA was degraded. Illumina Stranded Total RNA Prep was used to convert RNA into sequencing-ready libraries and the Illumina Ribo-Zero Plus rRNA Depletion Kit was used to remove abundant RNA by enzymatic depletion. RNA from each of 12 samples were normalized, and then progressed using the Illumina bcl2fastq 2.20.0.422 pipeline to generate the sequence data by NovaSeq 6000 System (Illumina, San Diego, CA, USA). Sequence libraries were prepared at 150bp paired end sequencing, with a depth of 50 million reads. The data generated met the predetermined quality standards, and the resultant FASTQ files were used for data analysis. RNA-seq was performed by Australian Genome Research Facility.

Quality control and differential gene expression analysis

The RNA-seq data was analysed using the Galaxy web platform at *usegalaxy.org* (9). As shown in Supplementary Figure 2, FASTQ files were generated after quality control assessment on the Galaxy platform using the 'FastQC' tool (10). The raw reads were trimmed using 'Trimmomatic' to remove the adaptors (11). The quality of the raw reads was again assessed using FastQC and aligned individually to the human reference genome Hg38 (Human Dec. 2013 GRCh38/hg38), using 'HISAT2' (version 2.2.1) (12). The mapped reads were then quantified using 'Feature Counts' and differentially expressed genes (DEGs) between D^{+Comps} and D^{-Comps} were analyzed using 'Limma-Voom' (version 3.50.1), in which the selected contrast of interest was D^{+Comps} relative to D^{-Comps}. Furthermore, genes were normalized, and filtered out for those less than 1 count per million. Limma-Voom analyzed the DEGs with the following criteria: log2-fold-change, and adjusted p-value <0.05 using the Benjamini-Hochberg (BH) method (13, 14). The list of DEGs were then sorted to identify significantly up or down-regulated genes, using a cut-off value of ≥1.5 absolute fold-change (FC) (BHp<0.05). The Panther 17.0 Classification System was used to independently evaluate the list of significantly up and down-regulated genes for gene ontology (GO) enrichment analysis using the over-representation test and functional classification of biological processes (15, 16).

The 'Genes of Interest' (GOI) data was generated as per Figure 1 and 2. In order to identify the interaction between individual GOI, a PPIs network was established using the search tool for retrieval of interacting genes (STRING) database and the network was visualized by Cytoscape (version 3.9.1) (17, 18). Nodes in the PPIs network represent genes, while the edges represent interactions between genes. In this study, nodes with the greatest number edges in connection with others, were termed 'hub' genes.

GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted individually on the up and down-regulated GOI, as well as the combined GOI list. The top 5 most significant enriched 'GO' categories were reported (false discovery rate FDR<0.05).

Functional Phenotyping by Mass Cytometry

Cell preparation and staining with antibodies for mass cytometry

Blood sample was stored in the Smart Tube Proteomic Stabiliser and underwent erythrocyte lysis using the Smart Tube Thaw-Lyse Kit as per manufacturer's protocol (Smart Tube Inc., Las Vegas, NV, USA). After lysis, white blood cell pellets were resuspended in FACS buffer (0.5% BSA, 2mM EDTA and 0.02% Sodium Azide in PBS) and a total of $2x10^6$ cells were blocked with heparin to reduce non-specific eosinophil staining (19). Samples were then incubated with anti-CD45 antibody to barcode samples as ^{106}Pd-CD45 for D^{+Comps} and ^{108}Pd-CD45 for D^{-Comps} samples. Paired D^{+Comps} and D^{-Comps} samples were then stained with cell surface markers as shown in Supplementary Table 1. DNA intercalator Iridium in Perm Buffer with Paraformaldehyde was used to stain DNA in the cells.

Subsequently cells were counted and resuspended at approximately $1x10^6$ cells/mL with EQ calibration beads. Samples were then filtered, and data acquisition was performed with a time-of-flight mass cytometer (Helios™, CyTOF®system, Fluidigm, San Francisco, CA, United States).

Manual gating of CD163+ monocyte subsets

Mass cytometry data was analysed with FlowJo (FlowJo LLC., Oregon, USA). The manual gating strategy used to profile CD163+ monocytes with cell surface markers indicating monocyte function is shown in Supplementary Figure 3. Briefly, normalization beads were excluded, then cell singlets were gated on event length against DNA. The paired D^{+Comps} and D^{-Comps} were de-barcoded as individual samples and negative selection was sequentially processed to exclude platelets, granulocytes, T-cells, B-cells and NK cells then positive selection applied using HLADR. Subsequently CD163+ monocytes were gated under CD14+ monocytes. Finally, gating was used under CD163+ monocytes to identify various cell function markers and results expressed as a percentage of CD163+ monocytes. These studies were undertaken in a blinded manner to the group source of the CD163+ monocytes (complications present or absent).

Gene-Protein Combined PPIs Network

To investigate the interaction between genes and cellular surface markers, the significantly different cell surface markers across D^{+Comps} and D^{-Comps} were included in the GOI list, and a PPIs network was analysed as described above. The nodes with the greatest number of edges in connection with others, were termed 'hub node(s)'.

Statistical Analysis

Data analysis of clinical characteristics and the immunophenotyping of CD163+ monocytes was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Continuous data were checked for normality and presented as mean ± standard deviation (SD) for parametric data and median and IQR or proportion for non-parametric data as indicated. Grouped data were compared by either Welch's t-test for parametric data, or Mann-

Whitney test for non-parametric data or Fisher's exact test to identify differences between groups.

For RNA-seq data, the DEGs were accepted as significant when adjusted p-value BHp was <0.05. The over-representation test identified significantly enriched biological processes and was determined using Fisher's exact test ($p<0.05$) with FDR correction. Finally, the PPIs network utilized an FDR <0.05 to identify enriched GO categories and KEGG pathways.

Data and Resource Availability

The datasets generated and/or analysed during the current study are not publicly available due to restrictions in the ethics permission provided by participants.

Results

Clinical Characteristics of Individuals with Diabetes with or without Complications

Clinical characteristics of individuals with diabetes and with (D^{+Comps}, n=6) or without (D^{-Comps}, n=6) any microvascular or macrovascular complications, are presented in Table 1. There was no significant difference in age, BMI, HbA1c, and lipid profile between the groups.

RNA Profiling of CD163+ Monocytes

As shown in Figure 1 and 2, a total of 10,868 DEGs were identified in a comparison between D^{+Comps} and D^{-Comps}. Analysing the DEGs according to an FC ≥1.5 or ≤-1.5 revealed 885 significantly up-regulated genes (FC≥1.5; BHp<0.05), and 190 significantly down-regulated genes (FC≤-1.5; BHp<0.05). This included mRNA, microRNA (miRNA), tRNA, pseudogenes, antisense RNA, and long non-coding RNAs (IncRNAs).

Gene over-representation analysis

The over-representation test found that the genes associated with 'regulation of centrosome cycle' were 6.7-fold enriched in D^{+Comps} when compared to reference genome (FDR<0.05), these included *PLK2, CENATAC, RBM14, CCNL2, KAT2A, CCNL1, XRCC3, CEP295NL, CEP131, CDK1B* and *CDK11A*.

Gene functional classification analysis

When the complete lists of 885 up and 190 down-regulated genes were functionally classified according to biological processes, 677 up-regulated and 185 down-regulated genes from CD163+ cells were identified in D^{+Comps} compared to D^{-Comps} (Figure 1). The biological processes used were: biological adhesion, immune system process, localization, response to stimulus and signalling. These were selectively screened for genes relating to diabetes, inflammation, immune system functions or monocyte functions and resulted in a total of 13 up-regulated GOI including *ZAP70, CD5, NLGN3, MAPK81P3, MAPK13, IL11RA, ZBTB17, TRAF1, TRPV1, ZBTB25, NFKB2, IRS2* and *TRAF31P2-AS1* and 11 down-regulated genes including *EMB, CD86, TLR4, NCK1, CD244, CD1C, PALLD, DSC2, TGFA, CX3CR1* and *PPBP,* being identified (Figure 1 and Figure 2).

Screening the list of 'Genes of Interest'

The process and results of selectively screening for GOI are detailed in Figure 2**.** The top 20 most up-regulated and down-regulated DEGs were compiled into a new list: GOI with 13 up-regulated and 11 down-regulated genes identified by their selected biological processes described above. In addition, the DEGs were rescreened again based upon their functions relating to diabetes, monocyte function, inflammation, cytokines/chemokines and matrix metalloproteinases (MMPs). After discounting genes already identified, this analysis revealed

a further 13 up-regulated genes (FC ≥1.5; BHp<0.05) which included *MAPK81P1, LOC286059, AMIGO3, APOA2, ADGRL1, MMP24, NFKBID, CIQTNF-AS1, ADGRB1, SMAGP, FASN, RELT*, and *TRAF4*, and these were added onto the GOI list. There were no further down-regulated genes found which met the cut off value ≥1.5. This three-pronged screening process resulted in the final list of 75 GOI shown in Supplementary Table 2. Forty-six GOI were up-regulated in which microRNAs: *MIR27A, MIR3648-1 and MIR23A* had the greatest increase in D^{+Comps} (FC>6-fold, BHp<0.01, BHp<0.05, BHp<0.01, respectively). There were 29 down-regulated GOI in which *CD200R1* was the most reduced (FC -3.7-fold, BHp<0.05). Figure 3 depicts the spread of these genes in a volcano plot.

Gene interaction network analysis

To further understand potential functional associations between the genes in the curated list of GOI, the interactional PPIs networks were created for up-regulated GOI (A), down-regulated GOI (B) and all GOI (C), as shown in Figure 4. Within the 46 up-regulated genes from CD163+ monocytes in D^{+Comps}, STRING identified a network of 8 nodes and 8 edges including *RELT, TRAF1, NFKB2, NFKBID, CD5, CD7, ZAP70* and *MAPK13* which contained interconnected genes relating to T cell function such as *RELT, NFKBID, CD5, CD7* and *ZAP70* (Figure 4A). *CD5* was the 'hub' gene with 3 edges, connected to 3 different nodes and *CD7* was the most up-regulated gene by 5.1-fold. Within the 29 down-regulated genes, STRING identified a network of 10 genes and edges including *CALM2, CYP1B1, PTGS2, CD244, CD86, TLR4, CD1C, PPBP, CX3CR1* and *CD200R1* (Figure 4B). *CD86* and *TLR4* were the 'hub-genes', each with 6 edges connected to 6 nodes. *CD200R1* was the top down-regulated gene by -3.7-fold. This network of down-regulated genes demonstrated a connection between monocyte activation genes *CD86, TLR4* and *CD244*, and the gene associated with monocyte migration, *CX3CR1*.

Within the 75 combined all GOI, STRING identified the largest network of 26 genes (Figure 4C). The 'hub-genes' with the most connections included *TLR4* with 10 edges and *CD86* with 9 edges. *CD7* was the most up-regulated gene, and *CD200R1* the most down-regulated. Furthermore, *IRS2* was connected to *FASN*, and *MAPK81P3* was connected to *MAPK81P1*.

Functional Phenotyping of CD163+ Monocytes

The expression of cell surface markers representing various monocyte functions were quantified as a subset of CD163+ identified monocytes (Table 2). The proportion of chemokine receptor CCR5+ cells was 1.3-fold lower and CD11b+, CD11c+, and CD31+, indicators of cell adhesion and trans-endothelial migration, were reduced 4.5, 2.2 and 8.7-fold respectively in CD163+ cells in D^{+Comps} compared to D^{-Comps} (all $p<0.05$). In addition, CD39 and CD86 which can promote immune regulation were significantly decreased in D^{+Comps} (3.1 and 3.9-fold respectively; both $p<0.05$). Interestingly, the positive proportion of the toll-like receptors CD282 and CD284 and activation marker CD38 were very low in CD163+ cells in both groups with no difference between them. The marker involved in phagocytosis and clearance, CD68, was elevated in CD163+ cells in both groups and was slightly lower in D^{+Comps} compared to D^{-Comps}.

Gene-protein Interaction Network

A gene-protein interaction network was created combining the 75 GOI in addition to the significantly different cell surface markers including CCR5, CD11b, CD11c, CD31, CD39, CD86 and CD68. This analysis revealed a network of 32 nodes in which CD11b and *TLR4* were 'hub-nodes' with 16 edges each connected to 16 nodes and CD86 with 14 edges connected to 14 nodes which was found down-regulated at both gene and cell surface levels

(Figure 5). The 'hub-node' CD11b was connected to genes including *TLR4*, *PPBP, PTGS2,* and *ZAP70* as well as various cell surface markers. *TLR4* was linked to genes *TRPV1, NFKB2, TRAF1, CD200R1, CX3CR1, PPBP* and surface markers CD86, CD68, CD31, CD39 (Figure 5). CD86 was in connection with genes *CD244, PTGS2, ZAP70* and cell surface markers CD31 and CD39.

CD7 was most up-regulated by 5.1-fold which was connected to 8 nodes, including proteins CD11c, CD11b, CD68, CCR5 and genes *CD1C, CD5*, *CD244* and *ZAP70*. CD31 was most down-regulated by -8.7-fold and was connected to 12 nodes, including genes *MMP24*, *PTGS2, CD1c, CD5* and *NK1*.

There were two notable low-density 'hub-genes' within this network including *TRPV1* connected to *TLR4, CALM2, BBS10, WHRN* and *LPRN* and the pro-inflammatory gene *NFKB2* connected to *TLR4, TRAF1*, *PTGS2,* and *CD5*.

Discussion

Diabetes is a chronic metabolic disease, that is increasing in incidence and prevalence. As the duration of diabetes extends, individuals commonly develop accompanying microvascular or macrovascular complications which are progressive in nature and are responsible for increased morbidity and mortality, lowering the quality of life of individuals with the disease (1). As such these factors warrant the need for therapeutic measures which can either alleviate or halt the progression of diabetes-related complications resulting in an increased capacity to manage their diabetes. Chronic low-grade inflammation is a common feature found in diabetes individuals and may be a driver for the progressive nature of diabetes complications (2). The anti-inflammatory myeloid scavenger receptor CD163 has been reported to be decreased in individuals with diabetes complications (3). Using RNA-seq and mass cytometry, we observed that both the RNA and phenotypic profiles of circulating CD163+

monocytes were different in those with diabetes and complications compared to those without. More specifically, in the context of complications, we report the enriched centrosome cycle genes and up-regulated miRNAs, suggesting increased cell apoptosis therefore resulting in decreased numbers of anti-inflammatory CD163+ monocytes (Figure 6). Additionally, in individuals with diabetes-related complications, CD163+ monocytes were characterised to suggest reduced abilities for cell recruitment and trans-endothelial migration, along with dysregulated activation of their adaptive immune system (Figure 6). These findings strongly support the concept that the anti-inflammatory nature and functionality of the CD163+ monocytes are diminished in diabetes complications, and this may reduce the potential to resolve inflammation.

Deep RNA profiling of circulating CD163+ monocytes in our study revealed that in individuals with diabetes-related complications, the genes associated with the 'regulation of centrosome cycle' were up-regulated and enriched. Similarly, Wang and colleagues reported enriched centrosome amplification within peripheral blood mononuclear cells of individuals with T2DM, although the complication status of the participants was unknown (20). The centrosome and its associated components determine the geometry of microtubule arrays throughout the cell cycle which influence cell shape, polarity, motility, chromosome segregation and cell division (21). Dysregulation in the centrosome cycle, can implicate cellular apoptosis (22) which would correspond with the up-regulated microRNAs *MIR23A* and *MIR27A* found in this study in CD163+ cells, as they also contribute to increased cellular apoptosis (23, 24). Our finding of potentially increased cellular apoptosis of circulating CD163+ monocytes would explain the previous finding of decreased circulating peripheral CD163+ monocytes in individuals with diabetes-related complications (3). The reduced presence of anti-inflammatory CD163+ monocytes in the systemic circulation would support

the concept of a prolonged inflammatory state in the systemic environment of individuals with diabetes-related complications (Figure 6).

A significantly distinct characteristic profile of CD163+ monocytes was observed in this study in the individuals with diabetes and complications compared to those without. Specifically, genes and cell surface markers involved in trans-endothelial migration and recruitment of CD163+ monocytes were significantly suppressed in diabetes complications and the expression of chemokine receptors, *CX3CR1*, *CXCL7 (PPBP)* and CCR5 were down-regulated. When coupled with the decreased proportion of the adhesion markers CD11b, CD11c and CD31, it would be indicative of a reduced ability of CD163+ monocytes to adhere to the endothelium of vasculature and extravasate into local tissues (Figure 6). Furthermore, the association between the up-regulated gene *MMP24* and down-regulated protein CD31 which we discovered from the interaction network, suggested that the reduced cell surface expression of CD31 may possibly be due to the action of MMP24. MMP24, is a membrane-type MMP expressed on cell surface and has previously been found to attenuate the cell surface expression of CD31 by mediating ecto-domain shedding in animal studies of diabetic retinopathy (25).

In this study, we report that in the anti-inflammatory CD163+ monocyte, the function of antigen recognition may be decreased in individuals with diabetes and complications compared to those without, although a very low proportion of TLR4+ cells was detected in the CD163+ cells from both groups. The down-regulated *TLR4* would be consistent with a diminished capacity of the CD163+ monocytes to recognize and internalize antigens such as pathogen-associated molecular pattern molecules (PAMPs) and damage associated molecular pattern molecules (DAMPS), leading to an inadequate immune response, impaired pathogen

clearance (26) and continued inflammation (Figure 6). The down-regulated *TLR4* in CD163+ cells, which is a 'hub-node' as depicted in Figure 5, raises the proposition that the reduced TLR4 functionality would trigger a cascade of events involving both monocyte function and immune response and thereby contribute to the development of complications (27). On the other hand, an earlier study in a rat model of diabetic retinopathy had reported increased TLR4 in retinal extracts was associated with disease progression (28) and that the blockade of TLR4 signalling pathway reduced inflammation (28-30). Indicating that the TLR4 signalling must be tightly regulated to prevent excessive inflammation.

The ability of the CD163+ monocytes to clear and regulate extracellular ATP was indicated to be diminished in individuals with diabetes and complications and this was potentially due to the decreased expression of the ectonucleotides CD39 and CD73, as illustrated in Figure 6. A dysregulation of extracellular ATP may impair the supply and formation of adenosine, an anti-inflammatory mediator, which would lead to a pro-inflammatory environment (31). Similarly, the lowest CD39 expression in circulating lymphocytes was observed in individuals with diabetes-related complications, compared to those without complications (32). Reduced CD39 expression also has been implicated in reduced monocyte-mediated lymphocyte activation (33). This presents the possibility that the unresolving nature of diabetes-related complications may be due to the dysregulated interaction between the antigen-presenting CD163+ monocytes and the adaptive immune system (Figure 6).

We observed an increase in the expression of the gene *CD5* in CD163+ monocytes and CD5 has been reported to suppress the activation of peripheral T cells through inhibition of T cell receptors at the immunological synapse (34). Similarly, CD86 was also down-regulated in the CD163+ monocytes from the complications group. CD86 is a co-stimulatory molecule

necessary for effective T cell activation and survival (35). As depicted in Figure 6, our results therefore indicate, that CD163+ monocyte mediated T cell activation was down-regulated in individuals with diabetes-related complications, due to increased expression of *CD5* and decreased CD86. However we report, an alternative pathway for T cell activation involving the up-regulated expressions of *CD7* and *ZAP-70* which are both implicated in initiating T cell responses via the antigen receptor (36, 37). This may indicate a possible connection point between the non-specific immune response driven by monocytes and the adaptive and specific immune response mediated by T cells.

Furthermore, we found that in diabetes-related complications CD163+ monocyte mediated immuno-suppression was likely reduced due to decreased expression of the immune inhibitory receptor *CD200R1* (Figure 6). The *CD200* interaction with its receptor *CD200R1* typically evokes an immune-suppressive response (38). Our study therefore supports the concept that long-duration of diabetes with complications was the result, of reduced immuno-suppression, leading to prolonged inflammation at organ sites. Interestingly, in our study we demonstrated that *CD200R1* was connected to the down-regulated monocyte function markers CD86 and *TLR4* (Figure 4b), implicating the *CD200-CD200R1* immuno-suppressive pathway as responsible for the decrease in monocyte mediated T cell activation (Figure 6).

In this study, a satellite network was identified with the up-regulated *TRPV1* gene in individuals with diabetes-related complications. Earlier studies have reported that blockade of *TRPV1* in rodent T2DM halted disease progression, indicating that a *TRPV1* antagonist may be a therapeutic target of diabetes disease progression, improving glucose intolerance and correcting dysfunctional insulin secretion (39).

A limitation of the current study is the small sample size. Despite generating an extensive amount of data pertaining to all aspects of cellular function, we have constrained the differential gene analysis strictly to areas of our interests. Future studies will focus on identifying genomic mechanisms of lipid, insulin or glucose dysregulation in CD163+ monocytes and expand the sample size in a larger cohort study.

In conclusion, our study is the first to comprehensively characterize the RNA profile and phenotype of CD163+ monocytes in individuals with long-term diabetes and complications, compared to those without complications, using advanced techniques of RNA-seq and mass cytometry. Our study presents new implications for the role of CD163+ monocytes in diabetes-related complications. This study identified a set of genes that are more abundant in CD163+ monocytes which are involved in centrosome cycle and miRNAs that are associated with cell death, consistent with the decreased CD163+ cells. Additionally, the study observed potentially decreased activity in genes and cell surface markers involved in monocyte activation, recruitment, and immune regulation. These results suggest that CD163+ monocytes have unique characteristics in diabetes-related complications and it is their potential loss of function and programmed death that leads to increased tissue complications of diabetes. Prospective research is now needed to build upon our data to define the role of these cells in the development of complications in diabetes.

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Author Contributions

E.S. performed the experiment, data analysis and drafted the manuscript. J.W. and B.B. acquired the clinical samples and clinical data. D.S. and C.B. assisted with mass cytometry and N.D. assisted with RNA-seq data analysis. S.M. assisted with the study cohort design. S.T. contributed to data interpretation, and revision of the manuscript. D.M. contributed to study design, performed part of experiment, supervision and revision of the manuscript. D.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to review/edit and approved the final version for submission.

Duality of Interest

The authors declare that no other potential conflicts of interest relevant to this article were reported.

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Table 1: Clinical characteristics of individuals with diabetes and with and without complications.

	D^{+Comps} (n=6)	D^{-Comps} (n=6)	P-value
Age (years)	73.2 ± 5.2	67.2 ± 18.3	ns
Men/Women (n)	4/2	3/3	ns
Duration of diabetes (years)	22.0 ± 5.9	28.0 ± 4.9	ns
BMI (kg/m^2)	30.5 ± 5.9	26.7 ± 3.9	ns
HbA_{1c} (mmol/mol)	64 (55 – 66)	61 (40 – 64)	ns
HbA_{1c} (%NGSP units)	8.0 (7.2-8.2)	7.7 (5.8-8.0)	ns
Total Cholesterol (mmol/L)	4.3 ± 0.9	3.7 ± 0.6	ns
HDL-c (mmol/L)	1.4 ± 0.1	1.4 ± 0.4	ns
LDL-c (mmol/L)	2.3 ± 0.9	1.7 ± 0.5	ns
Triglycerides (mmol/L)	1.5 ± 0.8	1.4 ± 0.2	ns

Results are expressed as mean±SD or median with interquartile range (IQR) or proportion. Significance was accepted at $p<0.05$ and no significance indicated by ns. Complications was defined by the presence of any diabetic retinopathy in each participant in D^{+Comps}.

Table 2: Functional phenotyping of CD163+ monocytes in individuals with diabetes and with or without complications.

Function	Positive % of CD163+ cells	D^{+Comps} (n=6)	D^{-Comps} (n=6)	Fold Change	P value
Activation	**CD282 (TLR2)**	0.4 (0.1 – 3.7)	0.45 (0.2 – 0.8)	-0.1	ns
	CD284 (TLR4)	0.3 (0.2 – 3.6)	0.5 (0.3 – 0.7)	-1.9	ns
	CD38	0.4 (0.2 – 3.1)	2.4 (1.7 – 4.1)	-6.0	p=0.06
Chemokine receptors	**CD192 (CCR2)**	2.6 (1.7 – 4.8)	0.9 (0.5 – 3.6)	2.6	ns
	CD195 (CCR5)	59.6 (45.9 – 70.9)	78.4 (72.9 – 78.7)	-1.3	p<0.05
	CX3CR1	0.8 (0.7 – 2.1)	0.8 (0.4 – 0.9)	-1.1	ns
	CXCR3	9.6 ± 7.3	8.2 ± 9.6	0.8	ns
Adhesion & migration	**CD11b**	12.1 (7.8 – 22.2)	54.4 (29.9 – 58.6)	-4.5	p<0.05
	CD11c	27.8 ± 11.5	60.1 ± 22.3	-2.2	p<0.05
	CD31 (PECAM-1)	4.7 (3.5 – 13.9)	41.0 (18.8 – 52.3)	-8.7	p<0.05
Immune regulation	**CD39**	4.2 ± 2.4	13.0 ± 6.8	-3.1	p<0.05
	CD73	0.7 (0.5 – 3.6)	1.3 (0.9 – 2.1)	-1.9	p=0.06
	CD80	1.8 (1.3 – 3.1)	1.3 (0.8 – 1.6)	0.7	ns
	CD86	3.2 ± 1.1	12.5 ± 7.2	-3.9	p<0.05
	CD206	0.1 (0.05 -1.05)	0.25 (0.03 – 1.9)	-2.8	ns
Phagocytosis & Clearance	**CD36**	99.2 (82.2 – 99.6)	92.0 (65.7 – 99.5)	1.1	ns
	CD68	97.3 (93.7 – 98.2)	98.8 (98.1 – 99.7)	-1.0	p<0.05

Results are expressed as mean ± SD or median with interquartile range (IQR) or proportion. Folder changes were expressed as D^{+Comps} compared to D^{-Comps} and significance accepted at $p<0.05$. No significance indicated by *ns*.

Figure 1

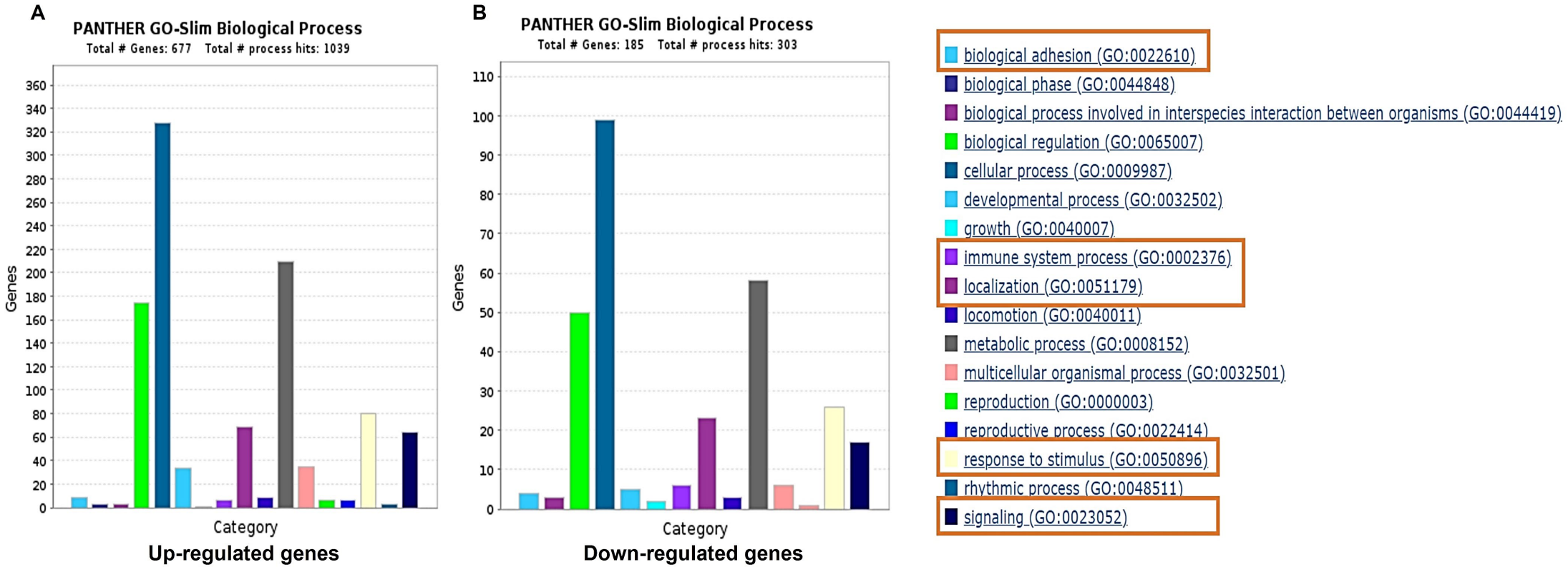


Figure 1

Functional characterization of up and down-regulated genes (FC ≥1.5, BHp<0.05) in D^{+Comps} compared with D^{-Comps} according to biological process. The orange box indicates biological processes that were selectively screened for genes relating to processes of our interest.

Figure 2

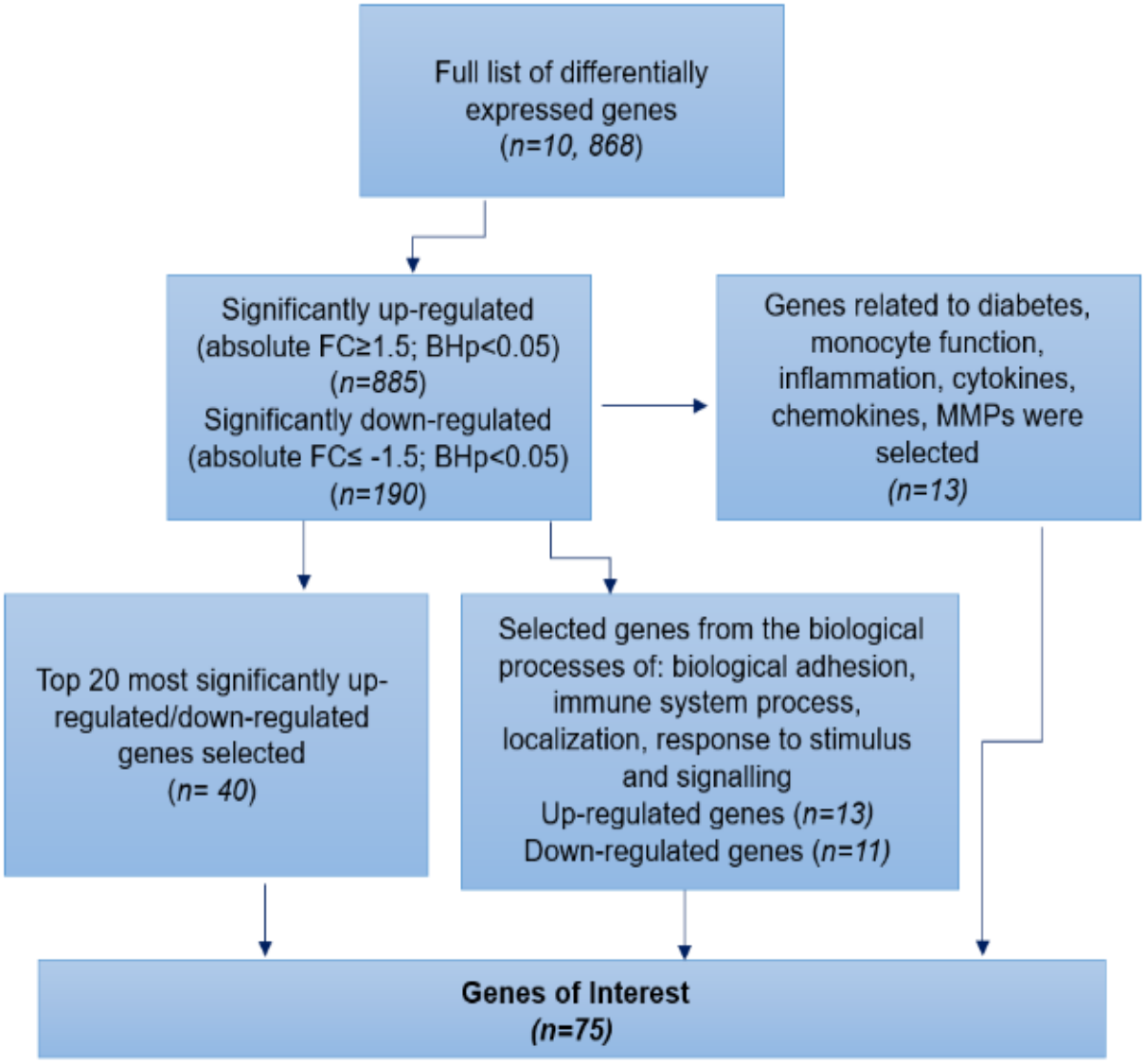


Figure 2

A flow-chart outlining the approach for identifying significant and relevant differentially expressed genes between individuals with long-term diabetes and complications (D^{+Comps}) and those without complications (D^{-Comps}). The top 20 most significantly up and down-regulated genes were combined with selected genes identified from functional characterization on PANTHER, as well as manual searching for genes related to monocyte function, cytokines, chemokines or MMPs, to generate a final list of 'Genes of Interest'.

Figure 3

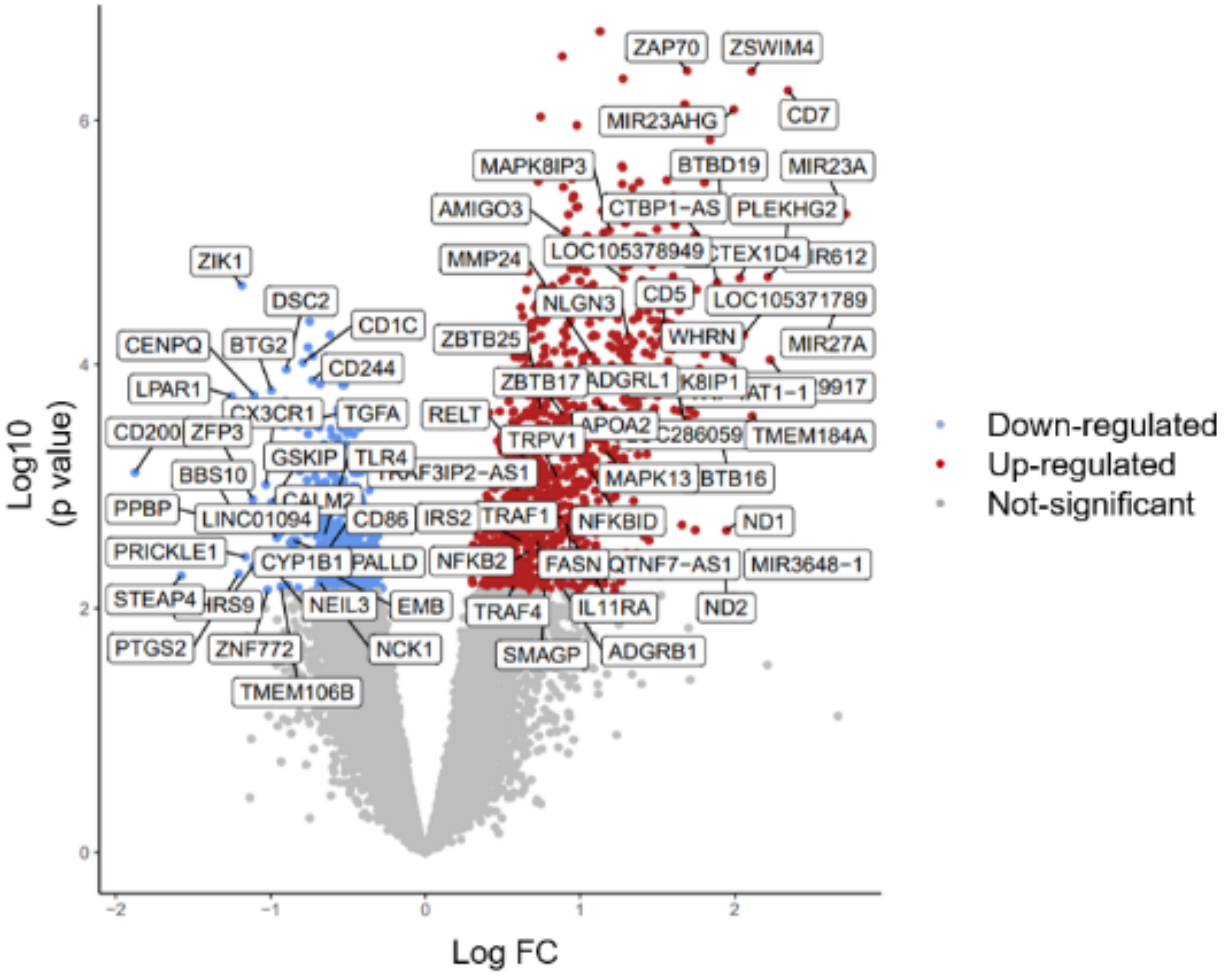


Figure 3

A volcano plot illustrating the selected 75 ‘Genes of Interest’ (FC≥1.5 & FC ≤-1.5, BHp<0.05).

Figure 4

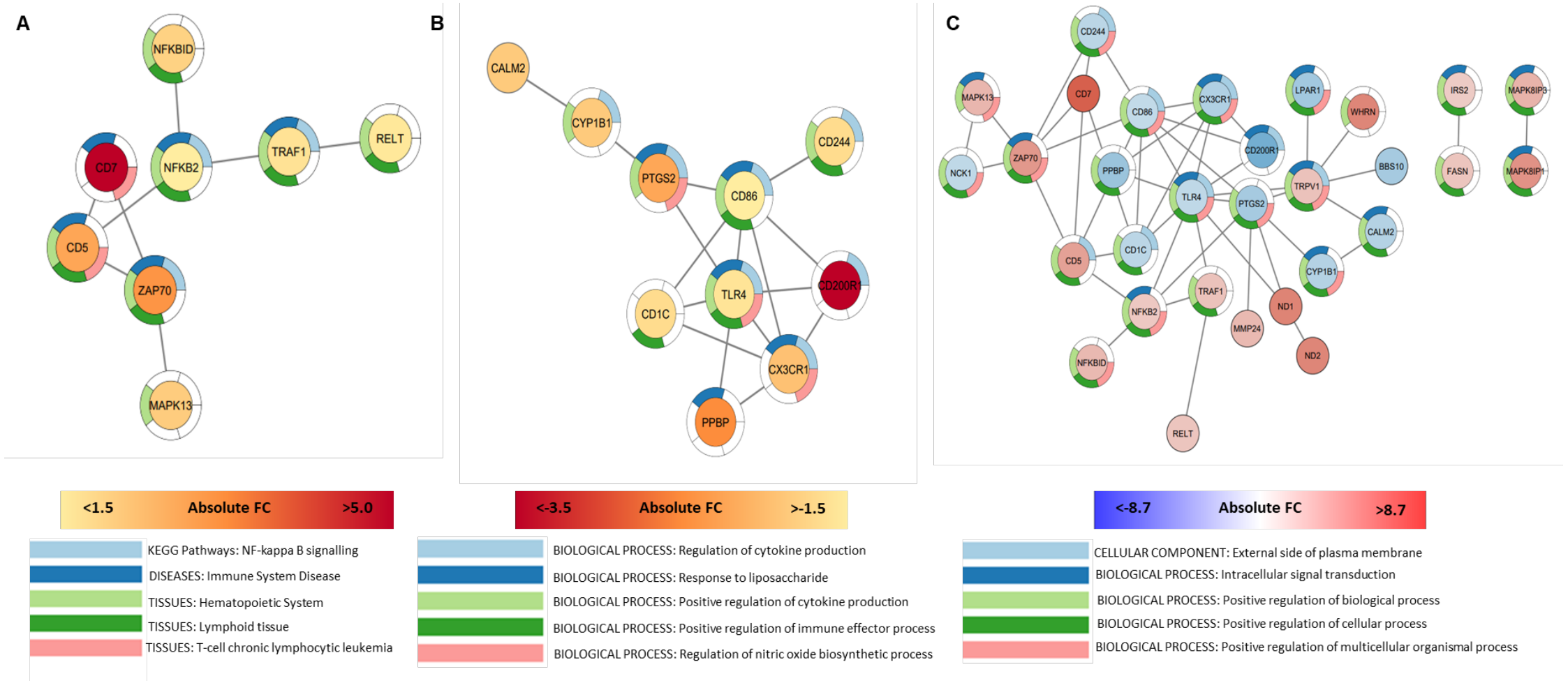


Figure 4

Gene Interactions network illustrating the largest connected network of genes in: a) up-regulated genes, b) down-regulated genes, and c) combined up and down-regulated genes, in individuals with long-term diabetes and complications (D^{+Comps}) and those without complications (D^{-Comps}). Genes were considered significantly up or down-regulated if the fold change was ≥1.5, and significance determined at BHp<0.05. The colour of the circular node indicates the intensity of the fold-change, confirmed by the legend below. The sectioned multicolour outline of each node indicates the involvement of respective genes with the top 5 most enriched GO categories including KEGG pathways.

Figure 5

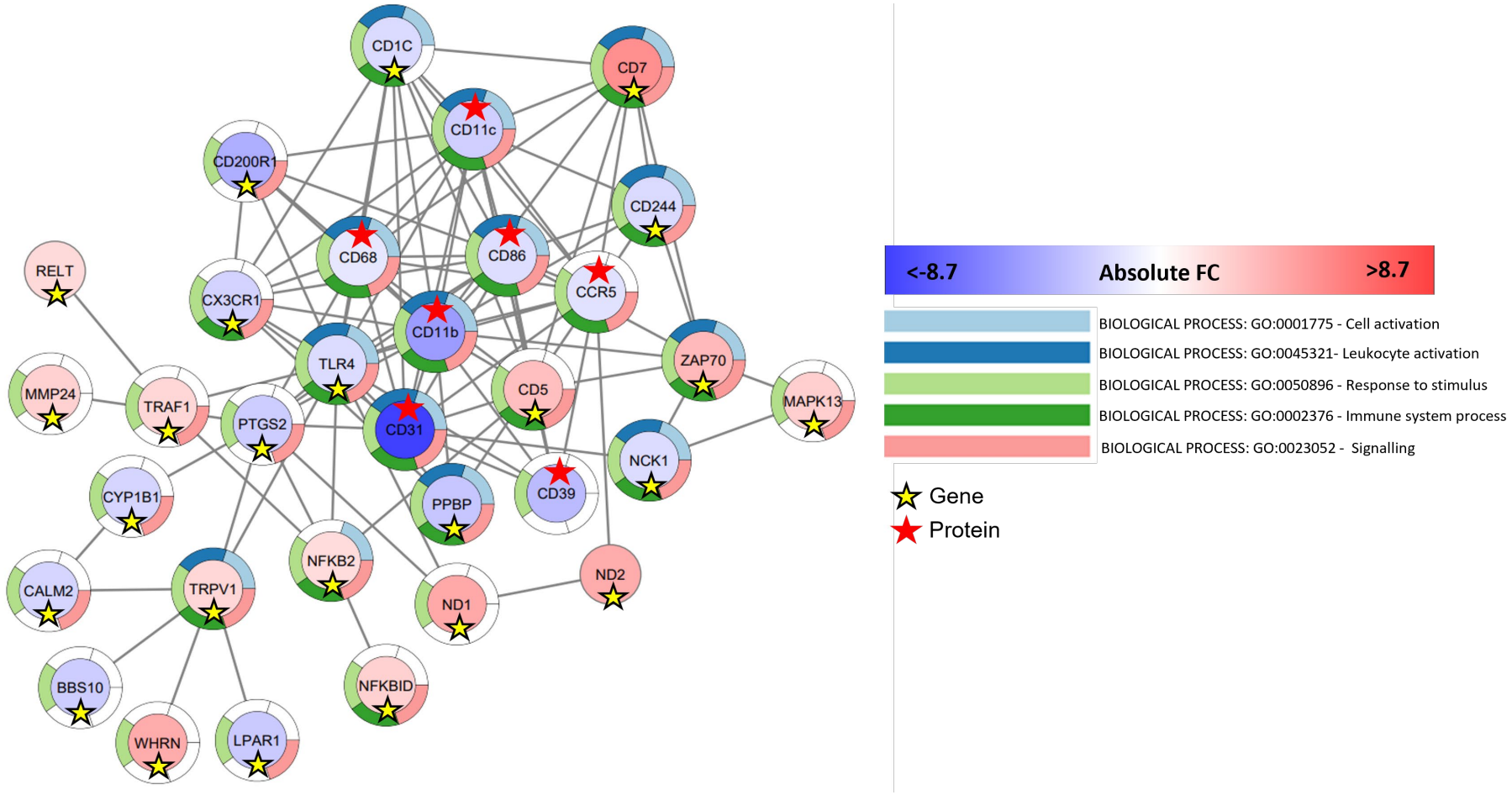


Figure 5

Gene-Protein Interactions network illustrating the largest connected network of 'Genes of Interest' combined with significantly different cell surface markers in CD163+ monocytes between individuals with long-term diabetes and complications (D^{+Comps}) and those without complications (D^{-Comps}). Genes were considered significantly down or up-regulated if the fold change was ≥1.5 (BHp<0.05). Proteins were considered significantly different at p<0.05. The colour of the circular node indicates the intensity of the fold-change, confirmed by the legend below. The sectioned multicolour outline of each node indicates the involvement of respective genes with the top 5 most enriched GO categories including Biological Processes. Yellow star indicates genes, whereas red star indicates protein.

Figure 6

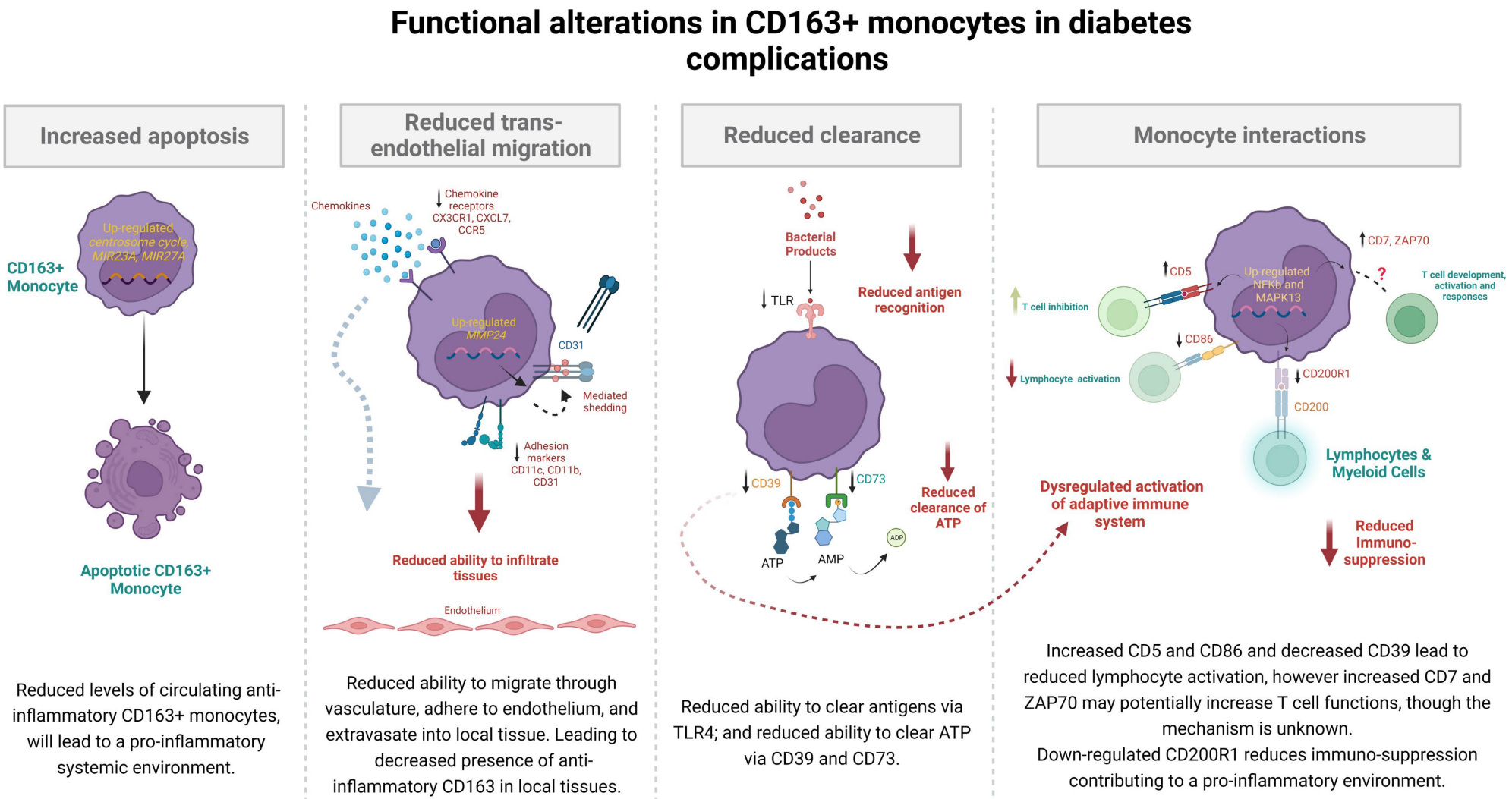
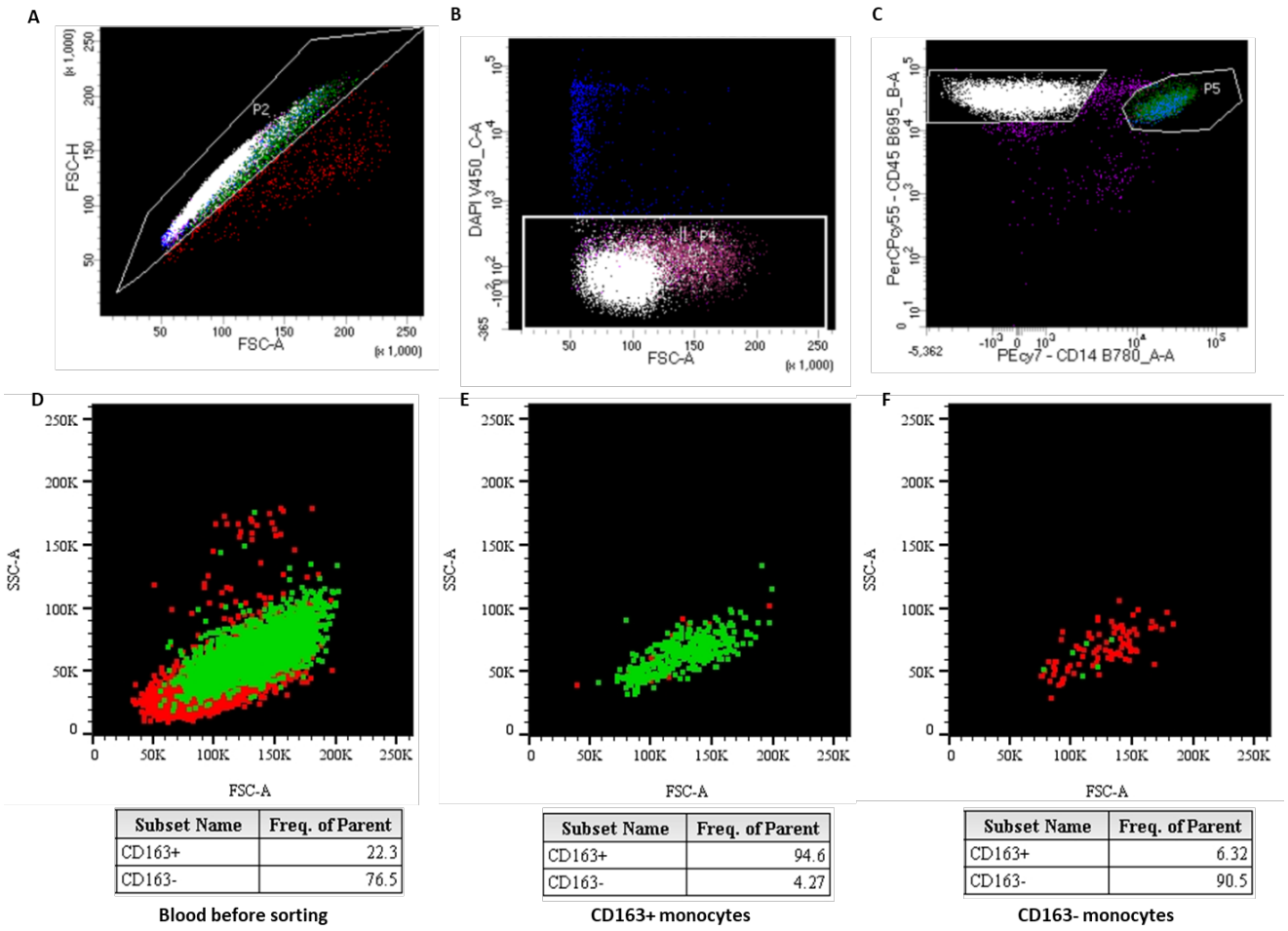


Figure 6

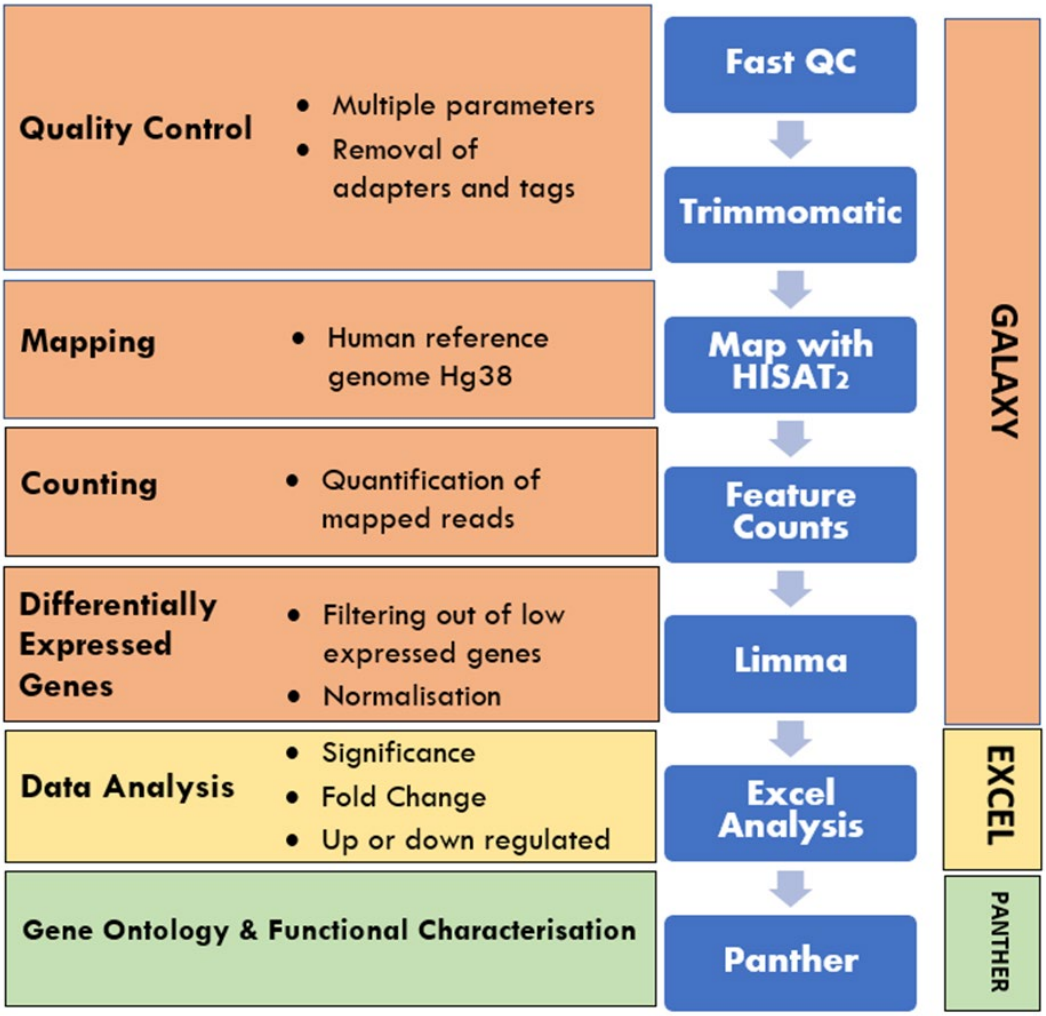
An infographic depicting the RNA and protein level functional alterations in the CD163+ monocytes in individuals with diabetes-related complications. The first panel describes mechanisms of increased apoptosis, the second panel depicts mechanisms involved in reduced trans-endothelial migration, the third panel depicts reduced CD163+ monocyte function, whilst the fourth panel depicts monocyte interactions.



Supplementary Figure 1

Isolating and detecting the purity of the CD163+ monocytes by flow cytometry. The proportion of CD163+ and CD163- monocytes were detected from blood by flow cytometry: (A) selection of singlets (B) live cells identified as DAPI- (C) CD45+CD14+ monocytes (D) before cell sorting by FACSAria, and (E & F) after sorting from (E) the isolated CD163+ cells (F) the isolated CD163- cells. Green dots indicate CD163+ cells and red dots indicate CD163- cells.

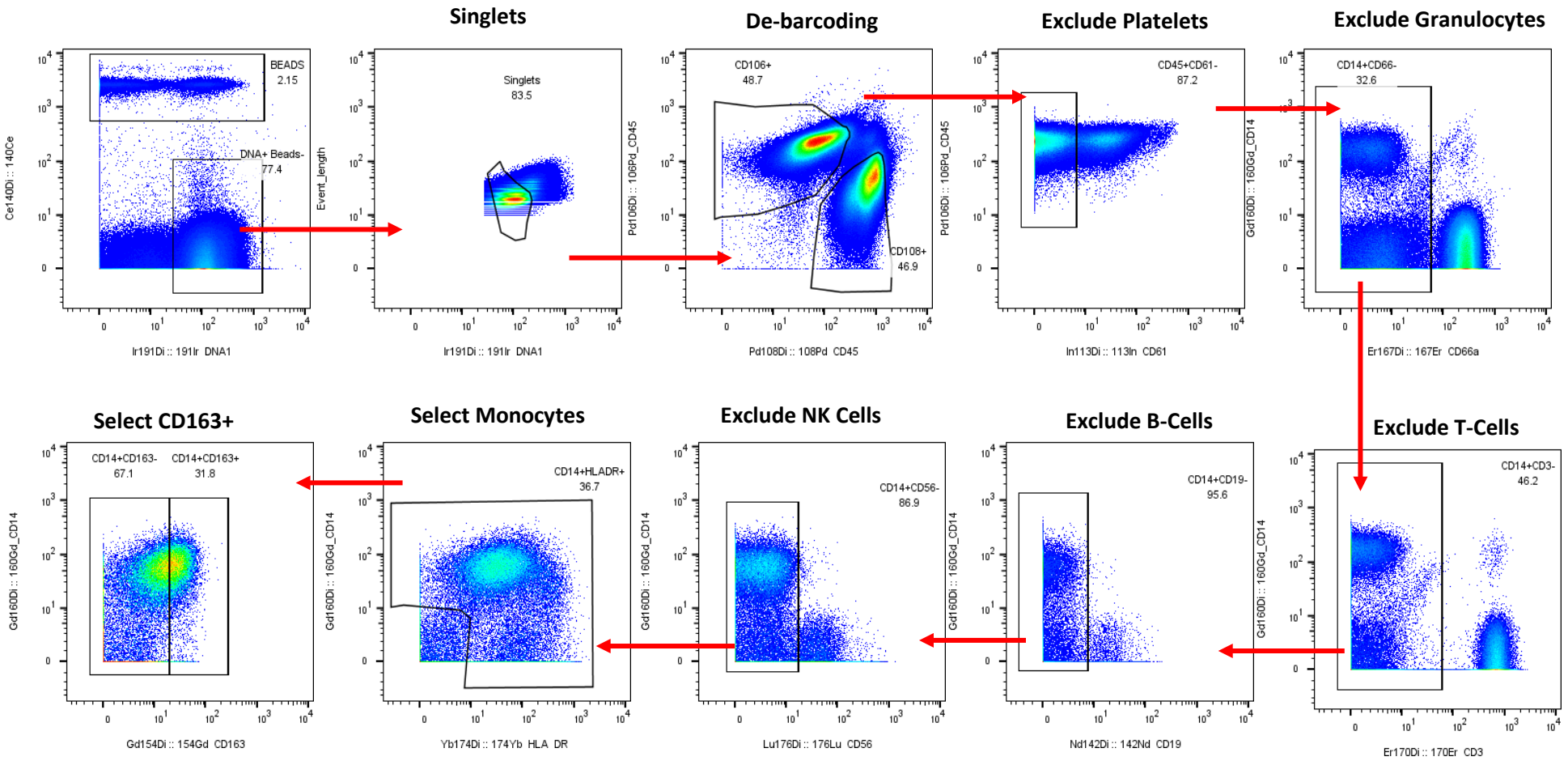
Supplementary Figure 2



Supplementary Figure 2

The RNA-sequencing analysis pipeline to identify differentially expressed genes, gene ontology and functional characterization in CD163+ monocytes in D^{+Comps} and D^{-Comps} using Galaxy and PANTHER classification tools, is shown.

Supplementary Figure 3



Supplementary Figure 3

The gating strategy established to target the CD163+ monocytes is shown using a negative selection to exclude platelets, granulocytes and lymphocytes, from CD45+ white blood cells.

Supplementary Material for Review

Supplementary Table 1. The list of mass cytometry immunophenotyping panel used in this study including the target CD marker, it's conjugated rare-earth metal isotype, the company details of the antibodies, and purpose of the marker within this panel.

Metal		Clone	Company	Target	Marker purpose
^{106}Pd	Palladium	HI30	Biolegend	CD45	Lineage/Barcode
^{108}Pd	Palladium	HI30	Biolegend	CD45	Lineage/Barcode
^{113}In	Indium	VI-PL2	Biolegend	CD61	Lineage
^{167}Er	Erbium	YTH71.3	Abcam	CD66	Lineage
^{170}Er	Erbium	UCHT1	Biolegend	CD3	Lineage
^{142Nd}	Neodymium	HIB19	Biolegend	CD19	Lineage
^{176}Yb	Ytterbium	REA196	Miltenyi	CD56	Lineage
^{160}Gd	Gadolinium	M5E2	BD	CD14	Lineage
^{174}Yb	Ytterbium	L243	Biolegend	HLA-DR	Lineage
^{154}Sm	Samarium	GHI/61	Biolegend	CD163	Anti-inflammatory
^{171}Yb	Ytterbium	TL2.1	Biolegend	CD282 (TLR-2)	Activation
^{149}Sm	Samarium	HTA125	BD	CD284 (TLR-4)	Activation
^{172}Yb	Ytterbium	HIT2	BD	CD38	Activation
^{151}Eu	Europium	K036C2	Biolegend	CD192(CCR2)	Chemokine receptor
^{144}Nd	Neodymium	HEK/1/85a	Bio-Rad	CD195(CCR5)	Chemokine receptor
^{164}Dy	Dysprosium	2A9-1	Biolegend	CX3CR1	Chemokine receptor
^{163}Dy	Dysprosium	REA232	Miltenyi	CXCR3	Chemokine receptor
^{209}Bi	Bismuth	ICRF44	Biolegend	CD11b	Adhesion
^{115}In	Indium	Bu15	Biolegend	CD11c	Adhesion
^{155}Gd	Gadolinium	WM59	BD	CD31	Adhesion
^{175}Lu	Lutetium	A1	Biolegend	CD39	Immune regulation
^{173}Yb	Ytterbium	AD2	Biolegend	CD73	Immune regulation
^{162}Dy	Dysprosium	L307.4	BD	CD80	Immune regulation
^{156}Gd	Gadolinium	IT2.2	BD	CD86	Immune regulation
139La	Lanthanum	15-2	Biolegend	CD206	Immune Regulation
^{152}Sm	Samarium	5-271	Biolegend	CD36	Phagocytosis & Clearance
^{153}Eu	Europium	KP1	Biolegend	CD68	Phagocytosis & Clearance

Supplementary Table 2. List of the 'Genes of Interest' including the up and down-regulated genes (≥1.5 FC; BHp<0.05).

Gene Symbol	Absolute FC	Adjusted P value	Gene Function
MIR23A*	6.6	0.002	MicroRNAs
MIR3648-1*	6.4	0.041	MicroRNAs
MIR27A*	6.3	0.003	MicroRNAs
CD7*	5.1	0.001	T-cell interactions and also in T-cell/B-cell interaction during early lymphoid development
PLEKHG2*	5.0	0.002	Regulates lymphocyte chemotaxis
LOC100129917*	4.7	0.005	-
MIR612*	4.6	0.002	MicroRNAs
TMEM184A*	4.3	0.008	Heparin receptor and mediates anti-inflammatory responses
ZSWIM4*	4.3	0.001	Predicted to enable zinc ion binding activity
LOC105371789*	4.2	0.004	An RNA Gene, and is affiliated with the lncRNA class.
TCTEX1D4*	4.1	0.002	Involved in microtubule-based movement
MIR23AHG*	4.0	0.001	An RNA Gene, and is affiliated with the lncRNA class
TRI-TAT1-1*	3.9	0.005	An RNA Gene, and is affiliated with the tRNA class
ND1*	3.9	0.026	Enables NADH dehydrogenase activity. Involved in mitochondrial electron transport.
ND2*	3.8	0.042	Enables NADH dehydrogenase activity. Involved in mitochondrial electron transport.
WHRN*	3.8	0.004	Organization and stabilization of sterocilia elongation and actin cystoskeletal assembly
ZBTB16*	3.8	0.010	Involved in cell cycle progression
BTBD19*	3.8	0.002	-
LOC105378949*	3.7	0.002	-
CTBP1-AS*	3.6	0.002	An RNA Gene, and is affiliated with the lncRNA class
MAPK8IP1Φ	3.4	0.005	Regulator of the pancreatic beta-cell function, susceptibility gene for T2DM
ZAP70†	3.2	0.001	Plays a role in T-cell development and lymphocyte activation
LOC286059Φ	3.1	0.006	TNF receptor superfamily member pseudogene
CD5†	2.9	0.004	Act as a receptor to regulate T-cell proliferation
NLGN3†	2.5	0.004	Neuronal surface protein
AMIGO3Φ	2.4	0.002	Cell adhesion
MAPK8IP3†	2.3	0.002	Regulate the activity of numerous protein kinases of the JNK signaling pathway
MAPK13†	2.2	0.011	Integration point for biochemical signals, and processes such as proliferation, differentiation, transcription regulation and development

APOA2[Φ]	2.2	0.006	High density lipoprotein particles
ADGRL1[Φ]	2.2	0.005	Cell adhesion and signal transduction
MMP24[Φ]	2.1	0.004	Breakdown of extracellular matrix in embryonic development, reproduction, and tissue remodeling
NFKBID[Φ]	2.1	0.014	Enable NF-kappaB binding activity, T cell signaling and functions and regulation of gene expression
IL11RA[†]	1.9	0.028	Encodes the IL-11 receptor, a member of the hematopoietic cytokine receptor family
C1QTNF7-AS1[Φ]	1.9	0.024	Identical protein binding activity, collagen trimmer
ZBTB17[†]	1.9	0.007	Zinc finger protein involved in the regulation of c-myc.
TRAF1[†]	1.8	0.023	Mediates Signal transduction
ADGRB1[Φ]	1.8	0.048	Cell adhesion
TRPV1[†]	1.8	0.015	Detection and regulation of body temperature and nociception, mediates detection of noxious environmental stimuli
SMAGP[Φ]	1.7	0.048	Cell adhesion
ZBTB25[†]	1.7	0.007	Regulation of transcription by RNA polymerase II
FASN[Φ]	1.7	0.029	Catalyse synthesis of long chain saturated fatty acids
RELT[Φ]	1.7	0.014	Activates NF-kappaB pathway, selectively binds TRAF1, stimulates T cell proliferation
NFKB2[†Φ]	1.6	0.033	Functions as a central activator of genes involved in inflammation and immune function.
IRS2[†]	1.5	0.029	Mediates effects of insulin, insulin-like growth factor 1, and other cytokines
TRAF3IP2-AS1[†]	1.5	0.017	RNA Gene, and is affiliated with the lncRNA class.
TRAF4[Φ]	1.5	0.047	Mediates signal transduction
EMB[†]	-1.5	0.042	Involved in cell growth and development by mediating interactions between the cell and extracellular matrix
CD86[†]	-1.5	0.031	Pathogen recognition and activation of innate immunity
TLR4[†]	-1.6	0.027	Pathogen recognition and activation of innate immunity
NCK1[†]	-1.6	0.046	Signaling and transforming proteins
CD244[†]	-1.7	0.006	Expressed on NK cells and some T cells that mediate non-major histocompatibility complex (MHC) restricted killing
CD1C[†]	-1.7	0.005	Mediate the presentation of primarily lipid and glycolipid antigens of self or microbial origin to T cells
PALLD[†]	-1.8	0.029	Organizing the actin cytoskeleton
DSC2[†]	-1.9	0.005	Cell-cell junctions

TGFA†	-1.9	0.008	Growth factor which activates a signaling pathway for cell proliferation, differentiation and development
TMEM106B*	-1.9	0.048	Involved in dendrite morphogenesis and lysosome localization
CALM2*	-1.9	0.027	Calcium binding protein that plays a role in signaling pathways, cell cycle progression and proliferation
CYP1B1*	-1.9	0.038	Catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids
LINC01094*	-1.9	0.028	RNA Gene, and is affiliated with the lncRNA class
GSKIP*	-2.0	0.020	Involved as a negative regulator of GSK3-beta in the Wnt signaling pathway
BTG2*	-2.0	0.006	Antiproliferative properties, involved in the regulation of the G1/S transition of the cell cycle
NEIL3*	-2.0	0.038	Initiate the first step in base excision repair by cleaving bases damaged by reactive oxygen species
ZNF772*	-2.0	0.049	Regulation of transcription by RNA polymerase II
CX3CR1*†	-2.0	0.016	Involved in the adhesion and migration of leukocytes
CENPQ*	-2.2	0.006	Involved in assembly of kinetochore proteins, mitotic progression and chromosome segregation.
DHRS9*	-2.2	0.039	Dehydrogenase/reductase
ZFP3*	-2.2	0.019	Regulation of transcription by RNA polymerase II
PRICKLE1*	-2.2	0.035	Negative regulator of the Wnt/beta-catenin signaling pathway
BBS10*	-2.3	0.025	Member of the Bardet-Biedl syndrome (BBS) gene family
ZIK1*	-2.3	0.003	Regulation of transcription by RNA polymerase II
PTGS2*	-2.3	0.042	It is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis.
LPAR1*	-2.4	0.006	Proliferation, platelet aggregation, smooth muscle contraction, chemotaxis, and tumor cell invasion
PPBP*†	-2.6	0.023	Potent chemoattractant and activator of neutrophils
STEAP4*	-3.0	0.043	Involved in adipocyte development and metabolism
CD200R1*	-3.7	0.014	The receptor-substrate interaction may function as a myeloid downregulatory signal

*Indicates the gene is from the 20 most up or down-regulated

† Indicates the gene is from selected biological processes

ϕ Indicates the gene is from manual search of original DEG list based on our interest

Supplementary Table 3. STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page number
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	84
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	85
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	87
Objectives	3	State specific objectives, including any prespecified hypotheses	88
Methods			
Study design	4	Present key elements of study design early in the paper	88
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	88
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	88
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	92
		(*b*) Describe any methods used to examine subgroups and interactions	92

		(*c*) Explain how missing data were addressed	
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	93
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	93
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	93-97
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	97
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	102
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	97-102

Generalisability	21	Discuss the generalisability (external validity) of the study results	97-102
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	103

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Chapter 6: Immunophenotyping Reveals Immune Dysfunction in Diabetes Complications

This chapter presents the findings from the mass cytometry immunophenotyping of circulating white blood cells in individuals with and without diabetes complications. The findings from Chapter 5 suggest different functional characterisation of the CD163+ monocytes between individuals with and without diabetes complications. As such we endeavoured to investigate whether other circulating white blood cells were also functionally profile different in those with and without diabetes complications. The results from this investigation suggested that various immune cell populations have diminished abilities to adhere to the endothelial wall in the process of trans-endothelial migration into the tissues. We also report decreased proportions of specific T cell and B cell subsets, which can represent potential mechanisms of immune dysfunction or suppression; and therefore be implicated in the progression of diabetes complications. This work has been submitted to Australasian Diabetes Congress 2023.

6.1 Abstract

Diabetes and its complications are a growing modern health challenge, due in part to the global obesity epidemic. The progressive nature of microvascular and macrovascular complications contributes to much of the morbidity and mortality associated with diabetes. Additionally, there is a lack of therapeutic measures that can alleviate or halt the progression of these complications. Chronic systemic inflammation is a characteristic feature of diabetes complications and this study therefore aimed to immunophenotype circulating immune cells to investigate whether there are different immune cell phenotypes in individuals with diabetes-related complications.

Mass cytometry was utilised to phenotype the white blood cell populations of adults with duration of diabetes greater than 10 years and with (D^{+Comps}, n=8) or without (D^{-Comps}, n=13) complications. Data were analysed by Flow Jo, and statistical analysis performed using GraphPad Prism.

There was no significant difference in the proportion of circulating monocytes, mDendritic cells, granulocytes, T cells, B cells and NK cells between diabetes with and without complications. However, the adhesion markers were decreased across majority of immune cell populations in D^{+Comps}. In addition, CD 4+ T conventional cells, CD8+ T cells and their subsets CD8+ memory T cells and CD127+ subset, as well as immune regulation marker CD73+ in B cells, were all decreased in individuals with complications compared to those without.

These results suggest that immune cell populations may have reduced capacity to adhere to the endothelial wall in the process of trans-endothelial migration into the tissues. A decrease in the phenotype of specific T cell and B cell subsets indicates immune dysfunction or suppression, which would worsen the progression of diabetes complications. Having

identified these changes future investigations are required to determine the cause and significance of these findings.

6.2 Introduction

Diabetes and its consequent microvascular and macrovascular complications are increasing in prevalence. The progressive and unresolving nature of diabetes complications reduces the quality of life, and imposes a heavy health and economic burden (216). Chronic low-grade systemic inflammation contributes to the local tissue inflammation at affected organ sites of diabetes complications and is a characteristic feature of diabetes (75, 217, 218). For instance in diabetic nephropathy, the infiltration of leukocytes leads to the subsequent greater production of pro-inflammatory cytokines and contributes to worsening kidney damage (219). Whilst macrophage infiltration is a feature of the tissue inflammation in diabetes complications, obtaining suitable tissue samples for macrophage studies is challenging. As a result, circulating monocytes, the precursor of macrophages, which have been used as a study substitute, have been also found to be altered in diabetes (220-222). Min and colleagues reported decreased circulating myeloid cells including CD16+ monocytes and CD163+ anti-inflammatory monocytes in individuals with diabetes complications (75). Similarly, other leukocyte phenotypes have also been reported to be changed in individuals with diabetes complications (9-11). For instance, circulating neutrophils were increased, and monocytes and lymphocytes were decreased in individuals with diabetes macular oedema when compared to those without macular oedema (223). Additionally, specific T cells, CD4+CD25+ T regulatory cells (T regs) were significantly decreased in individuals with diabetes duration greater than 10 years, compared to those with diabetes duration less than 10 years (224). Furthermore it has been reported that in T2DM, interstitial infiltration of CD4+ T cells correlated with the degree of proteinuria in DN (225). These recent studies suggest that diabetes can be implicated in the change in populations and phenotypes of circulating

immune cells which may exacerbate tissue damage and contribute to the pathophysiology of diabetes complications. However, despite the extensive research on diabetes, no study has been able to comprehensively profile both the innate and adaptive immune cells in individuals with diabetes complications. This study seeks to address this gap by investigating and characterizing the immune cell phenotypes in individuals with long duration of diabetes with and without complications.

In addition, in my CD163 study as described in Chapter 5; we characterised CD163+ monocytes and suggested a reduced ability to transmigrate into the tissues, reduced capacity to clear antigens, and also dysregulated interaction with the adaptive immune system in individuals with diabetes complications. In this investigation, we examined whether there are any phenotype differences in other leukocyte populations. This would tie the investigation of the innate and adaptive immune cell populations including monocytes, granulocytes, myeloid dendritic cells, NK cells, T cells and B cells and their phenotypes as well as the expression of activation and function markers to diabetes complications. We hypothesise that phenotypic alterations in circulating immune cells in individuals with diabetes complications contribute to inadequate functioning of their immune system.

6.3 Methods

6.3.1 Study Subjects and Experimental Design

Individuals with diabetes for greater than 10 years were recruited from the Diabetes Centre, Royal Prince Alfred Hospital. The participants were then further sorted according to those with (D^{+Comps}; n=8) or without (D^{-Comps}; n=13) any microvascular or macrovascular traditional complications. The demographics of the groups have been described in Chapter 2. The Human Ethics Review Committee of this institution approved this study, which was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. All participants gave their written informed consent.

Fresh whole blood was collected from each individual and stored with Smart Tube Proteomic Stabiliser (Smart Tube Inc., California, USA) at -80˚C (details shown in Chapter 2).

6.3.2 Mass Cytometry

Using the *Helios* mass cytometry *System* (Fluidigm, California, USA), the immune cells were immunophenotyped. A list of CD markers was chosen that allowed the identification of monocytes, dendritic cells, granulocytes, T cells, B cells, NK cells and their subsets including various function and activation markers. The full panel of cell surface CD markers are presented in Table 6.1

6.3.3 Red Blood Cell Lysis Procedure After Thawing Fixed Blood Samples

The full methodology for this section is described in Chapter 2. Briefly smart tube fixed cells were thawed at 4˚C, then thoroughly mixed with TLB1 (Smart Tube Inc., California, USA). The solution was then filtered through a 70µm cell strainer and incubated with 10mL TLB1 at room temperature. Solutions were then centrifuged for 5 mins at 600 x g at 4˚C and pellet

resuspended in TLB1. The sample was washed with TLB1 followed by TLB2, and finally the pellet resuspended in 1mL FACS buffer, and stored on ice.

6.3.4 Antibody Staining

The full methodology for this section is described in Chapter 2. In short, 2.0 x 10^6 cells were suspended in FACS buffer and centrifuged at 500 x g at room temperature. The cell pellet was resuspended in FACS buffer with heparin and incubated for 20 minutes at room temperature. After two washing cycles in FACS buffer, the samples were barcoded with either ^{106}Pd-CD45 or ^{108}Pd-CD45 and incubated on ice for 30 minutes. Following three FACS buffer washing cycles, each barcoded sample was combined with a differently barcoded sample, and paired samples were stained with cell surface antibodies as listed in Table 6.1 and incubated on ice for 30 minutes. Following three final washes in FACS buffer, DNA intercalator was added to samples, incubated at room temperature for 20 minutes, followed by 12 hours at 4˚C.

Following the 12-hour incubation, the samples were washed once with FACS buffer and once with ultrapure water before being resuspended in cell acquisition solution. Subsequently cells were counted using trypan blue and resuspended at 1 x 10^6 cells/mL with EQ calibration beads in ultrapure water. Samples were then filtered into a FACS tube and all cell events were acquired on the time-of flight mass cytometer, *Helios*.

6.3.5 Data Analysis – Manual Gating of White Blood Cell Subsets

The output data file was analysed on Flow Jo (FlowJo LLC., Oregon, USA). A manual gating strategy was developed and utilised for the quantification of each major leukocyte population including monocytes, dendritic cells, granulocytes, T-cells, B-cells and NK cells and the various activation and function CD markers were quantified by ‘frequency of parent’ and classified into subsets. Figure 6.1 depicts a representative manual gating strategy for the

identification of monocytes (CD14+HLADR+). Briefly, calibration beads were excluded, then single cells were gated on event length against DNA. The paired D^{+Comps} and D^{-Comps} were gated for ^{106}Pd-CD45 and ^{108}Pd-CD45 respectively to identify individual samples. Within individual samples, CD45+ and CD61- cells were chosen as the leukocyte population, and the remaining cells were manually gated by selecting positive and negative cells of specific leukocyte populations and excluding other cell types. In this way, we aimed to separate the desired leukocyte populations. The manual gating strategies we established to identify leukocytes and their subtypes (including monocytes, dendritic cells, granulocytes, T-cells, B-cells and NK cells) were as follows:

CD14+ total monocytes populations were gated following the selection of CD45+CD61- cells, for CD14+ by selecting the following groups CD14±CD66- → CD14±CD3- → CD14±CD19- → CD14±CD56- → CD14+HLADR+ cells.

After the selection of CD45+CD61- cells, CD11c+ mDendritic cells were gated by selecting into the following groups CD11c±CD66- → CD11c±CD3- → CD11c±CD19- → CD11c±CD56- → CD11c±CD14- → CD11c+ populations.

For CD66+ granulocytes, following the selection of CD45+CD61- cells, CD66+ cells were gated by selecting CD66±CD3- → CD66±CD19- → CD66±C56- → CD66±CD14- → CD66±CD11c- → CD66+ populations.

For CD3+ T cells, following the selection of CD45+CD61- cells, CD3+ cells were gated by selecting CD3±CD66- → CD3±CD19- → CD3±C56- → CD3±CD14- → CD3±CD11c- → CD3+ T cells.

For CD19+ B cells, following the selection of CD45+CD61- cells, CD19+ cells were gated by selecting CD19±CD3- → CD19±CD66- → CD19±C56- → CD19±CD14- → CD19±CD11c- → CD19+ B cells.

For CD56+ NK cells, following the selection of CD45+CD61- cells, CD56+ cells were gated by selecting CD56±CD3- → CD56±CD19- → CD56±C66- → CD56±CD14- → CD56±CD11c- → CD56+ NK cells.

Various activation and function markers were gated as a subset of each leukocyte population and quantified as a 'frequency of parent". Gated populations were then compared for cell frequency between the experimental groups.

6.3.6 Data Analysis – Statistical Analysis

Data analysis of clinical characteristics of those in the D^{+Comps} and D^{-Comps} groups was performed using GraphPad Prism 9 statistical package (GraphPad Software, San Diego, CA, USA). Continuous data were checked for normality and presented as mean ± standard deviation (SD) for parametric data, median and IQR or proportion for non-parametric data as indicated. Grouped data were compared by either Welch's t-test for parametric data, or Mann-Whitney test for non-parametric data or Fisher's exact test to identify differences between groups. Similarly, the statistical analysis of the data from the immunophenotyping of leukocytes was conducted as described above, using GraphPad Prism 9. Significance was accepted when $p<0.05$ and indicated as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Table 6.1 Mass cytometry panel used to immunophenotype white blood cells from fixed whole blood.

Metal		Clone	Company	Target	Staining Conc. (µg/mL)	Marker purpose
^{106}Pd	Palladium	HI30	Biolegend	CD45	6	Lineage/Barcode
^{108}Pd	Palladium	HI30	Biolegend	CD45	8	Lineage/Barcode
^{143}Nd	Neodymium	HI100	BD	CD45RA	2	Activation
^{147}Sm	Samarium	UCHL1	Biolegend	CD45RO	2	Activation
^{113}In	Indium	VI-PL2	Biolegend	CD61	0.5	Lineage/platelet
^{167}Er	Erbium	YTH71.3	Abcam	CD66	1	Lineage
^{160}Gd	Gadolinium	M5E2	BD	CD14	4	Lineage
^{148}Nd	Neodymium	B73.1	Biolegend	CD16	1	Lineage
^{170}Er	Erbium	UCHT1	Biolegend	CD3	2	Lineage
^{168}Er	Erbium	SK3	Biolegend	CD4	1	Lineage
^{145}Nd	Neodymium	RPA-T8	Biolegend	CD8a	4	Lineage
^{142Nd}	Neodymium	HIB19	Biolegend	CD19	1	Lineage
^{176}Yb	Ytterbium	REA196	Miltenyi	CD56	0.5	Lineage
^{174}Yb	Ytterbium	L243	Biolegend	HLA-DR	1	Lineage
^{169}Tm	Thulium	M-A251	Biolegend	CD25	2	Activation
^{165}Ho	Holmium	A019D5	Biolegend	CD127	4	Activation
^{171}Yb	Ytterbium	TL2.1	Biolegend	CD282 (TLR-2)	4	Activation
^{149}Sm	Samarium	HTA125	BD	CD284 (TLR-4)	4	Activation
^{172}Yb	Ytterbium	HIT2	BD	CD38	2	Activation
^{151}Eu	Europium	K036C2	Biolegend	CD192(CCR2)	2	Chemokine receptor
^{144}Nd	Neodymium	HEK/1/85a	Bio-Rad	CD195(CCR5)	2	Chemokine receptor
^{164}Dy	Dysprosium	2A9-1	Biolegend	CX3CR1	1	Chemokine receptor
^{163}Dy	Dysprosium	REA232	Miltenyi	CXCR3	2	Chemokine receptor
^{209}Bi	Bismuth	ICRF44	Biolegend	CD11b	4	Adhesion
^{115}In	Indium	Bu15	Biolegend	CD11c	2	Lineage and adhesion
^{155}Gd	Gadolinium	WM59	BD	CD31	2	Adhesion

^{152}Sm	Samarium	5-271	Biolegend	CD36	4	Phagocytosis and clearance
^{153}Eu	Europium	KP1	Biolegend	CD68	4	Phagocytosis and clearance
^{175}Lu	Lutetium	A1	Biolegend	CD39	1	Immune Regulation
^{173}Yb	Ytterbium	AD2	Biolegend	CD73	2	Immune regulation
^{162}Dy	Dysprosium	L307.4	BD	CD80	1	Immune regulation
^{156}Gd	Gadolinium	IT2.2	BD	CD86	1	Immune regulation
^{139}La	Lanthan	15-2	Biolegend	CD206	8	Immune regulation
^{159}Tb	Terbium	2416C	R&D	ADIPOR1	4	Metabolic Function
^{166}Er	Erbium	Polyclonal	Abcam	ADIPOR2	4	Metabolic Function
^{141}Pr	Praseodymium	HI98	Biolegend	CD15	0.5	Activation
^{158}Gd	Gadolinium	A16085I	Biolegend	VEGFR2	4	Adhesion

Column details include the rare earth element isotypes used for antibody conjugation, clone, manufacturer, targeted CD marker, titrated staining concentration and purpose for inclusion in panel.

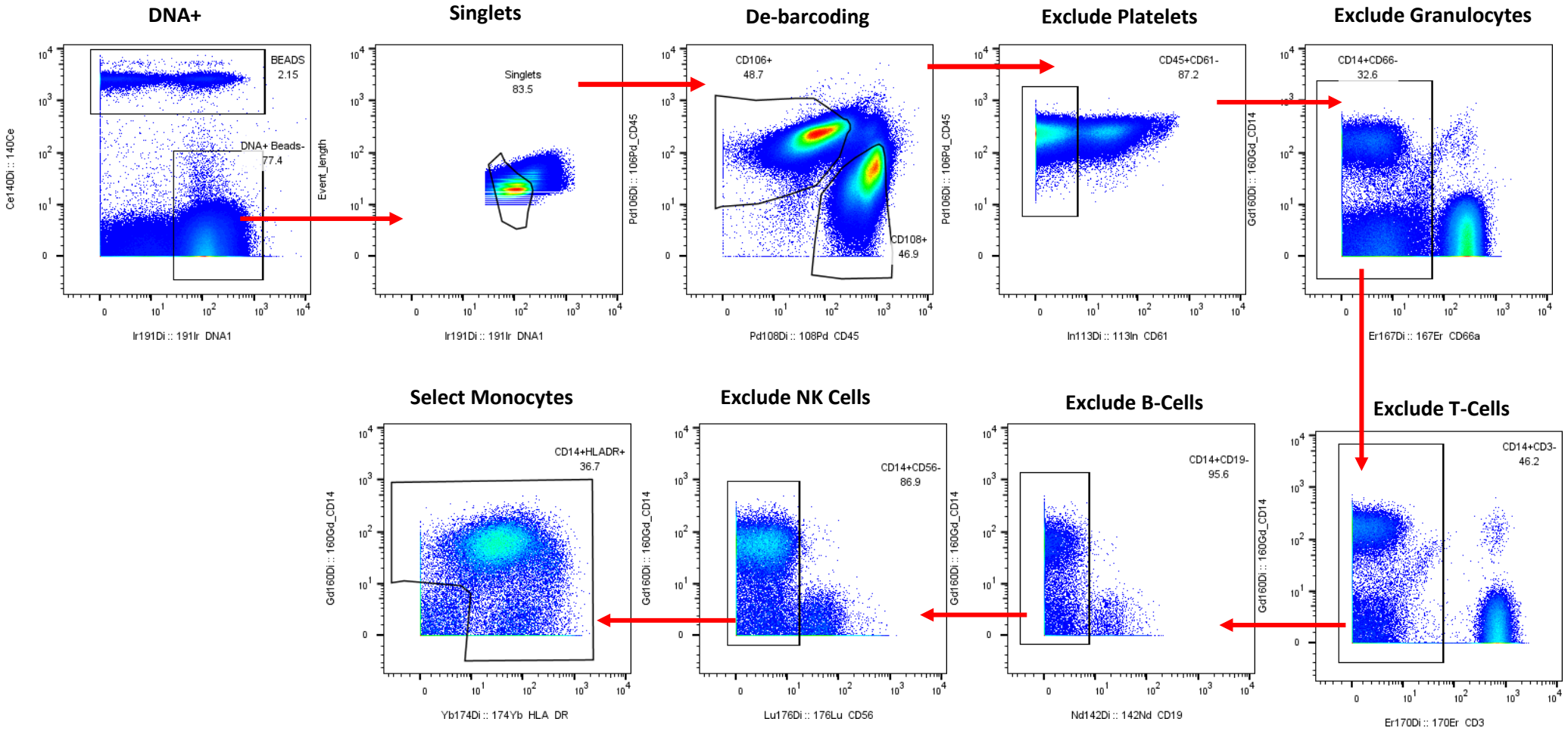


Figure 6.1: A flow diagram of the manual gating strategy used to identify monocytes (CD14+HLADR+). DNA+ events were selected, and thus normalisation beads were excluded, singlets were then gated. Total cells were separated into individual patient samples by de-barcoding. Platelets (CD61+) were excluded and then the leukocyte population of investigation (monocytes) was positively gated with other leukocyte subtypes excluded in a negative selection process.

6.4 Results

6.4.1 Participant Clinical Characteristics

Clinical characteristics of individuals with diabetes with (D^{+Comps}, n=8) or without (D^{-Comps}, n=13) any diabetes complications are presented in Table 6.2. There was no difference in the age of individuals and proportion of male and female between D^{+Comps} and D^{-Comps}. Individuals with diabetes complications had a longer duration of diabetes ($p<0.05$), however there was no difference in HbA_{1c}, total cholesterol, HDL-c, LDL-c, and triglycerides between D^{+Comps} and D^{-Comps} (Table 6.2).

Table 6.2: Clinical characteristics of individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

	D^{+Comps} (n=8)	**D^{-Comps} (n=13)**	**P value**
Age (years)	69.0 ± 16.6	70.5 ± 7.1	ns
Sex (M/F) (n)	6/2	7/6	ns
Duration of Diabetes (years)	25.5 (24.5 - 32.0)	20.2 (19.5 - 25.2)	p<0.05
T2DM /total cohort (%)	6/8 (75%)	12/13 (92%)	ns
BMI (kg/m^2)	26.1 (21.1 – 28.4)	28.4 (25.2 - 31.6)	ns
HbA_{1c} (%NGSP units)	8.0 (7.2 – 8.2)	7.0 (6.1 - 8.1)	ns
Total Cholesterol (mmol/L)	4.0 (3.2 - 4.1)	4.1(3.3 - 5.1)	ns
HDL-c (mmol/L)	1.5 (0.9 - 1.7)	1.3 (1.2 - 1.5)	ns
LDL-c (mmol/L)	1.8 (1.2 - 2.2)	1.9 (1.4 - 2.2)	ns
Triglycerides (mmol/L)	1.5 (1.2 - 1.6)	1.5 (0.9 - 1.6)	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at $p<0.05$ and no significance indicated by ns.

6.4.2 Characterization of Circulating Leukocyte Phenotypes

Within the CD45+ leukocyte population, major lineage leukocytes including monocytes (CD14+), myeloid dendritic cells (mDCs; HLA-DR+CD11c+), granulocytes (CD66+), T cells (CD3+), B cells (CD19+) and NK cells (CD56+) were not different between individuals with diabetes with or without complications (Table 6.3).

Table 6.3: The proportion of leukocyte phenotype in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

Cell Population (% of CD45+ cells)	D^{+Comps} (n=8)	D^{-Comps} (n=13)	P Value
Monocytes (CD14+)	6.1 ± 2.6	5.9 ± 2.5	ns
mDCs (HLA-DR+CD11c+)	0.6 (0.2 – 1.0)	1.0 (0.4 – 1.2)	ns
Granulocytes (CD66+)	66.6 ± 15.1	68.6 ± 9.6	ns
T Cells (CD3+)	18.3 ± 9.5	17.2 ± 6.1	ns
B Cells (CD19+)	1.6 ± 1.4	1.9 ± 1.3	ns
NK Cells (CD56+)	2.3 ± 0.9	2.9 ± 1.4	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at $p<0.05$ and no significance indicated by ns. mDCs: myeloid dendritic cells.

6.4.2.1 The Profile of Monocytes

As shown in Figure 6.2, CD14+ monocytes were classified into three subsets including classical ($CD14^{hi}$ $CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical monocytes ($CD14^{lo}CD16^{+}$). The majority (around 80%) of total monocytes belonged to the classical ($CD14^{hi}$ $CD16^{-}$) subset. However, the three monocyte subsets were not significantly different between D^{+Comps} and D^{-Comps} (Figure 6.2).

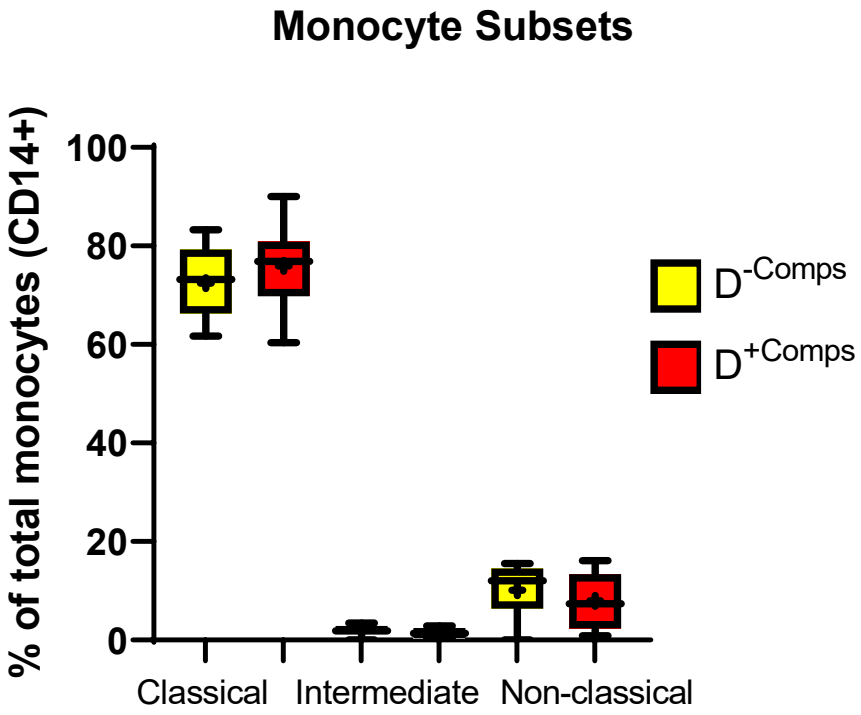


Figure 6.2: The proportion of classical, intermediate and non-classical monocytes as a % of total monocytes. Yellow bars indicating individuals with diabetes without complications (D^{-Comp}), and red indicating individuals with diabetes with complications (D^{+Comps}).

The expression of activation and function markers on the CD14+ monocyte is displayed in Table 6.4. Adhesion markers CD11b+ and CD11c+ were decreased 2.6- and 4.3-fold in D^{+Comps} compared to D^{-Comps} respectively (both $p<0.001$). Chemokine receptors CCR2+, CX3CR1+ and CXCR3+ were decreased 1.4-fold, 1.4-fold and 1.7-fold respectively ($p<0.05$; $p<0.05$ and $p<0.01$), but CCR5+ increased 1.6-fold in D^{+Comps} ($p<0.05$). However, there were less proportion of CCR5+ and CX3CR1+ cells within CD14+ monocytes. Activation makers CD282+ and CD284+ were increased 2-fold and 1.7-fold in D^{+Comps} ($p<0.01$ and $p<0.05$). Markers of immune regulation CD80+ was increased 3.0-fold ($p<0.01$) and CD86 decreased 3-fold ($p<0.0001$) in D^{+Comps}, respectively.

Table 6.4: The expression of monocyte activation and function markers on CD14+ monocytes in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

Function	Subset % of CD14+	D^{+Comps} (n=8)	D^{-Comps} (n=13)	P Value
Adhesion & migration	CD11b+	20.6 (14.3-27.1)	53.1 (41.3-60.6)	p<0.001
	CD11c+	11.3 (9.2-18.7)	48.9 (31.3-55.7)	p<0.001
	CD31+	10.2 ± 4.8	11.9 ± 3.7	ns
Chemokine Receptors	CCR2+	34.9 ± 10.7	47.8 ± 9.1	p<0.05
	CCR5+	2.6 (2.3 – 5.7)	1.6 (0.6 – 2.5)	p<0.05
	CX3CR1+	0.5 (0.3 – 0.8)	0.7 (0.5 – 1.1)	p<0.05
	CXCR3+	31.8 (9.3 – 52.1)	54.1 (49.1- 63.1)	p<0.01
Activation	CD282+	4.1 (2.6 – 7.7)	1.9 (1.3 – 2.5)	p<0.01
	CD284+	2.7 (2.1 – 3.6)	1.6 (1.2 – 1.6)	p<0.05
	CD38+	1.0 (0.7-2.4)	1.4 (1.1-3.1)	ns
Phagocytosis & clearance	CD36+	80.2 (76.4-84.8)	86.8 (77.4-90.6)	ns
	CD68+	89.2 (86.3 – 91.9)	94.2 (88.3 – 96.0)	p=0.07
Immune regulation	CD39+	81.3 ± 8.0	85.6 ± 5.1	ns
	CD73+	1.1 (0.8- 1.8)	1.2 (0.3 – 1.2)	ns
	CD80+	3.0 (1.7 – 3.6)	1.0 (0.7 – 2.3)	p<0.01
	CD86+	9.2 ± 20.5	28.1 ± 11.0	p<0.0001
	CD206+	0.1 (0.04 – 0.3)	0.2 (0.08 – 1.7)	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at p<0.05 and no significance indicated by ns.

The profile of classical monocytes

The expression of activation and function markers on $CD14^{hi}$ $CD16^{-}$ classical monocytes is shown in Table 6.5. Adhesion markers CD11b+, CD11c+ and CD31+ were all significantly decreased in D^{+Comps} compared to D^{-Comps} ($p<0.01$; $p<0.01$ and $p<0.001$ respectively). Chemokine receptor CCR2+ was decreased 1.4-fold ($p<0.01$), however CCR5+ was increased 3.7-fold in D^{+Comps} ($p<0.01$). Activation marker CD38+ was decreased 7-fold in D^{+Comps} ($p<0.05$) but at a very low proportion. Phagocytosis and clearance markers CD36+ and CD68+ were slightly reduced in D^{+Comps} ($p=0.05$, $p<0.01$ respectively), though expressed at high proportion in both D^{+Comps} and D^{-Comps}. Immune regulation markers CD39+ and CD86+ were decreased 2.8-fold ($p<0.05$) and 6.1-fold ($p<0.001$) in D^{+Comps} respectively, however CD80+ was increased 1.3-fold ($p<0.05$) but at very low proportion of cell population.

Table 6.5 The expression of monocyte activation and function markers on CD14^{hi} CD16^{-} classical monocytes in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

	Subset % of *classical monocytes*	**D^{+Comps} (n=8)**	**D^{-Comps} (n=13)**	**P Value**
Adhesion & migration	CD11b+	18.7 (11.1 – 24.6)	59.6 (50.6 – 66.5)	p<0.01
	CD11c+	26.8 (24.0 – 37.9)	71.6 (56.2 – 78.6)	p<0.01
	CD31+	3.2 (3.1 – 5.9)	52.6 (28.9 – 69.3)	p<0.001
Chemokine receptors	CCR2+	42.7 ± 12.6	60.6 ± 9.4	p<0.01
	CCR5+	3.4 (2.3 – 9.0)	0.9 (0.4 – 2.6)	p<0.01
	CX3CR1+	0.8 (0.6 – 1.2)	0.6 (0.3 – 0.9)	p=0.06
	CXCR3+	4.2 (2.6 – 5.6)	0.2 (0.004 – 4.6)	p=0.07
Activation	CD282+	0.4 (0.2 – 1.1)	0.2 (0.2 – 0.4)	ns
	CD284+	0.96 (0.7 – 1.7)	0.9 (0.6- 1.4)	ns
	CD38+	0.3 (0.2 – 2.5)	2.1 (1.0 – 5.2)	p<0.05
Phagocytosis & clearance	CD36+	99.1 (96.1 – 99.6)	99.8 (99.4 – 99.9)	p=0.05
	CD68+	98.7 (95.8 – 99.3)	99.8 (99.4 – 100.0)	p<0.01
Immune Regulation	CD39+	4.4 ± 2.4	12.3 ± 9.1	p<0.05
	CD73+	0.4 (0.4 – 1.0)	0.6 (0.5 – 1.0)	ns
	CD80+	0.8 (0.6 – 1.2)	0.6 (0.3 – 0.7)	p<0.05
	CD86+	2.1 (1.6 – 2.5)	12.8 (5.7 – 16.3)	p<0.001
	CD206+	0.1 (0.1 – 0.4)	0.3 (0.1 – 2.1)	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at p<0.05 and no significance indicated by ns.

The profile of intermediate monocytes

Within $CD14^{+}CD16^{+}$ intermediate monocytes, adhesion markers CD11b+, CD11c+ and CD31+ were all decreased in D^{+Comps} compared to D^{-Comps} at 2.6, 1.6 and 1.5-fold respectively ($p<0.01$; $p<0.05$ and $p<0.05$). Chemokine receptors CCR2+ and CCR5+ were not different between individuals with or without complications, however CX3CR1+ was increased 3-fold ($p<0.05$) and CXCR3+ increased by 29.5-fold ($p<0.001$) in individuals with complications. Activation markers CD282+ was reported to be increased by 5.3-fold ($p<0.05$), and CD284+ was found trending increased by 3-fold ($p=0.07$) in those with complications. Markers involved in phagocytosis and clearance including CD36+ and CD68+ were both decreased in D^{+Comps} (both $p<0.05$). Immune regulation markers CD39+ and CD86+ were decreased by 3.3-fold ($p<0.05$) and 3.2-fold in D^{+Comps} ($p<0.001$), respectively (Table 6.6).

Table: 6.6 The expression of monocyte activation and function markers on $CD14^{+}CD16^{+}$ intermediate monocytes in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

	Subset % of intermediate monocytes	D^{+Comps} (n=8)	D^{-Comps} (n=13)	P Value
Adhesion & migration	CD11b+	6.2 (3.9 – 10.2)	16.1 (8.9-26.2)	p<0.01
	CD11c+	58.0 (47.7 –74.7)	92.1 (65.5 – 93.5)	p<0.05
	CD31+	47.3 ± 25.3	71.6 ± 15.7	p<0.05
Chemokine receptors	CCR2+	4.6 (2.7 – 17.5)	8.0 (5.1 – 14.7)	ns
	CCR5+	6.2 ± 5.1	3.0 ± 2.2	ns
	CX3CR1+	5.4 ± 4.2	1.8 ± 1.1	p<0.05
	CXCR3+	29.5 (4.7 – 40.3)	0.0 (0.0 – 15.4)	p<0.001
Activation	CD282+	5.8 (1.1 - 7.1)	1.1 (0.2 - 4.5)	p<0.05
	CD284+	4.5 (1.6 - 6.4)	1.5 (0.5 - 4.3)	p=0.07
	CD38+	0.6 (0.0 – 1.5)	0.3 (0.0 – 0.8)	ns
Phagocytosis & clearance	CD36+	79.2 (64.1 - 90.9)	95.2 (89.6 - 97.2)	p<0.05
	CD68+	95.4 (93.5 - 98.6)	99.9 (95.6 - 100.0)	p<0.05
Immune Regulation	CD39+	5.9 (4.4 - 12.3)	19.7 (9.6 - 33.05)	p<0.05
	CD73+	3.5 (1.2 – 4.8)	2.8 (0.7 – 5.5)	ns
	CD80+	1.4 (0.7 – 3.2)	1.5 (0.3 – 2.6)	ns
	CD86+	7.8 ± 3.1	24.7 ± 14.1	p<0.001
	CD206+	0.2 (0.0 – 0.7)	0.9 (0.1 – 1.9)	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at p<0.05 and no significance indicated by ns.

The profile of non-classical monocytes

The expression of activation and function markers within the $CD14^{lo}CD16^{+}$ non-classical monocyte population is displayed in Table 6.7. Adhesion markers CD11c+ and CD31+ were decreased 1.5 and 1.2-fold in D^{+Comps} ($p<0.01$ and $p<0.05$) compared to D^{-Comps}. Chemokine receptors CCR5+, CX3CR1+ and CXCR3+ were increased in D^{+Comps} 5.3, 2.6 and 27-fold respectively (all $p<0.001$). Interestingly, CD11b+ cells and CCR2+ cells were lost in this subset in both groups. Immune regulation marker CD39+ was decreased 2.5-fold ($p<0.05$) and CD86+ decreased 3.1-fold (both $p<0.001$), however CD80+ was found at very low proportion although 4-fold ($p<0.001$) increased in D^{+Comps} compared to D^{-Comps}.

Table 6.7 The expression of monocyte activation and function markers on $CD14^{lo}CD16^{+}$ non-classical monocyte in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

	Subset % of non-classical monocytes	D^{+Comps} (n=8)	D^{-Comps} (n=13)	P Value
Adhesion & migration	CD11b+	0.0 (0.0 – 0.04)	0.1 (0.01 – 0.2)	p=0.05
	CD11c+	61.1 (53.7 – 65.5)	89.7 (69.7 – 95.30	p<0.01
	CD31+	80.5 (58.3-91.2)	95.5 (90.4-97.3)	p<0.05
Chemokine receptors	CCR2+	0.0 (0.0-0.05)	0.02 (0.0-0.1)	ns
	CCR5+	2.1 (1.1-4.3)	0.4 (1.5-1.3)	p<0.001
	CX3CR1+	1.6 (1.1-2.1)	0.6 (0.4-0.9)	p<0.001
	CXCR3+	13.5 (1.9-33.0)	0.1 (0.0-1.3)	p<0.001
Activation	CD282+	0.06 (0.0 - 0.2)	0.2 (0.03 - 0.2)	ns
	CD284+	0.4 ± 0.2	0.3 ± 0.2	ns
	CD38+	0.5 ± 0.6	0.3 ± 0.3	ns
Phagocytosis & clearance	CD36+	11.3 (9.2-23.1)	15.3 (10.3-59.4)	ns
	CD68+	98.4 ± 1.1	99.2 ± 0.7	ns
Immune Regulation	CD39+	0.8 ± 0.7	2.0 ± 1.7	p<0.05
	CD73+	0.1 (0.0 - 0.2)	0.2 (0.09 – 0.4)	ns
	CD80+	0.8 (0.5 – 1.4)	0.2 (0.2 – 0.4)	p<0.001
	CD86+	6.6 (4.3-8.0)	20.4 (9.6 -37.7)	p<0.001
	CD206+	0.1 (0.0 – 0.5)	0.1 (0.1 – 1.3)	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at $p<0.05$ and no significance indicated by ns.

6.4.2.2 The Profile of mDendritic Cells

The CD11c+ mDendritic cells were classified into the CD16+ inflammatory dendritic cells (infDCs) and CD16- conventional dendritic cells (cDCs) which were not different between D^{-Comps} and D^{+Comps} (Figure 6.3).

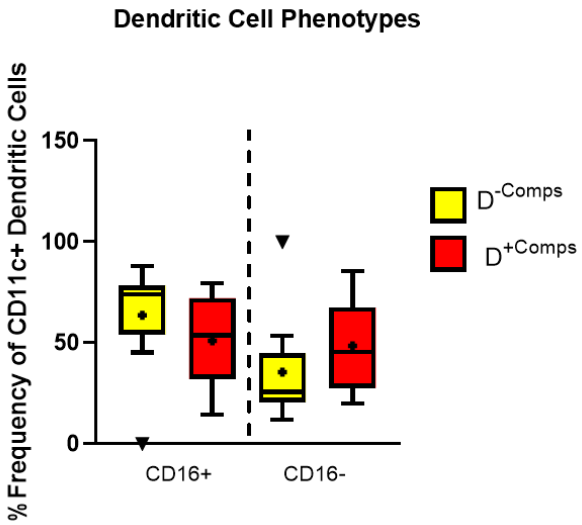


Figure 6.3: The proportion of CD16+ and CD16- subsets of CD11c+ dendritic cells. Yellow bars indicating individuals with diabetes without complications (D^{-Comps}), and red indicating individuals with diabetes with complications (D^{+Comps}). Outliers are indicated by ▼

The activation and function markers of CD11c+ dendritic cells are displayed in Table 6.8. Adhesion maker CD31+ was decreased by 1.6-fold ($p<0.05$) in D^{+Comps} compared to D^{-Comps}. Additionally, in those with complications the migration markers CX3CR1+ and CXCR3+ were increased 2.8-fold and 2.3-fold respectively (both $p<0.001$) and CCR5+ was trending increased by 1.8-fold ($p=0.06$) compared to those without complications. Activation marker CD68+ was decreased by 1.1-fold ($p<0.01$) in those with complications. CD86+, which is involved in immune regulation was decreased 2.1-fold ($p<0.001$) in D^{+Comps}, however CD80+, CD25+, ADIPOR2 and VEGFR2+ were all increased in D^{+Comps} 1.7, 1.6, 1.4 and 1.5-fold respectively (all $p<0.05$) (Table 6.8).

Table 6.8: The expression of activation and function markers on CD11c+ dendritic cells in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

Function	Subset % of CD11c+ cells	D^{+Comps} (n=8)	D^{-Comps} (n=13)	P Value
Adhesion	CD31+	46.0 (20.2 - 61.9)	73.4 (59.7 - 75.9)	p<0.05
Migration	CCR2+	3.9 (2.3 - 9.2)	8.9 (5.5 - 9.5)	ns
	CCR5+	7.6 ± 4.4	4.1 ± 2.3	p=0.06
	CX3CR1+	1.7 (1.2 - 2.6)	0.6 (0.4 - 1.0)	p<0.001
	CXCR3+	66.9 ± 11.7	28.6 ± 25.5	p<0.001
Activation	CD68+	78.6 ± 7.9	89.6 ± 4.5	p<0.01
	CD38+	1.6 (0.5 - 2.8)	1.8 (1.1 - 3.7)	ns
Immune Regulation	CD73+	0.3 (0.1 - 1.1)	0.8 (0.3 - 1.1)	ns
	CD39+	55.4 (48.7 - 68.4)	59.3 (52.8 - 63.9)	ns
	CD80+	21.5 (17.8-26.8)	12.5 (7.0-16.4)	p<0.05
	CD86+	14.2 (8.1 - 16.3)	30.2 (22.5 - 44.3)	p<0.001
	CD25+	1.1 (0.8 - 2.6)	0.7 (0.5 - 1.5)	p<0.05
	CD15+	1.8 (0.8 - 2.6)	0.4 (0.1 - 12.2)	ns
	ADIPOR1+	12.5 (9.3 - 19.9)	7.2 (4.6 - 11.5)	ns
	ADIPOR2+	7.9 (5.8 - 16.6)	5.7 (4.4 - 7.6)	p<0.05
	CD309/ VEGFR2	31.8 ± 13.2	20.5 ± 6.6	p<0.05

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at p<0.05, and no significance indicated by ns.

6.4.2.3 The Profile of Granulocytes

Within CD66+ granulocytes, adhesion markers CD11b+, CD11c+ and CD31+ were all decreased in D^{+Comps} by 1.6, 4 and 100-fold respectively (all $p<0.0001$). There was no significant difference in phagocytosis marker CD68+ and activation marker CD282+ between individuals with or without complications. Immune regulation marker CD39+ was decreased by 3.3-fold in those individuals with complications ($p<0.05$) but expressed at very low proportions in both groups (Table 6.9).

Table 6.9: The expression of activation and function markers on the CD66+ granulocytes in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

	Subset % of CD66+	**D^{+Comps} (n=8)**	**D^{-Comps} (n=13)**	**P Value**
Adhesion	CD11b+	61.1 (40.7 – 77.9)	98.4 (91.9 – 99.3)	p<0.0001
	CD11c+	5.3 (3.6 – 13.0)	21.3 (18.5 – 26.0)	p<0.0001
	CD31+	0.2 (0.007 – 0.9)	21.6 (3.4 – 62.5)	p<0.0001
Phagocytosis & clearance	CD68+	57.1 ± 22.9	61.3 ± 25.1	ns
Activation	CD282+	0.2 ± 0.1	0.1 ± 0.1	ns
Immune Regulation	CD39+	0.03 (0.01-0.05)	0.1 (0.05-0.3)	p<0.05

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at $p<0.05$, and no significance indicated by ns.

6.4.2.4 The Profile of T cells

The CD3+ T cells were further separated into CD4+ (CD4+CD8-) and CD8+ (CD4-CD8+) T cells. The proportion of CD4+ T cells (T helper cells) was not different between individuals with diabetes with or without complications; however the CD8+ cells (cytotoxic T cells) were decreased 3.2-fold in D^{+Comps} ($p<0.001$) (Figure 6.4).

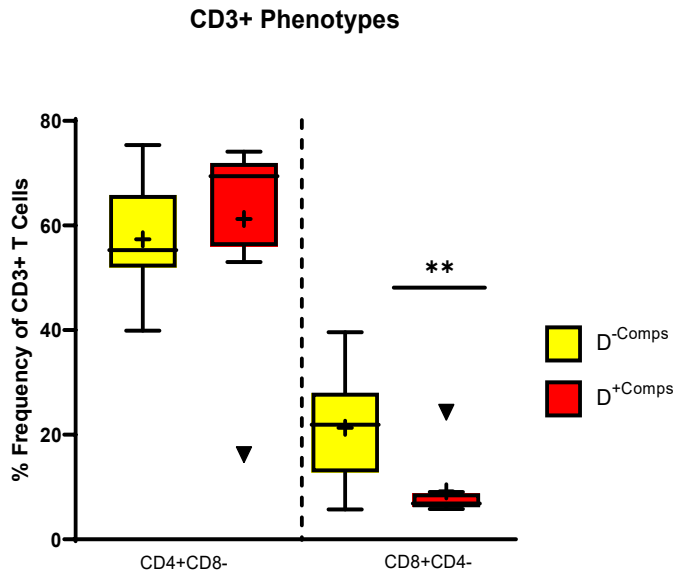


Figure 6.4 The T cell phenotypes including CD4+ (CD4+CD8-) and CD8+ (CD8+CD4-) T cells in individuals with diabetes and with (D^{+Comps}) and without (D^{-Comps}) complications. Yellow bars indicating D^{-Comps} and red bars indicating D^{+Comps}. ▼ *indicates outliers.*

*** indicated by $p<0.001$.*

The profile of CD4+ T cells

The subsets of CD4+ T cells are described in Table 6.10. T conventional (Tcon) cells ($CD127^{hi}CD25^{lo}$) were 2.5-fold decreased ($p<0.0001$) and T regulatory (Treg) cells ($CD127^{lo}CD25^{hi}$) were trending increased by 1.3-fold ($p=0.06$) in D^{+Comps}. Naïve (CD45RA+CD45RO-) CD4+ T cells were not different between D^{+Comps} and D^{-Comps}, however CD4+ memory (CD45RO+CD45RA-) T cells were 1.6-fold trending increased in D^{+Comps} ($p=0.06$). There was no difference in the proportion of CD45RA+ or CD45RO+ in T

conventional cells or T regulatory cells between D^{+Comps} and D^{-Comps}. Interestingly, a high proportion of CD45RO+ was observed from both T conventional cells and T regulatory cells from both diabetes groups (Table 6.10).

Table 6.10. The CD4+ T cell subsets in individuals with diabetes and with (D^{+Comps}) or without (D^{-Comps}) any traditional diabetes complications.

	CD4+ T Cell Subsets	**D^{+Comps} (n=8)**	**D^{-Comps} (n=13)**	**P Value**
Subset % of CD4+ Cells	$CD127^{hi}CD25^{lo}$ (T conventional cells)	18.5 ± 4.7	46.7 ± 16.8	p<0.0001
	$CD127^{lo}CD25^{hi}$ (T regulatory cells)	4.5 ± 1.4	3.3 ± 0.9	p=0.06
	CD45RA+CD45RO- (CD4+ Naïve T cells)	12.3 (8.1-16.3)	19.7 (13.1- 42.3)	ns
	CD45RO+CD45RA- (CD4+ Memory T cells)	60.1 (49.8-65.5)	38.1 (21.3-53.0)	p=0.06
Subset % of $CD127^{hi}CD25^{lo}$ (T conventional cells)	CD45RA+	9.3 ± 4.7	9.9 ± 6.5	ns
	CD45RO+	72.4 (58.3-80.6)	76.2 (65.8 – 79.8)	ns
Subset % of $CD127^{lo}CD25^{hi}$ (T regulatory cells)	CD45RA+	3.0 (1.2 – 6.7)	2.5 (1.1 – 5.3)	ns
	CD45RO+	73.3 ± 7.8	80.5 ± 11.6	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). Significance was accepted at p<0.05, and no significance indicated by ns.

The Profile of CD8+ T cells

The CD8+ cytotoxic T cells were further classified into CD8+ memory T cells (CD45RO+ CD45RA-) and CD8+ naïve T cells (CD45RA+CD45RO-) shown in Figure 6.5. The CD8+ memory T cells (CD45RO+CD45RA-) were 1.6-fold decreased in the D^{+Comps} compared to D^{-Comps} ($p<0.05$), and CD8+ naïve T cells (CD45RA+CD45RO-) were not different between the groups (Figure 6.5). Within the CD8+ T cells, the proportion of CD127+ was decreased 3.2-fold in D^{+Comps} compared to D^{-Comps} [2.3 (1.0-3.8) vs 7.5 (1.2-16.9), $p<0.001$].

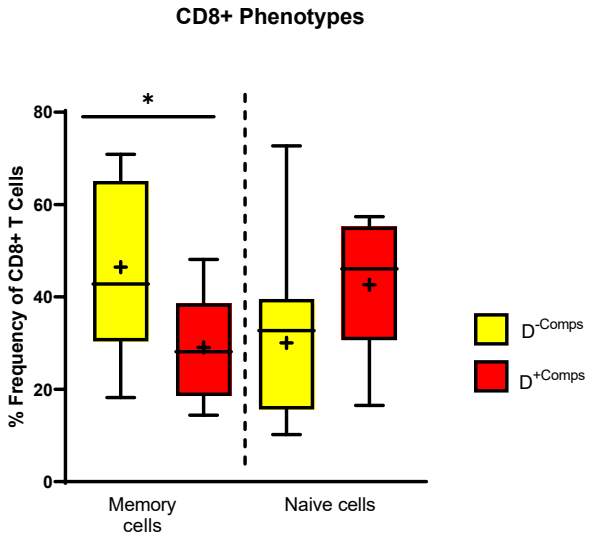


*Figure 6.5 The CD8+ memory and naïve T cells in individuals with diabetes and without (D^{-Comps}) and with (D^{+Comps}) complications. Yellow bars indicate D^{-Comps} and red bars indicate D^{+Comps}. * indicated significance at $p<0.05$.*

6.4.2.5. The Profile of B cells

The active (CD38+) and naïve (CD38-) B cells are displayed Figure 6.6. Active B cells were 1.2-fold trending decreased and naïve B cells were 1.3-fold trending increased in D^{+Comps} compared to D^{-Comps} (both p=0.06). Within CD19+ B cells, only immune regulation marker CD73+ was found significantly decreased in D^{+Comps} by 1.7-fold (25.2 ± 11.8 vs 44.2 ± 21.1, $p<0.05$).

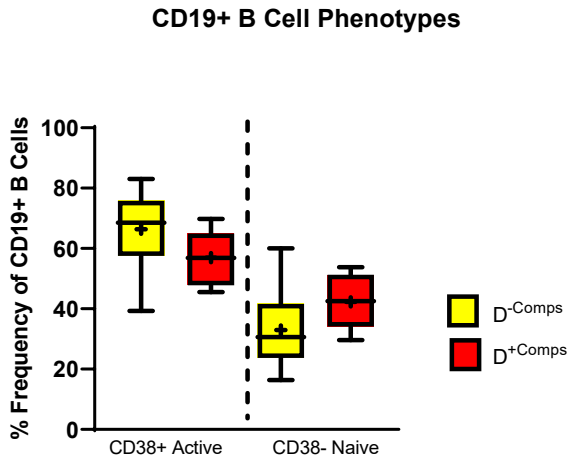


Figure 6.6 The CD38+ active and CD38- naïve B cells in individuals with diabetes and without (D^{-Comps}) and with (D^{+Comps}) complications. Yellow bars indicate D^{-Comps} and red bars indicate D^{+Comps}.

6.4.2.6 The Profile of NK cells

Mature (CD16+) and naïve (CD16-) NK cells were not significantly different between D^{+Comps} and D^{-Comps} (Figure 6.7). Within CD56+ NK cells, adhesion markers CD11b+ was decreased in D^{+Comps} by 12.6-fold (0.3 ± 0.2 vs 3.8 ± 2.6; $p<0.001$) and CD11c+ was decreased in D^{+Comps} by 4-fold [2.1 (1.6-2.6) vs 8.5 (3.1 – 13.0); $p<0.001$) compared to D^{-Comps}.

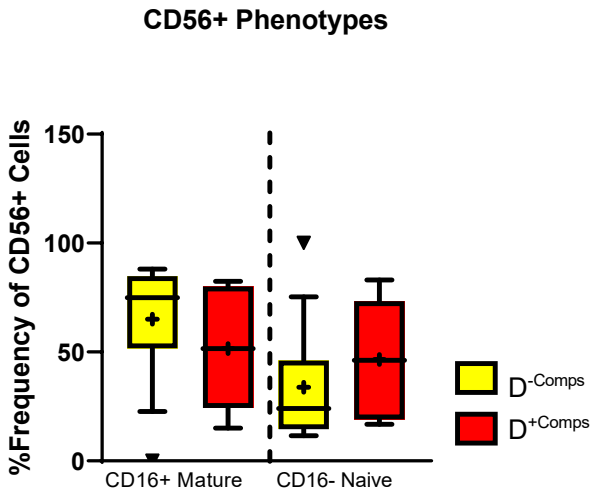


Figure 6.7 The proportion of CD16+ mature and CD16- naïve cells of CD56+ NK cells. Yellow bars indicate individuals with diabetes without complications (D^{-Comps}) and red indicates individuals with diabetes with complications (D^{+Comps}). ▼ indicates outliers.

6.5 Discussion

In current times the global prevalence of diabetes is increasing at pandemic proportions, which foreshadows the consequential rise of diabetes-related complications. The progressive nature of diabetes complications greatly affects the quality of life of those with these complications, and also contributes to the morbidity and mortality associated with the diabetes (226). Therefore, there is great need for therapeutic targets that can result in management of these diabetes-related complications. Diabetes is characterised by underlying chronic systemic inflammation. Additionally, dysregulation of the immune system may be a contributing factor towards the unresolving pathological mechanisms associated with diabetes-related complications in local tissues. In this chapter, we have endeavoured to investigate the immunophenotypic profiles of circulating immune cells and their specific phenotypes including activation and function markers, in individuals without and with diabetes complications.

Individuals with diabetes-related complications in this study had a longer duration of diabetes compared to those without complications. Although there was no significant difference in the proportion of circulating monocytes, mDendritic cells, granulocytes, T cells, B cells and NK cells between two diabetes groups; this study reveals significant differences in the expression of activation and function markers in immune cell subsets between individuals with and without diabetes complications. These differences in the characterisation of immune cell subsets suggest that alterations in the immune cells functionality may play a crucial role in the pathophysiology of diabetes complications.

After analysing the gene profile and cell surface markers of CD163+ monocytes from individuals with and without diabetes complications, as described in Chapter 5; we found that in those with diabetes complications, CD163+ monocytes had reduced expression of

adhesion markers, potentially reduced capacity to clear antigens, and dysregulated interaction with the adaptive immune system. However, it was unclear if a similar profile is present in the other immune cells. Therefore, we investigated the phenotypes of various circulating immune cells and their subsets in those with and without diabetes complications.

This study showed that there was no significant difference in the proportion of monocyte subsets; including classical ($CD14^{hi}$ CD16-), intermediate (CD14+CD16+) and non-classical ($CD14^{lo}$CD16+) monocytes between those with and without complications. However, similar to the CD163+ monocytes, classical, intermediate and non-classical subsets also demonstrated reduced expression of adhesion markers in D^{+Comps}, indicating that these monocyte subsets may also lose their ability to adhere to the vascular endothelial wall in order to extravasate from the circulation into the tissues where they differentiate into tissue macrophages. The lack of macrophages at tissue sites can therefore influence inflammatory responses within the tissue, and may contribute to the prolonged inflammation at tissue sites (227). In addition, the non-classical subset in both diabetes groups exhibited loss of CD11b, but increased CD31 expression suggesting that this monocyte subset maybe in the process of transmigration or extravasation through the endothelial barrier and also indicating these cells maybe undergoing differentiation into macrophages (228-230).

CCR2 is a receptor for the chemokines MCP-1, -2, -3, primarily expressed on monocytes and involved in monocyte recruitment to sites of inflammation, particularly in response to tissue injury (231). A low proportion of CCR2+ cells was found within CD163+ cells but there was no difference between diabetes with or without complications (Chapter 5). Contrastingly, approximately 50% of the classical monocytes were CCR2+ and significantly decreased in the complications group. Classical monocytes are involved in the initial response to infection and inflammation and express high levels of CCR2, by which they are recruited to sites of active inflammation to respond to stimuli generated by damaged or infected tissue (232) . Our

finding of decreased CCR2+ on the classical monocytes in D^{+Comps} suggested an impaired immune response to pathogens or other insults in diabetes complications. This decrease in CCR2 may potentially reduce the recruitment of these monocytes into inflamed tissues, thereby limiting the ability to resolve the tissue damage (232).

Compared to classical monocytes, in the intermediate subset, the proportion of CCR2+ cells was reduced and further absent in the non-classical monocytes in both diabetes groups. The observed proportions of CCR2+ cells across classical, intermediate and non-classical monocytes in our study of diabetes complications is consistent with data previously reported (233).

In contrast, CCR5, another chemokine receptor expressed on various immune cells (234) and binds to the chemokines RANTES, MIP-1α, and MIP-1β; was found to be increased within the classical and non-classical monocyte subsets in those with complications, which is completely opposite to CCR2. However, the proportion was very low in both diabetes groups compared it's proportions within CD163+ cells (Chapter 5). Previous studies have also observed CCR5 independently of CCR2, as implicated in the recruitment of monocytes to sites of tissue damage and inflammation (178). Typically, rather than classical and non-classical monocytes, intermediate monocytes express higher CCR5 (235), which is in concordance with our findings. Whilst CCR2 and CCR5 are both chemokine receptors, that bind chemokines involved in the recruitment of immune cells to sites of inflammation, CCR2 is also involved in the activation of monocytes, leading to the production of pro-inflammatory cytokines and other mediators of inflammation (236). Therefore, CCR2 and CCR5 would differ in their expression on specific monocyte subsets in diabetes complications, consistent with their different specificities in response to certain stimuli to promote different monocyte subsets. This may involve them in different responses after exposure to certain cytokines or growth factors and allow them to play different roles in immune function.

The chemokine receptors CX3CR1 and CXCR3 have been recognised as having functions additional to immune cell migration, including roles in tissue homeostasis, and in pathologic conditions, and roles in disease pathogenesis (237). Typically, CX3CR1 is expressed at low levels by classical monocytes, and at high levels in non-classical monocytes (238). Whilst in Chapter 5 we found that CX3CR1+ and CXCR3+ subsets were not different within CD163+ monocytes between individuals with and without complications; here we found they were increased in intermediate and non-classical monocytes in D^{+Comps} compared to D^{-Comps}. Apart from being involved in the regulation of immune cell trafficking within tissues, CX3CR1 has been reported to play a role in tissue repair and resolution of inflammation (239). Furthermore, CX3CR1 has been linked to monocyte maturation and the production of anti-inflammatory mediators (240). The greater expression of CX3CR1 and CXCR3 in monocytes could therefore indicate enhanced anti-inflammatory effects or immune response (240). However, the result of upregulating CX3CR1 and CXCR3 expression on monocytes can be intricate and influenced by various contextual factors. Moreover, it remains unresolved how mechanistically this increase in CX3CR1 and CXCR3 may contribute to the development of diabetes-related complications. More research is needed to fully understand their expression in different diabetes status.

In relation to monocyte activation, toll-like receptor CD284 (TLR-4) and CD282 (TLR-2) were not different between those with and without complications in CD163+ cells, however there was a trend for CD38 to be decreased (Chapter 5). Similarly in classical monocytes CD38 was also found decreased in those with diabetes complications. CD38 expression which enables the activation and proliferation of cells, has been reported robustly increased and associated with inflammatory conditions (241). The decreased CD38 expression in D^{+Comps} in this study may possibly be due to diabetes induced anti-CD38 autoimmunity (242), however it is not known yet how anti-CD38 antibodies could lead to decreased CD38+ cells.

Our results suggests that the presence of diabetes may cause reduced CD38, and that diabetes complications may exacerbate this reduction resulting in diminished monocyte activation, and other decreased effector functions in diabetes complications.

Interestingly, in the intermediate monocyte subset, TLR-2 was increased in individuals with complications compared to those without. Studies have confirmed that TLR-2 and TLR-4 are elevated in monocytes in individuals with diabetes (243). More specifically, other studies of tissue injury have reported that increased TLR-2 and TLR-4 on intermediate monocytes contributes to poorer healing and inflammation resolution (244); suggesting that TLR-2 and TLR-4 may be associated with the severity of inflammation.

Immune regulation markers CD39 and CD86 have been found decreased in CD163+ monocytes in D^{+Comps} compared to D^{-Comps}; and similar findings were observed in classical, intermediate and non-classical monocyte subsets. CD39, implicated in the clearance of adenosine triphosphate (ATP) and formation of extracellular adenosine, was down-regulated in those with complications; suggesting reduced adenosine production affected immunosuppression, and reduced ability of monocytes to activate the adaptive immune system (245, 246). Similarly the decrease in CD86 across all monocyte subsets indicated reduced lymphocyte activation (247). However, CD80 another immune regulation marker was found to be increased in classical and non-classical monocytes but was not different in CD163+ monocytes in D^{+Comps} (Chapter 5). Interestingly, it represented a very lower proportion in the monocyte subsets of both diabetes groups. Therefore, similar to CD163+ monocytes in diabetes complications, classical, intermediate and non-classical monocyte subsets also displayed dysregulated activation of adaptive immune system due to altered expression of various function and activation markers.

Phagocytosis and clearance marker CD68 was decreased in CD163+ and also in classical and intermediate monocytes in individuals with diabetes complications. In addition, the scavenger receptor CD36, was also found to be decreased in classical and intermediate monocytes in those with complications. CD36 recognises and binds glycated proteins (advanced glycated end-products; AGEs) that are formed in the presence of sustained hyperglycaemia such as in poorly controlled diabetes (248, 249). AGEs are known to act as a pro-inflammatory molecule in the pathophysiology of diabetes and are also involved in the suppression of the innate immune system (250). Furthermore, AGEs are known to accumulate in the serum in relation to the severity of diabetes (251). Therefore, decreased CD36 expression is indicative of a characterisation that may involve a reduced capacity to clear the pro-inflammatory molecule and a reduced capacity to suppress the innate immune system; both of these phenomena are consistent with ongoing and unresolving inflammation in diabetes complications. Additionally, we observed a non-significant trend for decreased CD36 expression in the "patrolling" non-classical monocytes. A study recently showed that CD36 was essential for the function of non-classical monocytes and regulated their patrolling speed and distance (248). Thus a decrease in CD36 expression would impair the monocytes ability to clear pathogens, therefore prolong inflammation in diabetes complications.

Similar to monocytes, in CD11c+ dendritic cells (mDCs), the adhesion marker CD31+ was decreased in those with complications compared to those without, suggesting a reduced ability to transmigrate across the vascular endothelial wall into the tissues. Despite the reduced ability to adhere to the vascular endothelial wall, the chemokine receptors CX3CR1 and CXCR3 were increased, suggesting increased migratory capacity. Whilst CX3CR1 and CXCR3 are typically known as migration-associated chemokine receptors in monocytes and macrophages, their role extends beyond typical functions. In a mouse model, CX3CR1-expressing mDCs have been found to be involved in the pathophysiology of an inflammatory

condition, specifically contributing to steatohepatitis (252, 253). This suggests that increased CX3CR1 expression on mDCs instead of being linked to increased trans-endothelial migration, may be involved in the pathophysiology of diabetes complications similar to the progression of steatohepatitis (252, 253), however the mechanism of which has not been elucidated to date. Supporting this, the markers involved in immune regulation and activation of the adaptive immune system were found dysregulated in mDCs in individuals with diabetes complications. Typically, mDCs that express CD25 and co-stimulatory markers CD80 and CD86, are considered fully mature and are involved in the co-stimulation of T cells and therefore the adaptive immune system (254). However, here we found CD80 and CD25 increased and CD86 decreased in D^{+Comps}, suggesting that mDC maturation and mDC mediated activation of the adaptive immune system could be dysregulated in those with diabetes complications. Supporting this, VEGFR2 which was increased in D^{+Comps}, (through the binding of VEGF), inhibits the functional maturation of mDCs, disrupts mDC differentiation and inhibits the ability of mature mDCs to simulate T cells (255). As such it can be suggested that in individuals with diabetes complications mDC mediated immune regulation and activation of T cells may be dysregulated. Furthermore, VEGF has been identified as the initiator of proliferative diabetic retinopathy (256), as such our finding of increased VEGFR2 expression presents a possible pathway by which VEGF may contribute to the persistent and worsening inflammation in DR.

In CD66+ granulocytes and CD56+ NK cells, (which are both granulated innate immune cells), adhesion markers were reduced in individuals with complications. Granulocytes are the first to infiltrate inflamed tissue and promote the recruitment of other leukocytes such as monocytes, (257). Similar to NK cells, granulocytes have the capacity to destroy invading microorganisms by degranulation, producing reactive oxygen species and forming neutrophil extracellular traps, all of which are effector functions that initially rely on adhesion integrins

such as CD11b and CD11c to adhere and “crawl” along the vascular wall before transmigration into the tissue (258, 259). Interestingly, the expression of CD11b is also associated with the maturation of NK cells which allows for greater effector responses (260). Therefore, our results suggest that in individuals with diabetes complications granulated innate cells, CD66+ granulocytes and CD56+NK cells would not be able to effectively transmigrate across the vascular cell wall into tissues, where they would exact effector responses. Additionally, CD56+ NK cells may not be able to reach maturation and reduce effector responses in diabetes complications.

Although the proportion of CD4+ T cells were not different in those with and without complications, within CD4+ T cells, we found Tcon cells were decreased and Treg cells trended to be increased in those with complications. Tcon cell populations include CD4+ helper cells, which are involved in the activation of B cells to secrete antibodies; the activation of macrophages to engulf and destroy microbes; and in the activation of cytotoxic T cells to kill infected target cells (160, 195). The significant decrease in Tcon cells we found in individuals with diabetes complications, would imply a decrease in the activation and function of the adaptive immune system. Supporting this, Tregs, which act to suppress the immune response through the inhibition of T cell proliferation, secretion of suppressive cytokines, cytotoxicity, metabolic disruption and suppression of macrophage mediated tissue repair (261, 262), showed a trend to be increased in individuals with diabetes complications compared to those without. Therefore, this suggests that in individuals with diabetes complications there is decreased T cell immune response, and increased suppression of the adaptive immune response.

On another hand, the CD8+ cytotoxic T cells and its subset CD8+ memory T cells as well as CD127+ CD8+ T cells were decreased in individuals with complications. CD8 cytotoxic T cells are involved clearance of tumour cells and host cells infected with intracellular

pathogens (263). As such, the decreased clearance of intracellular pathogens in diabetes complications may contribute to ongoing inflammation in diabetes complications, but it requires further investigation. Interestingly, CD8+ deficiency has been linked to various autoimmune and chronic inflammatory conditions including multiple sclerosis (264, 265), rheumatoid arthritis (266, 267) and T1DM (268, 269). Thus it is plausible that CD8+ deficiency may contribute to the unresolving nature of diabetes complications (270).

CD8+ memory cells are residual CD8+ antigen specific activated T cells, which are able to recognise previously encountered microbial antigens rapidly and mount a stronger immune response (263). The decreased CD8+ memory T cells in individuals with diabetes complications points to a weak immune response to microbial challenges in individuals with diabetes complications. CD127 plays a critical role in the survival and maintenance of CD8+ memory T cells by binding IL-7, a cytokine that is essential for the development and survival of T cells (271). Thus the decreased expression of CD127 supports reduced survival and maintenance of CD8+ memory T cells in diabetes complications (271).

CD19+ B cells are involved in the adaptive immune system as they serve as antigen presenting cells, provide costimulatory signals to produce cytokines for the effector functions of T cells (272). Within CD19+ B cells, we report a trend for decreased active B (CD19+CD38+) cells in diabetes complications, which corresponded to another investigation where CD38+ CD19+ B cells were decreased in individuals with diabetic retinopathy (273). CD38+ CD19+ B cells act to suppress immune response, by contributing to the maintenance of tolerance and limiting ongoing immune responses to re-establish immune homeostasis (274). Therefore, it is consistent that a decrease in these B cells might possibly contribute to the ongoing inflammation in diabetes complications. Additionally, we found that naïve B cells were trending increased in D^{+Comps}, suggesting that the necessary antigen presentation by T helper cells may be dysregulated, and that the further downstream effector functions of

B cells are also dysregulated in diabetes complications (160). Furthermore, CD73, which is involved in immunosuppression via the production of adenosine (275), was found to be significantly decreased in D^{+Comps}; indicating that B cell mediated immunosuppression may be decreased in individuals with diabetes complications.

To our knowledge this is the first study to immunophenotype various circulating immune cells in individuals with long duration of diabetes and complications. The small sample size of each group, however, is a significant limitation and it did not allow any comparative analysis between different complications. Future investigations can benefit with a larger cohort study. Additionally, with the aim of revealing potential therapeutic targets to alleviate inflammation related tissue damage; this study identifies the profile differences of immune cell subsets between those without and with complications, based on the expression of their cell surface markers. Future investigations will need to expand on this study by investigating the differential expression of intracellular markers within immune cell subsets, particularly concerning the intracellular production of cytokines and chemokines, which play such a critical role in the systemic and local inflammatory milieu. Another limitation of this study is that it is observational in nature, and therefore it cannot be determined whether the changes in the immune cell profiles are causing the progression of diabetes complications or whether the immune changes are in response to the complications. Future studies could investigate the immunophenotype of cells at multiple time points, to gain an understanding of immune changes overtime, thus establishing trends and any association with worsening progression.

In conclusion, this study found significant differences in the immunophenotype of circulating immune cells of individuals with diabetes complications compared to those without. Another significant result was the observation of the potentially decreased ability of certain innate immune cells to transmigrate into local sites of inflammation in those with complications compared to those without. Furthermore, in individuals with diabetes complications

alterations in the T cell and B cell subsets of the adaptive immune system indicated a characteristic profile that may be consistent with immune dysfunction or suppression, which would contribute to ongoing pathophysiology of diabetes complications. Future studies need to involve larger cohorts, include the investigation of intracellular markers in the mass cytometry panels to validate the current findings and allow identification of more subtle changes in immune cell function in individuals with and without diabetes complications.

Chapter 7: Associations between CD163 and Circulating Chemokines, Cytokines and Matrix-metalloproteinases in Diabetes Complications

This chapter presents the findings of multiplex immunoassays, measuring the level of various circulating cytokines, chemokines and MMPs in individuals with and without diabetes complications; and further reports on the correlation between these circulating proteins with the proportion of circulating CD163+ monocytes and sCD163 level. In consideration of the decreased proportion of CD163+ monocytes that had previously been reported in diabetes complications, we endeavoured to identify the potential cytokines, chemokines or MMPs involved in the regulation of the CD163+ monocytes, especially the relationship between circulating MMPs and sCD163. Our study found that cytokines IL-10 and IL-15 were increased in individuals with diabetes complications, however this was not correlated with the CD163+ monocyte proportion. Additionally, MMP-2, MMP-7 and MMP-10 may be implicated in the down-regulation of the CD163+ monocytes in individuals with diabetes complications, as being positively correlated with sCD163, suggesting these MMPs maybe involved in the cleavage of CD163 from cell surface. In addition, cytokine INF-α2, chemokine IP-10 and growth factor VEGFα were positively associated with sCD163; INF-α2 and VEGFα negatively with MMP-7 and/or MMP-2; and IP-10 positively with MMP-10 in diabetes complications, conveying a complex regulation network of CD163+ monocytes in diabetes complications, involving MMPs, cytokines, chemokines as well as growth factors.

7.1 Abstract

Chronic low-grade inflammation is a common feature of diabetes and may be a driving factor in the non-resolving nature of its complications. The anti-inflammatory CD163+ monocytes have been found to be decreased and possess phenotypic differences in individuals with diabetes complications which can contribute to the sustained inflammation caused diabetes complications. Although cytokines and matrix metalloproteinases (MMPs) have been implicated as factors involved in the regulation of CD163+ monocytes, there is a need for a definitive study of cytokines, chemokines and MMPs in diabetes complications. As such we aimed to identify cytokines, chemokines and MMPs involved in the regulation of CD163+ monocytes that are present in individuals with diabetes complications.

Multiplex immunoassays were used to quantify the plasma concentration of the selected cytokines, chemokines and MMPs present in individuals who had diabetes for greater than 10 years and with (D^{+Comps}) or without (D^{-Comps}) complications. The concentration of sCD163 was measured using a specific ELISA assay and mass cytometry was used to identify and determine the quantity of CD163+ monocytes in the white cell population from the collected blood samples. Statistical analysis was performed using GraphPad Prism.

We found that interleukin (IL)-10 and IL-15 were increased in D^{+Comps} compared to D^{-Comps}, however, there was no correlation found with either the proportion of CD163+ monocytes or the circulating sCD163 level in both diabetes groups. Additionally, MMP-2, MMP-7 and MMP-10, the cytokine INF-α2, chemokine IP-10 and growth factor VEGFα were all found positively correlated with sCD163 concentration in D^{+Comps}. Furthermore, INF-α2 and VEGFα were negatively correlated with MMP-7 and/or MMP-2 and IP-10 positive with MMP-10 in diabetes with complications. Our results indicate a complex relationship among these proteins wherein MMPs may be implicated in the regulation of the CD163+ monocytes,

as well as in the regulation of INF-α2 and VEGFα. Further investigation is needed to determine the underlying mechanisms and causative relationships between these findings.

7.2 Introduction

Diabetes is characterised by chronic low-grade systemic inflammation of tissues and organs that leads specific complications. Persistent inflammation causes an influx of inflammatory cells, particularly monocytes, into local tissue sites, resulting in monocytes maturing into macrophages at tissue sites. This contributes to or enhances damage and severity of the complication (226). Recently our group has reported that the circulating level of anti-inflammatory CD163+ monocytes was reduced in individuals with diabetes complications, suggesting that this reduction of CD163+ monocytes was a contributory factor in the worsening pathophysiology of diabetes complication (76).

In response to stimuli such as infection, inflammation or injury, circulating proteins such as cytokines are secreted by various leukocytes and these control the circulating level of CD163+ monocytes (106). These cytokines are important in regulating immune cell activation and differentiation as they promote inflammation, tissue repair, tissue regeneration and mediate the communication between cells (276-278). Glucocorticoids (GCs) and cytokines such as interleukin (IL)-6 and IL-10 have been found to up-regulate the expression of CD163; and other cytokines such as IL-4, tumour-necrosis factor -alpha (TNF-α) and interferon-gamma (IFN-ɣ) have been found to down-regulate CD163 expression (106, 112, 279). Additionally, MMPs may indirectly down-regulate the expression of CD163 via ecto-domain shedding to produce sCD163 (94, 106, 112). MMPs, are a family of enzymes involved in the breakdown and remodelling of extracellular matrix components. These proteinases can be produced by immune cells, and can trigger the release of growth factors, cytokines and chemokines (280, 281). In conditions of oxidative stress, or inflammatory

stimuli, MMPs have been reportedly involved in the proteolytic shedding of the CD163 protein from the monocyte cell surface, releasing the soluble form sCD163 into the circulation (94, 282). However, in diabetes complications, MMPs are responsible for the shedding of the membrane bound CD163 from the monocyte cell surface has yet to be identified.

In Chapter 5, we reported that the RNA and phenotypic profile of the CD163+ monocytes were characteristically distinct between those with and without diabetes complications and further may be indicative of reduced functionality which would contribute towards the pathophysiology of diabetes complications. With this premise, we aimed to define those cytokines and MMPs involved in regulating the circulating CD163+ monocytes and sCD163 by comparing individuals with and without diabetes complications. Additionally, in Chapter 5 we reported that the migratory capacity of CD163+ monocytes was different and the level of chemokine receptors CCR2 and CCR5 was different between individuals with and without diabetes complications. Chemokines are secreted proteins which function to regulate the recruitment and activation of immune cells to sites of infection, inflammation or injury (283). They are implicated in the development of diabetes nephropathy, retinopathy and neuropathy complications. The C-C motif of chemokine ligand 2 (CCL2) and C-X-C motif of chemokine ligands -1 (CXCL1) and -10 (CXCL10) are reported to play a role in the recruitment and activation of immune cells in the kidney, retina and peripheral nerves leading to inflammation fibrosis and tissue damage (284-287). This prompted us to investigate these specific chemokines whether they are altered in diabetes complications and contribute to the impaired migration of CD163+ monocytes into tissues.

Therefore, in this Chapter we quantified the circulating level of cytokines, chemokines and MMPs and explored their correlations with CD163+ monocytes and sCD163 in individuals with diabetes and with or without compilations.

7.3 Methods

7.3.1 Study Subjects

Adults with diabetes duration greater than 10 years were recruited from the Diabetes Centre, Royal Prince Alfred Hospital. The recruitment process and description of the inclusion and exclusion criteria for this set of participants is the same as described in section Chapter 2. Individuals were sorted according to those with (D^{+Comps}; n=6) or without (D^{-Comps}; n=12) any diabetes complications. The study cohort of this chapter is a sub-cohort from the initial cohorts utilised in Chapter 6. Fresh blood was collected in this study.

7.3.2 Multiplex Immunoassay Analysis of Cytokines, Chemokines and MMPs

A detailed description of the methodology in accordance with the manufacturer's protocol is conveyed in Chapter 2 section 2.3.4. Briefly, plasma level of cytokines, chemokines and MMPs were measured by bead-based multiplexed immunoassay using MILLIPLEX Multi-Analyte Profiling (MAP) Human Cytokine/Chemokine Kit and Human MMP Panel (Millipore, Massachusetts, USA) and was compared between the D^{+Comps} and D^{-Comps} groups. A list of all circulating proteins examined is displayed in Table 7.1.

Table 7.1 Cytokines, chemokines and matrix metalloproteinases (MMPs) in the study panel. Their sources and function in chronic inflammation from literature were shown in the table.

Group of Circulating Protein	Circulating Protein	Primary Cell Sources	Function in Chronic Inflammation
Cytokine	**IFN-α2**	Plasmacytoid dendritic cells, virally affected leukocytes	Modulates the anti-viral immune response (64).
	IL-1α	Various	Promotes activation, co-stimulation, and secretion of cytokines and other acute-phase proteins (64).
	IL-4	Th Cells	T and B cell proliferation (64).
	IL-6	Macrophages Th Cells, endothelial cells, adipocytes	Synthesis of acute phase proteins and activation of immune cells(64).
	IL-8	Macrophages, CD31+, T cells. Endothelial cells	Angeiogenesis and chemotaxis (64).
	IL-10	Macrophages and dendritic cells	Inhibition of Th1 inflammatory pathways in Th1 cells and macrophages (65).
	IL-12p70	Activated inflammatory cells including macrophages, neutrophils, microglia and dendritic cells	Cell differentiation and activation (66).
	IL-15	Many cell types, but specifically monocytes, DCs, epithelial cells, bone marrow stromal cells and fibroblasts	Activates NK cells, NKT cells, CD8+ cells (67).
	TNF-α	Many cell types	NFKβ pathway activation
Chemokine	**MDC**	Mature Macrophages and monocyte-derived dendritic cells	Chemoattractant for CCR4 expressing cells including lymphocytes, monocytes, monocytes derived dendritic cells, natural killer cells (68).
	MCP-1	Macrophages, dendritic cells, cardiac myocytes	Recruitment of leukocytes and monocytes (69).
	MCP-3	Endothelial cells, vascular smooth muscle cells and myelomonocytic cells	Chemotactic factor for monocytes and neutrophils (70).
	IP-10	Immune cells and non-immune cells	Chemoattractant for various immune cells such as

			activated T cells, NK cells, DCs and macrophages (71).
	MIP-1α	Monocytes, T cells, B cells neutrophils, dendritic cells and natural killer cells	Inflammation, wound healing (72).
	MIP-1ß		
Growth Factor	**VEGFα**	Many cell types	Endothelial cell proliferation, migration and angiogenesis (288).
Matrix Metalloproteinases	**MMP-1**	Many cell types including lymphocytes, granulocytes and activated macrophages	Degradation of extracellular matrix proteins and glycoproteins, membrane receptors, cytokines and growth factors (289).
	MMP-2		
	MMP-3		
	MMP-7		
	MMP-9		
	MMP-10		
	MMP-12		
	MMP-13		

The plates were run on MAGPIX with xPONENT 3.1 acquisition software (Luminex, Texas, USA). For each circulating protein analysed, output standard curve was generated with 4 PLOG, 5 PLOG graphs as the manufacturer's recommendations. Each standard curve option was reviewed, subsequently the optimal standard curve for analysis was established based on the following criteria: i) inter-assay %CV <15%; ii) R^2 close to 1; iii) recovery 70-130%; iv) sigmoid shaped curve. Based on the selected standard curve type, the absolute concentration of the analyte was generated automatically by the xPONENT 3.1 software.

7.3.3 Quantification of CD163+ Monocytes by CyTOF Mass Cytometry

The full methodology for this analysis is detailed in Chapter 2. In summary, the fixed blood samples were thawed and underwent lysis using Thaw-Lyse Buffers (Smart Tube Inc., California, USA) to remove red blood cells. The samples were then centrifuged and resuspended in FACS buffer wherein the cells were counted. Approximately 2 million cells per sample were then processed for mass cytometry including labelling with barcoding antibodies ^{106}CD45 and ^{108}CD45, followed by cell surface antibodies. The samples were then acquired using the 'Helios' mass cytometer (Fluidigm, California, USA).

After mass cytometry, the data was analysed using FlowJo (FlowJo LLC., Oregon, USA). A manual gating strategy was developed and utilised to quantify CD163+ monocytes, and the frequency of CD163+ monocytes as a % of total CD14+ monocytes was exported.

7.3.4 Quantification of Plasma sCD163 by ELISA

Plasma sCD163 was measured using the human soluble CD163 ELISA kit according to the manufacturer's protocols (Aviscera Bioscience, CA, USA). The samples were measured in duplicate and were diluted 1:200 to enable samples to be within the working range of the assay as per manufacturer's protocol. Resulting data was analyzed using a log-log standard curve with a coefficient of variation of ≤20%.

7.3.5 Data Analysis

Data analysis of the concentration of circulating cytokines, chemokines and MMPs, the proportion (%) of CD163+ circulating monocytes and the sCD163 level in the D^{+Comps} and D^{-Comps} groups was performed using GraphPad Prism 9 statistical package (GraphPad Software, San Diego, CA, USA). Continuous data was checked for normality and presented as mean ± standard deviation (SD) for parametric data and median and IQR or proportion for non-parametric data. Grouped data were compared by either Welch's t-test for parametric data, or Mann-Whitney test for non-parametric data or Fisher's exact test to identify differences between groups. Significance was accepted at $p<0.05$, and Bonferroni correction was used for multiple test correction to determine the adjusted p value. According to the normality test, the non-linear regression Spearman's correlation coefficient was used to verify the significance of correlations between CD163+ and circulating proteins, or sCD163 and circulating proteins.

7.4 Results

7.4.1 Clinical Characteristics

Clinical characteristics of individuals with diabetes and with complications (D^{+Comps}, n=6) or without (D^{-Comps}, n=12) are presented in Table 7.2. There was no difference in the age of individuals and proportion of male and female in the D^{+Comps} and D^{-Comps} groups. Individuals with diabetes complications had a longer duration of diabetes and higher HbA_{1c} however these did not meet significance.

Table 7.2: Clinical characteristics in individuals with (D^{+Comps}) or without (D^{-Comps}) any diabetes complications

Clinical characteristic	D^{+Comps} (n=6)	D^{-Comps} (n=12)	P value
Age (years)	67.6 ± 18.7	68.7 ± 9.3	ns
Sex (M/F) (n)	4/2	7/5	ns
Duration of diabetes (years)	25.5 (23.5 – 30.1)	20.3 (19.4 – 26.8)	ns
T2DM/ total cohort (%)	4/6	11/12	ns
BMI (kg/m^2)	25.5 ± 3.4	27.9 ± 3.2	ns
HbA_{1c} (%NGSP units)	7.6 (7.2 - 8.2)	6.9 (5.9 - 7.9)	ns
Total Cholesterol	3.8 (2.9 - 4.1)	3.9(3.2 - 4.8)	ns
HDL-c (mmol/L)	1.4 (0.9 - 1.7)	1.3 (1.2 - 1.6)	ns
LDL-c (mmol/L)	1.5 (1.1 - 2.2)	1.9 (1.4 - 2.2)	ns
Triglycerides (mmol/L)	1.4 (1.2 - 1.6)	1.4 (0.8 - 1.6)	ns

Results are expressed as median with interquartile range (IQR) or proportion (n). Significance was accepted when $p<0.05$ and no significance indicated by ns. BMI: body mass index; HbA1c: glycated haemoglobin; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol.

7.4.2. Circulating Level of CD163+ Monocytes and sCD163 in Individuals with Diabetes and with and without Complications

CD163+ monocytes were not significantly different between D^{-Comps} (n=12; 39.3 (33.8 – 53.4) %) vs D^{+Comps} (n=6; 34.4 (28.9 – 48.4) %) in this study cohort (Figure 7.1). In addition, the level of sCD163 was not different between D^{-Comps} (n=12; 173.1 (97.2- 204.9) ng/mL) vs D^{+Comps} (n=6; 233.2 (123.5 – 379.2) ng/mL) (Figure 7.2).

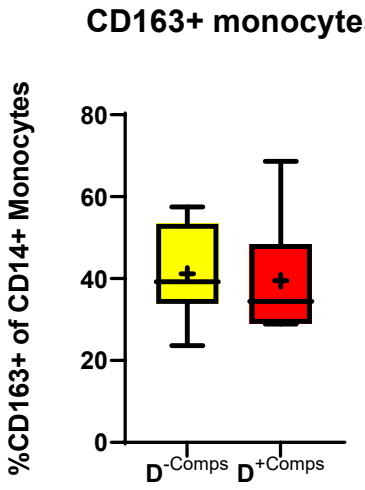


Figure 7.1: The level of CD163+ monocytes in individuals with diabetes and with (D^{+Comps}) and without complications (D^{-Comps}). D^{-Comps} indicated by yellow bar, and D^{+Comps}, indicated by red bar.

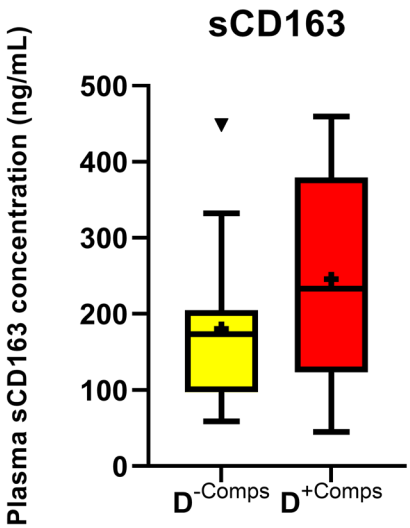


Figure 7.2: The plasma concentration of sCD163 in individuals with diabetes and with (D^{+Comps}) and without complications (D^{-Comps}). D^{-Comps} was indicated by yellow bar, and D^{+Comps} indicated by red bar. Outliers are indicated by ▼

7.4.3 Circulating Level of Cytokines and Chemokines in Individuals with Diabetes and with and without Complications

The expression of circulating cytokines and chemokines is presented in Table 7.3. IL-10 and IL-15 were increased 1.5-fold (p=0.01) and 1.6-fold (p=0.04) respectively in D^{+Comps}, but statistical significance disappeared after correction for multiple testing using the Bonferroni method with the adjusted p-value p<0.003. Additionally, the level of IL-1α, IL-4 and TNF-α was trending upward at 1.1-fold, 1.2-fold and 1.3-fold respectively in D^{+Comps} (all p=0.06) but failed to reach significance possibly due to the small numbers analysed.

Table 7.3 The circulating level of cytokines and chemokines in individuals with diabetes and with (D^{+Comps}) and without (D^{-Comps}) complications.

	Analyte (pg/mL)	D^{+Comps} (n=6)	D^{-Comps} (n=12)	P value
Cytokine	**INF-α2**	26.0 ± 8.2	19.4 ± 3.1	ns
	IL-1α	46.2 (42.5 - 54.3)	40.6 (39.5 - 44.7)	0.06
	IL-4	77.16 ± 15.33	62.3 ± 6.9	0.06
	IL-6	7.1 (3.2 - 17.9)	3.8 (2.1 - 6.4)	ns
	IL-8	8.9 ± 5.23	7.2 ± 2.8	ns
	IL-10	27.4 ± 6.2	18.1 ± 3.7	0.01*
	IL-12p70	11.1 ± 3.1	8.9 ± 1.9	ns
	IL-15	10.9 ± 3.6	6.9 ± 1.8	0.04*
	TNF-α	20.61 ± 5.4	15.3 ± 1.5	0.06
Chemokine	**MDC**	438.3 ± 421.8	466.7 ± 92.3	ns
	IP-10	152.0 (89.7 - 521.2)	134.2 (11.2 - 164.2)	ns
	MCP-1	124.5 (106.7 - 169.1)	115.7 (111.7 - 143.9)	ns
	MCP-3	32.2 (21.9 - 46.5)	22.1 (19.2 - 25.9)	ns
	MIP-1α	10.2 (9.4 - 12.1)	12.9 (9.7 - 15.9)	ns

	MIP-1β	33.7 (28.7 – 37.9)	30.1 (28.8 – 31.1)	ns
Growth Factor	**VEGFα**	46.9 (40.8 – 67.6)	45.7 (40.3 – 49.5)	ns

Results are expressed as mean and standard deviation or median with interquartile range (IQR). The significance was accepted at p<0.05 and ns indicated not significant.

7.4.4 Expression of Circulating MMPs in Individuals with Diabetes and with and without Complications

The circulating level of MMPs in individuals with diabetes and with or without complications is presented in Table 7.4. There were no significant difference in the plasma level of MMPs between D^{+Comps} and D^{-Comps}.

Table 7.4: The circulating level of MMPs in individuals with diabetes and with (D^{+Comps}) and without (D^{-Comps}) complications.

MMPs	D^{+Comps} (n=6)	D^{-Comps} (n=12)	P value
MMP-1 (pg/mL)	0.1 (0.04 - 0.1)	0.1 (0.1 - 0.2)	ns
MMP-2 (ng/mL)	5.3 (3.4 - 7.0)	4.9 (4.2 - 5.3)	ns
MMP-3 (ng/mL)	30.3 (19.7 - 36.6)	23.4 (15.2 - 30.7)	ns
MMP-7 (ng/mL)	0.5 (0.4 - 0.7)	0.5 (0.3 - 0.9)	ns
MMP-9 (ng/mL)	2.3 (1.6 - 4.0)	2.5 (2.1 - 3.8)	ns
MMP-10 (pg/mL)	0.03 (0.03 - 0.05)	0.03 (0.03 - 0.04)	ns
MMP-12 (ng/mL)	2.0 (1.2 - 2.4)	2.3 (1.8 - 2.6)	ns
MMP-13 (ng/mL)	3.6 (1.7 - 5.5)	4.8 (3.6 - 6.2)	ns

*Results are expressed as median with interquartile range (IQR). Significance was accepted at p<0.05 and indicated as *.*

7.4.5 Correlation of Circulating CD163+ Monocytes with Cytokines, Chemokines and MMPs in Individuals with Diabetes and with and without Complications

In individuals with diabetes complications (n=6), the proportion of circulating CD163+ monocytes was not correlated with any circulating cytokines, chemokines or MMPs from this study cohort (Figure 7.3).

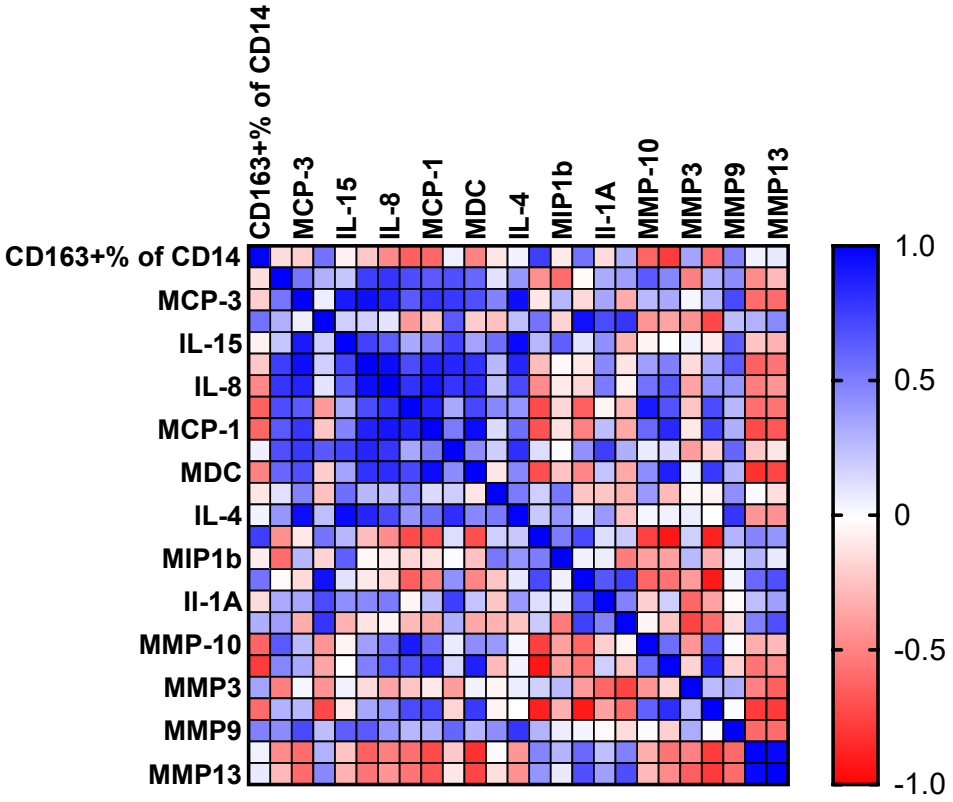


Figure 7.3: A heatmap of the Pearson R value depicting the correlation between CD163+% cells and cytokines, chemokines and MMPs in D^{+Comps}. Blue indicates positive and red represents negative R value.

In individuals with diabetes without complications (n=12), there were also no correlation being found between the proportion of circulating CD163+ monocytes and MMPs, except only a trend for MMP-10 (r=-0.5 [95%CI -0.8 – 0.03] p=0.06) (Figure 7.4).

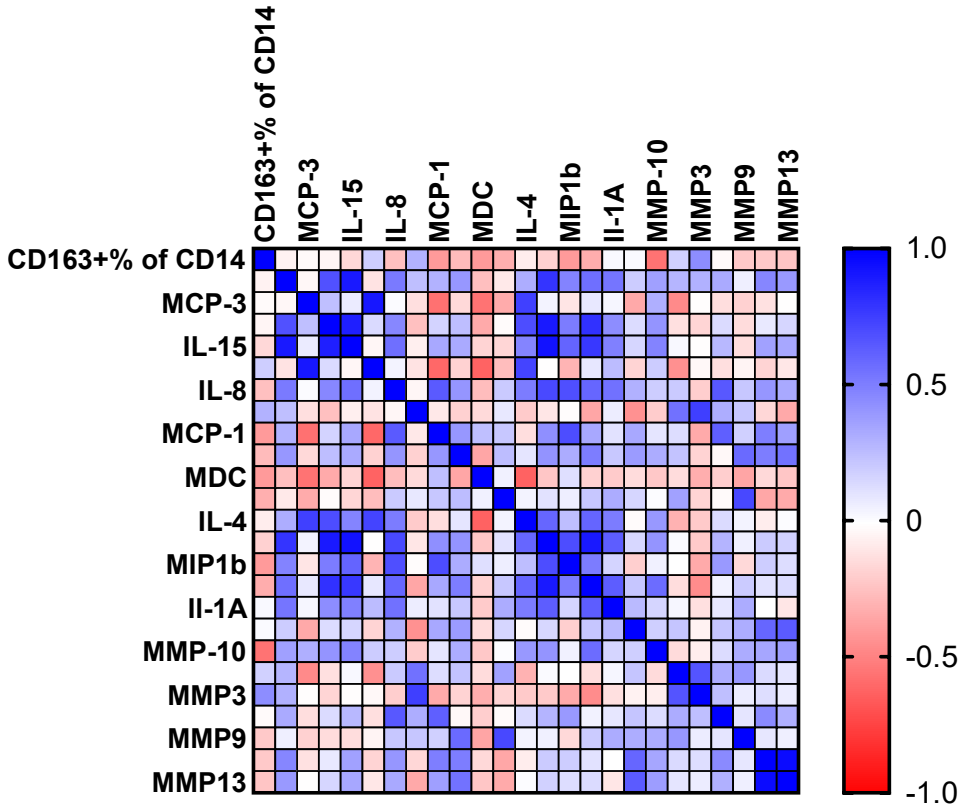


Figure 7.4: A heatmap of the Pearson R value depicting the correlation between CD163+% cells and cytokines, chemokines and MMPs in $D^{-Comps.}$ Blue indicates positive, and red represents negative R value.

7.4.6 Correlation of the Circulating Level of sCD163 with Cytokines, Chemokines and MMPs in Individuals with Diabetes and with and without Complications

The results for the correlation of plasma sCD163 to circulating cytokines, chemokines and MMPs levels in individuals with diabetes with and without complications are presented in Table 7.5. In D^{+Comps} group, sCD163 was positively correlated with cytokine INF-α2 (r=0.5 [95%CI 0.06 – 0.7]; p=0.02), chemokines IP-10 (r=0.5 [95%CI 0.2 – 0.8]; p=0.006;) and growth factor VEGFα (r=0.4 [95%CI -0.002 – 0.7]; p=0.04). Furthermore, sCD163 was positively correlated with MMP-2 (r=0.2 [95%CI 0.1-0.7]; p=0.01), MMP-7 (r=0.4 [95%CI-0.005 – 0.7]; p=0.05) and MMP-10 (r=0.5 [95%CI 0.1 – 0.7]; p=0.01). In individuals without diabetes complications, sCD163 was not found to be correlated with any of the circulating cytokines or chemokines, however it was correlated with MMP-12 (r=0.2 [95%CI -0.5 – 0.7]; p=0.02) and MMP-13 (r=-0.007 [95%CI -0.6 – 0.6]; p=0.03).

Table 7.5: The correlation of circulating sCD163 with the level of cytokines, chemokines and MMPs in individuals with diabetes and with and without complications.

	sCD163 vs circulating protein	D^{+Comps} (n=6)	P Value	D^{-Comps} (n=12)	P Value
Cytokines	**INF-α2**	0.5 (0.06-0.7)	0.02	-0.08 (-0.6 – 0.5)	ns
	IL-1α	-0.06 (-0.5 – 0.4)	ns	-0.1 (-0.6 – 0.5)	ns
	IL-4	-0.2 (-0.6 – 0.2)	ns	-0.3(-0.7 – 0.3)	ns
	IL-6	-0.1 (-0.5 – 0.3)	ns	-0.5 (-0.8 – 0.2)	ns
	IL-8	-0.02 (-0.4 – 0.4)	ns	-0.02 (-0.6 – 0.6)	ns
	IL-10	0.007 (-0.4 – 0.4)	ns	0.09 (-0.5 – 0.6)	ns
	IL-12p70	-0.008 (-0.4 – 0.4)	ns	-0.3 (-0.7 – 0.4)	ns
	IL-15	-0.2 (-0.6- 0.2)	ns	-0.018 (-0.6 – 0.6)	ns
	TNF-α	0.2 (-0.2 – 0.6)	ns	0.3 (-0.3 – 0.7)	ns
Chemokine	**MDC**	0.2 (-0.2 – 0.6)	ns	0.5 (-0.5 – 0.8)	ns
	IP-10	0.5 (0.2 – 0.8)	0.006	0.1 (-0.5 – 0.6)	ns
	MCP-1	0.3 (-0.2 – 0.6)	ns	0.4 (-0.2 – 0.8)	ns
	MCP-3	-0.2 (-0.6 – 0.3)	ns	-0.3 (-0.8 – 0.3)	ns
	MIP-1α	-0.2 (-0.5 – 0.3)	ns	-0.2 (-0.7 – 0.5)	ns
	MIP-1β	0.2 (-0.3 – 0.5)	ns	-0.03 (-0.6 – 0.6)	ns
Growth Factor	**VEGFα**	0.4 (-0.002 – 0.7)	0.04	-0.05 (-0.6 – 0.5)	ns
Metalloproteinases (MMPs)	**MMP-1**	-0.2 (-0.5 – 0.3)	ns	-0.04 (-0.6 – 0.5)	ns
	MMP-2	0.5 (0.1 – 0.7)	0.01	-0.01 (-0.6 – 0.6)	ns
	MMP-3	-0.2 (-0.6 – 0.2)	ns	-0.4 (-0.8 – 0.2)	ns
	MMP-7	0.4 (-0.005 – 0.7)	0.05	0.4 (-0.3- 0.8)	ns
	MMP-9	-0.4 (-0.7 – 0.009)	ns	0.2 (-0.4 – 0.7)	ns
	MMP-10	0.5 (0.1 – 0.7)	0.01	0.2 (-0.5 – 0.7)	ns
	MMP-12	0.1 (-0.3 – 0.5)	ns	0.2 (-0.5- 0.7)	0.02
	MMP-13	0.1 (-0.3- 0.5)	ns	-0.007 (-0.6 – 0.6)	0.03

Results are expressed as r (95%CI). Significance was accepted when adjusted $p<0.05$.

7.4.7 Correlation between the Circulating Level of MMPs, Cytokines and Chemokines in Individuals with Diabetes Complications

In individuals with diabetes complications (D^{+Comps}, n=6), INF-α2 was negatively correlated with MMP-2 (r= -0.9 [95%CI -0.9 - -0.4], p=0.01) and MMP-7 (r= -0.8 [95%CI -0.9 - -0.2], p=0.02). IP-10 was positively correlated with MMP-10 (r=0.8 [95%CI 0.3 – 0.9], p=0.01). Additionally, VEGFα was negatively correlated with MMP-7 (r=-0.8 [95%CI -0.9 - -0.3] p=0.01) (Figure 7.5). However, after Bonferroni correction none of these associations sustained their statistical significance at p<0.004, a possible consequence of the limited numbers used in the study.

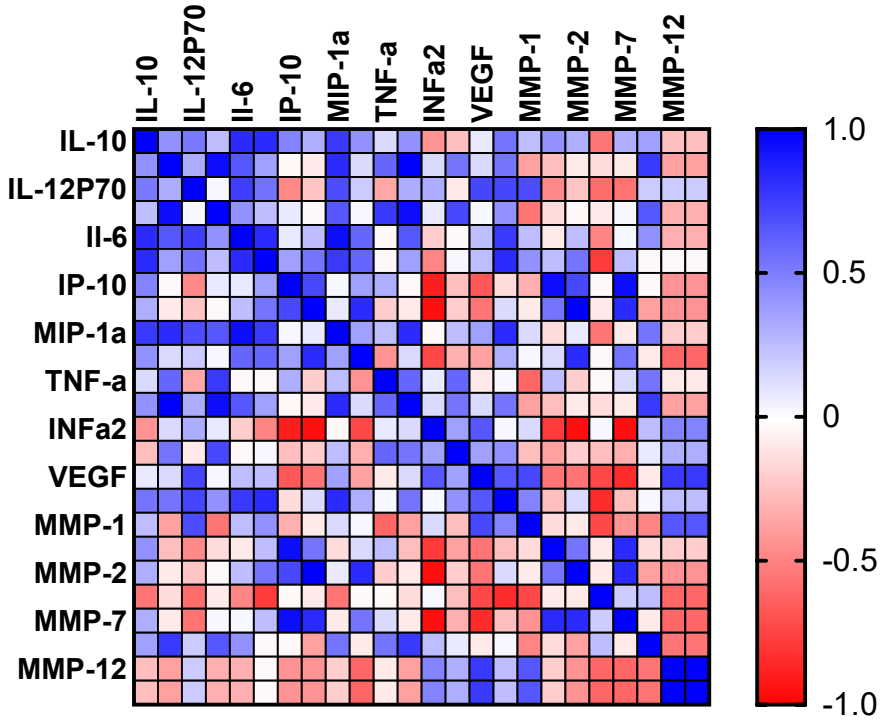


Figure 7.5: A heatmap of the Pearson R value depicting the correlation between level of circulating MMPs and cytokines and chemokines. Blue indicates positive, and red represents negative R value.

7.5 Discussion

Since diabetes is characterised by low-grade chronic inflammation which appeared to be responsible for the pathophysiology of diabetes complications (226), it was significant that the anti-inflammatory haemoglobin-haptoglobin scavenger receptor CD163 monocyte was decreased in individuals with diabetes complications compared to those with diabetes alone (76). In addition, in Chapter 5 we reported that there was differential expression of various functional and activation markers in the CD163+ monocytes of diabetes individuals with complications. More specifically, in those with complications CD163+ monocytes potentially displayed decreased trans-endothelial migration, decreased activation and dysregulated immune regulation (Chapter 5). This prompted our investigation of the expression of circulating cytokines, chemokines and MMPs involved in regulating the alteration of CD163+ monocytes in those with diabetes complications.

We found that cytokines IL-10 and IL-15 were increased in individuals with diabetes complications. This supports a previous study that found in individuals with more than three diabetic complications, IL-10 and IL-15 were both elevated compared to individuals with less than three complications (290). Therefore, this indicated that IL-10 and IL-15 may be involved in more severe diabetes complications status. IL-10, an anti-inflammatory cytokine produced by a myriad of immune cells including monocytes and macrophages, normally inhibits the production of pro-inflammatory cytokines and stimulates the production of anti-inflammatory cytokines by macrophages, enhancing phagocytic function, tissue repair and remodelling (291). Although IL-10 is suggested to play a protective role in T2DM, a study found that macrophages exposed to high glucose were less responsive or resistant to IL-10; thus suggesting that despite increased IL-10, decreased cellular responsiveness (hyporesponsive sensitivity to IL-10) may contribute to the chronic inflammation in diabetes, limiting anti-inflammatory and tissue reparative functions of IL-10 (292). Other

investigations have reported that the pro-inflammatory cytokine IL-15 was increased in the vitreous fluid from the eyes of individuals with proliferative diabetic retinopathy compared to non-proliferative diabetic retinopathy; suggesting that IL-15 was implicated in the progression of retinopathy (293).

Whilst plasma IL-10 and IL-15 levels were increased in individuals with diabetes complications, neither of these two cytokines correlated with the level of circulating CD163+ monocytes or with circulating sCD163 level in individuals with or without diabetes complications. This suggests that the mechanism by which IL-10 and IL-15 contribute to the developing diabetes complications, is independent of CD163. This contrasted with previous studies where IL-10 was known to induce and up-regulate CD163 on monocytes in diabetic mice (*in vivo*) and *in vitro* (112, 294).

Previous studies have identified that CD163 may be cleaved from the cell surface by MMPs in response to oxidative stress, inflammatory stimulus and other inflammatory conditions such as multiple sclerosis (94, 295). Although, we didn't find any correlations between the proportion of circulating CD163+ monocyte numbers with cytokines, chemokines or MMPs tested in both diabetes groups in this study, the sCD163 level has been observed positively correlated with plasma MMP-2, MMP-7 and MMP-10 in D^{+Comps}, suggesting that these MMPs may be involved in the cleavage of CD163 from the monocyte cell surface in individuals with diabetes complications. Except for degradation of ECM proteins, MMPs have multiple functions such as regulation of cytokines and growth factors (296). In addition, MMPs have been reported to cleave cell surface proteins, for example MMP-2 has been reported to shed the fibroblast growth factor from the cell surface to a soluble form and MMP-7 can cleave the ecto-domain of E-cadherin (297). MMP-10, stromelysin-2 can activate other MMPs which is less characterized (298). A recent study has reported that CD163 expression is a feature of the M2 polarised macrophage which plays a role in post

injury resolution by mediating matrix deposition and tissue remodelling. These M2 macrophages were reported co-expressing of CD163, MMP-2 and MMP-7 (299). This association suggested that in diabetes complications it would contribute to either the promotion or resolution of chronic inflammation. Furthermore another study found that individuals with diabetes with COVID infections had macrophages mainly polarised towards the M2 phenotype with significant up-regulation of pro-fibrotic marker MMP-7 (300). Therefore, these studies suggested that M2 macrophages with increased CD163 and MMP-2 or MMP-7 and MMP-2 or MMP-7 may potentially mediate the cleavage of CD163, thus may be associated with the sustained inflammation in diabetes complications, as circulating haemoglobin will not be internalised and endocytosed via the CD163 scavenger receptor and therefore causing oxidative stress, tissue damage and inflammation.

In D^{-Comps}, there is a positive correlation between sCD163 with MMP-12 and a very weak negative correlation with MMP-13, suggesting that in diabetes individuals without complications, there might be other mechanism by which sCD163 is generated. MMP-12, also known as macrophage metalloelastase has been associated with increased atherosclerotic burden in individuals with T2DM, implicating it in the inflammatory processes of macrovascular diabetes complications (301). MMP-13 was found upregulated and associated with neurotoxicity in an earlier investigation in a zebrafish model of diabetes (302), which also suggested an association between MMP-13 and diabetes complications. However, the very weak correlation with sCD163 detected in diabetes individuals without complications indicates MMP-13 may not directly link with the CD163 cleavage.

Cytokine INF-α2, chemokine IP-10 and growth factor VEGFα were found positively correlated with the circulating level of sCD163 in diabetes complications, thus suggesting that these circulating proteins may stimulate the generation of sCD163 (Figure 7.6). In a previous investigation, our group reported that IP-10 was negatively correlated with CD163+

monocytes in individuals with diabetes, indicating that IP-10 may also be involved in mediating the shedding of CD163 indirectly (76). It corroborates with our current finding of IP-10 being positively correlated to the production of sCD163. In addition, the positive correlation between IP-10 and MMP-10 in diabetes complications suggests that IP-10 mediated CD163 cleavage may be through MMP-10.

Interestingly in diabetes complications we found the level of INF-α2 negatively correlated with both MMP-2 and MMP-7 and VEGFα negatively correlated with MMP-7, implying that in diabetes complications MMP-2 and MMP-7 proteolytically regulate INF-α2 and VEGFα and this would lead to the cessation of further down-stream signalling pathways of INF-α2 and VEGFα, contributing towards the ongoing inflammation in diabetes complications. Previous studies have identified that MMP-12 by cleaving IFN-α at the C-terminus, led to the termination of downstream signalling pathways (303) but this has not been observed in diabetes complications. In addition, the cleavage of VEGF isoforms, especially VEGFα can be mediated by MMP-7 (304), and the cleaved products are reported to reduce the ability to stimulate mitogenesis (the generation of new cells) thus inhibiting the process of tissue repair and regeneration, causing ongoing inflammation (305). As such it can be hypothesised that in diabetes complications MMP-7 may be involved in the proteolytic cleavage of VEGFα, which contributes to ongoing inflammation, as VEGF is no longer able to stimulate the regeneration of tissues.

Alternatively, INF-α2 may cause the decreased synthesis of MMP-2 and MMP-7 or even inhibiting its proteolytic activity (306), similar to the studies in multiple sclerosis where they have shown that IFN-β can decrease the level of MMP-9 (306). However a positive correlation have found between sCD163 with either INF-α2 and, MMP-2 and MMP-7 in diabetes complications, negating the possibility that INF-α2 down-regulated MMP-2 and

MMP-7. The mechanism whereby MMPs enable this and the nature of their involvement requires further investigation.

We uniquely hypothesise that INF-α2, IP-10 and VEGFα are involved in the indirect regulation of the CD163+ monocytes in individuals with diabetes complications via the action of MMP-2, MMP-7 and MMP-10 on their cell surface. The formation of sCD163 acts as a proxy indicator of the degree of monocyte activation, but also indicates a reduced capacity of monocyte anti-inflammatory function, as once monocytes have lost CD163 from their cell surface, they are unable to reduce environmental oxidative stress produced by reduced processing of circulating haemoglobin, thus contributing to a prolonged inflamed state (Figure 7.6).

This study on the regulation of the CD163+ monocytes in individuals with or without diabetes is limited by its small sample size. Additionally, the study design is observational and only measures the level of circulating proteins at one time-point. Future investigations would benefit from a larger cohort in an investigation across multiple time-points, to correctly project the change in circulating cytokines and MMPs across the progression of diabetes. Furthermore, the findings of this investigation have led us to propose a mechanism for the regulation of CD163+ monocytes in diabetes complications. *In vitro* studies elucidating the direct effect of circulating cytokines, chemokines and MMPs on the expression and cleavage of CD163 from the cell surface would confirm these findings. Future investigations may focus on utilising animal studies to investigate whether the change in circulating cytokines, chemokines and MMPs between those with and without diabetes complications corresponds to the degree of inflammation at tissue sites; as it will require tissue samples from these sites to investigate the changes of CD163+ cells.

In conclusion, we report that circulating cytokines IL-10 and IL-15 are different between those with and without diabetes complications and the mechanism by which this drives inflammation may be independent of alteration of CD163+ monocytes. The cytokine INF-α2, chemokine IP-10 and growth factor VEGFα may be implicated in the level of circulating sCD163 in diabetes complications, which may be cleaved off due to the action of MMP-2, MMP-7 and MMP-10, which is a novel finding.

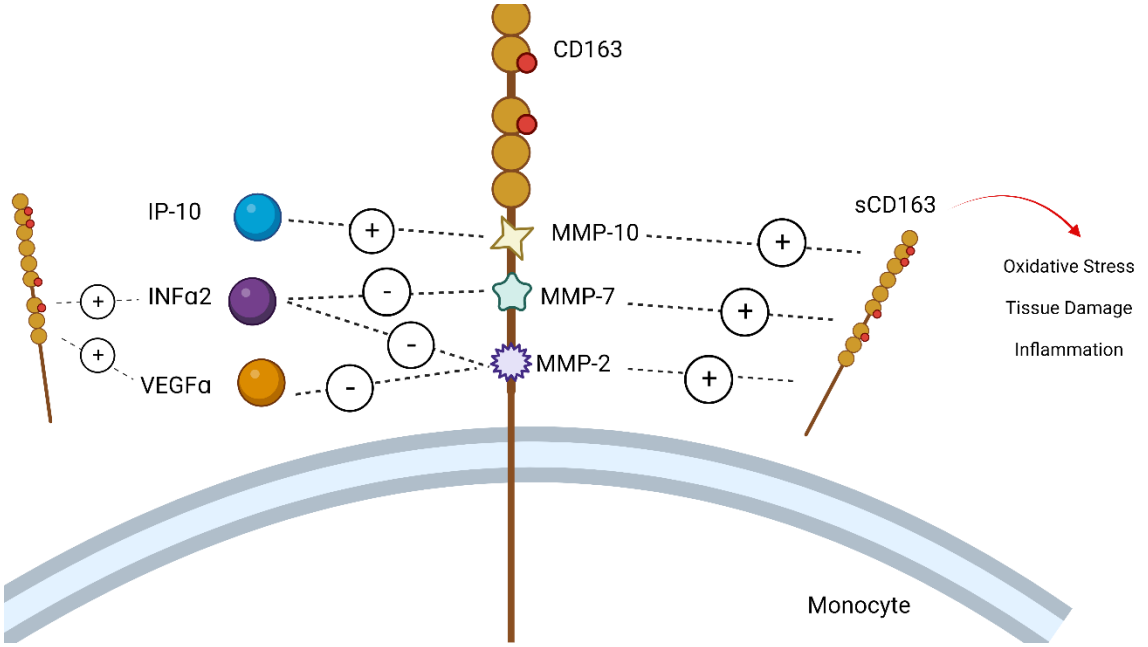


Figure 7.6: A diagram illustrating the proposed proteolytic action of MMP-2, MMP-7 and MMP-10 on the membrane bound CD163 receptor. MMP-2, -7 and -10 were all positively correlated with the levels of plasma sCD163 in individuals with diabetes complications. IP-10 was positively correlated with MMP-10, and also with sCD163. INFa2 and VEGFa were negatively correlated with MMP-7 and MMP-2, but positively with sCD163. The cleavage of sCD163 leads to oxidative stress, tissue damage and inflammation.

Chapter 8: Discussion, Future Directions and Conclusion

This thesis presents research from five related studies that investigated the literature, function and regulation of the CD163+ monocyte in diabetes complications, as well as utility of sCD163 as a disease status marker in diabetes complications, and the profile of circulating immune cells in diabetes complications. Chapters 3 to 7 of this thesis have comprehensive discussion of the key findings of the results arising from the study, this chapter will discuss how these studies address the central hypothesis of this thesis and highlight future research arising from the findings.

8.1 Decreased Circulating CD163+ Monocytes in Diabetes Complications

A previous study conducted by Min et al. reported the number of circulating CD163+ monocytes to be decreased in individuals with diabetes complications compared to those without complications (76). This study was the only one known to examine the CD163+ monocyte levels in the peripheral blood in diabetes complications and has been included in the systematic review conducted in Chapter 3. In the present study, there was no significant difference in the proportion of circulating CD163+ monocytes between individuals with and without diabetes complications. This might be due to a smaller cohort size in this study compared to the investigation by Min et al. Despite the incongruency concerning the proportion of circulating CD163+ monocytes in diabetes complications, the RNA-sequencing results from Chapter 5, support the concept of decreased CD163+ cells in diabetes complications. The analysis of differentially expressed genes in CD163+ cells between individuals with and without complications found that genes involved in the ‘regulation of the centrosome cycle’ were upregulated and enriched in those with complications. Dysregulation in the centrosome cycle can implicate cellular apoptosis (307). Additionally, our gene analysis revealed that microRNAs *MIR23A* and *MIR27A* were also up-regulated in

individuals with complications. These microRNAs have also been found to contribute towards increased cellular apoptosis (308, 309), and may therefore contribute to the decrease in circulating CD163+ monocytes in individuals with diabetes complications. Therefore, the decreased proportion of circulating CD163+ monocytes in diabetes complications could be the result of the apoptosis of CD163+ monocytes due to increased genes pertaining the 'centrosome cycle' and the up-regulation of specific microRNAs.

8.2 Regulation of CD163+ Monocytes in Diabetes Complications

Previous investigations have found that the level of circulating CD163+ monocytes can be decreased by pro-inflammatory mediators such as LPS, IFN-ɣ and TNF-α; and endogenous pro-inflammatory mediators such as IL-α, IL-1β as well as chemokine CXCL-8 (IL-8) (106, 112, 126) . Although these changes were not observed in this study shown in Chapter 7, the cytokine INF-α2, chemokine IP-10 and growth factor VEGFα were found associated with circulating sCD163 level in diabetes complications in this study, suggesting that these proteins may play as the key mediators responsible for the down-regulation of CD163+ monocytes in individuals with diabetes complications. It's worth noting the difference between studies, suggesting that various mediators are involved in the regulation of CD163 and the underlying mechanisms in diabetes complications may differ with other disease conditions which may involve distinct cytokine profiles.

Additionally, the decreased proportion of circulating CD163+ monocytes could also be caused by the proteolytic cleavage of CD163 by MMPs from cell surface forming sCD163. Previous studies have reported that in conditions of oxidative stress or in the presence of inflammatory stimuli, MMPs can proteolytically shed CD163 from the monocyte cell surface (94). In Chapter 7, we measured the levels of MMPs in individuals with diabetes and with and without complications and found a positive correlation between sCD163 with MMP-2,

MMP-7 and MMP-10 in individuals with diabetes complications. Instead, only MMP-12 was positively associated with sCD163 level in those without complications. This indicates that the regulation of CD163+ cells and consequent formation of sCD163 occurs via different MMPs between those with and without complications, suggesting different regulatory pathways in individuals with complications compared to those without.

8.3 sCD163 in Diabetes Complications

The decreased presence of circulating CD163+ monocytes has been found to correlate directly with increased sCD163 (127). Furthermore, our systematic review in Chapter 3 indicated that the increased sCD163 which reflects macrophage activation, was associated with worsening disease severity in certain diabetes complications (76, 157, 310). As such sCD163 may have functional utility as a biomarker or indicator of disease severity. As reported in the systematic review in Chapter 3, sCD163 was found increased in the plasma in DR and DN, and increased in the serum in DPN, CVD and PAD (76, 151, 156, 157, 311). Furthermore the increase of sCD163 in PAD was correlated with worsening complication status (157). Recent investigations have reported that sCD163 has also been found increased in individuals with NAFLD and NASH in a no diabetes focus study (158). Considering that NAFLD and NASH are growingly being recognised as co-morbid complications of diabetes, we sought to examine the ability of sCD163 to predict worsening disease progression of NAFLD and diabetes complications in cohort comprising of diabetes complications and NAFLD. In our investigation in Chapter 4, we found that sCD163 was increased in microvascular complications compared to individuals with diabetes but without microvascular complications. More specifically this increase was in individuals with DN, however sCD163 did not show a strong clinical value to detect DN.

In contrast, circulating sCD163 was increased in individuals with diabetes with advanced NASH fibrosis compared to those with less severe NASH fibrosis, and it also correlated with

progressing fibrosis severity, suggesting sCD163 may have utility as a biomarker of certain diabetes complications and disease severity in NASH fibrosis.

8.4 Functions of CD163+ Monocytes in Diabetes Complications

The previous finding that the proportion of circulating CD163+ monocytes is decreased in individuals with diabetes complications, indicated that the CD163+ monocyte may have protective and anti-inflammatory functions (76). We thus aimed to characterise the CD163+ monocytes at the gene and protein levels in individuals with and without diabetes complications. In Chapter 5, we found that in individuals with diabetes complications CD163+ monocytes exhibited a characteristic profile suggesting reduced abilities in cell recruitment and trans-endothelial migration. Chemokine receptors, *CX3CR1*, *CXCL7 (PPBP)* and CCR5 and adhesion markers CD11b, CD11c and CD31 were reduced in diabetes complications, therefore indicating a reduced ability of CD163+ monocytes to adhere to the vascular endothelium and extravasate into local tissues, possibly resulting in un-resolving and persistent inflammation in the tissue sites of diabetes complications.

Additionally, we found that in diabetes complications CD163+ monocytes were characterised to suggest a reduced ability to clear bacterial antigens due to decreased TLR-4 mediated antigen recognition, thus indicating a possible mechanism for systemic inflammation. Furthermore, the CD39/CD73 mediated internalisation of ATP and production of adenosine may be decreased, again suggesting another mechanism for greater systemic inflammation in individuals with diabetes complications compared to those without. Similarly, the decreased *CD200R1*, also implies decreased immunosuppression, suggesting another mechanism by which there may be greater systemic inflammation in individuals with diabetes complications compared to those without.

Furthermore, we found various mechanisms by which the CD163+ monocyte has dysregulated connections with the adaptive immune system. We found that the *CD200R* gene was down-regulated, possibly leading to decreased *CD200R/CD200* mediated immune-suppression. In a study in sarcoidosis, it was found that the CD200R expression is reduced, and furthermore is associated with increased pro-inflammatory cytokine release (312). This may suggest that decreased CD200R in diabetes complications may be associated with the increased pro-inflammatory cytokine IL-15 in diabetes complications as observed in Chapter 7. However, a direct association between decreased CD200R and increased IL-15 has not yet been established.

Thus, this study suggests that in individuals with complications the anti-inflammatory nature and functionally of the CD163+ monocytes are diminished which may reduce their potential to resolve inflammation.

8.5 Circulating Immune Cells in Diabetes Complications

In addition to CD163+ monocytes, various other circulating white blood cells have been found to be different between individuals with and without diabetes complications (224, 225). This suggests that diabetes can lead to change in the populations and phenotypes of circulating immune cells which may exacerbate systemic inflammation or tissue damage in diabetes complications. In Chapter 6, we immunophenotyped the white blood cells of individuals with and without diabetes complications. Although we found no difference in the proportion of circulating monocytes, mDendritic cells, granulocytes, T cells, B cells and NK cells, there was a significant difference in the expression of activation and function markers in immune cell subsets between those with and without complications. In congruence with the immunophenotyping of CD163+ monocytes in Chapter 5, we found that the classical, intermediate and non-classical subsets of monocytes all displayed reduced expression of

adhesion markers in diabetes complications. Similarly, in Chapter 6 we found lymphocyte activation and function markers to be different between individuals with and without complications. Specifically, the phenotype of T cells in diabetes complications included reduced Tcon cells and increased T reg cells, which together can been implicated to decrease the T cell immune response, increase the suppression of the adaptive immune response and thereby contribute to the progression of diabetes complications (160, 195, 261, 262). Additionally in diabetes complications active B cells were trending decreased and naïve B cells trending increased, this imbalance further illuminates another potential mechanism contributing to immune dysfunction or suppression in diabetes complications, which would worsen the progression of diabetes complications.

The immunophenotype of lymphocytes found in Chapter 6, can be linked to the CD163+ monocyte transcriptome found in Chapter 5. In diabetes complications we reported that CD163+ monocytes may be implicated in dysregulated activation of the adaptive immune system. The *CD5* gene which can suppress the activation of peripheral T cells (313) was found to be increased in CD163+ monocytes in diabetes complications, suggesting enhanced suppression of T cells in diabetes complications. This corroborates with the finding of decreased T con cells, suggesting that *CD5* may down-regulate T con cells via CD163+ monocyte in diabetes complications. Similarly, the reduced expression of co-stimulatory molecule CD86 on CD163+ monocytes, indicates another mechanism for the reduced activation of T cells, as CD86 is normally involved in the activation and survival of T cells (247). Therefore our results from Chapter 5 and 6 together, suggest that CD163+ monocytes may play an important role in the dysregulation of the adaptive immune system, which contributes to the progression of diabetes complications, however the pathways are not established.

8.6 Limitations and Future Research Directions

A key limitation of this thesis lies in the study design of the conducted investigations, particularly with regard to the inclusion and exclusion criterion applied to select study participants. In Chapter 4 the study participants, had both diabetes as well as NAFLD. The comorbidity of NAFLD present in the participants with and without diabetes complications, may have confounded the ability of sCD163 to be able to detect diabetes complications, as we found sCD163 to be correlated with LSM score, reflective of liver fibrosis severity. Whilst the presence of NAFLD in the diabetes cohort presented as experimental limitation for the ability of sCD163 to detect singular diabetes complications, it reflects a true, real-world diabetes population, wherein NAFLD coexists with diabetes such that NAFLD is present at 59.7% in individuals with T2DM. Another limitation of the study participants in this thesis was the small cohort size. In Chapter 4, the small total cohort size, resulted in small sub-groups, when the whole cohort was stratified according to specific types of diabetes complications. Similarly, in the investigations conducted in Chapters 5-7, the small cohort sizes affected the statistical power of the results when the groups were compared, and future investigations would benefit from larger cohort sizes.

The small cohort size however, did not impact the investigation conducted in Chapter 5, wherein mega-data was output from the RNA-sequencing of CD163+ cells from only 6 individuals in both groups. The RNA-sequencing investigation however was limited by the immune focused analysis approach. At the level of gene classification and ontology analysis, differentially expressed genes were selected for down-stream analysis if they were involved in immune function. For the purposes of immune analysis, we had purposefully excluded all other differentially expressed genes between those with and without diabetes complications. Recent investigations have proposed iron and lipid metabolism as being implicated in the

progression of diabetes complications (314, 315). As such future studies could examine the RNA-sequencing data of the CD163+ monocytes from the perspective of iron and lipid metabolism, revealing differences and dysregulation in iron and lipid metabolism between those with and without diabetes complications, thus identifying how these differences may pathologically contribute to the progressive nature of diabetes complications, and further reveal potential therapeutic targets in the progression of diabetes complications.

Similarly, the immunophenotyping investigations in Chapters 5 and 6 were intentionally designed to investigate the characteristic profile concerning immune function of CD163+ monocytes and other circulating white blood cells; thus these investigations were limited as the CD marker panel utilised was restricted to cell surface markers pertaining to immune cell function. Future studies would benefit from investigating the immunophenotype of the CD163+ cell at the cell surface and intra-cellular level with CD markers pertaining to iron and lipid metabolism.

Likewise, the multiplex immunoassay panel in Chapter 7, investigated the differences in circulating inflammation related proteins cytokines, chemokines and MMPs between those with and without diabetes complications, thus it was intentionally restrictive. However considering our finding of MMP-2, -7 and -10 being associated with sCD163 in diabetes complications, future investigations would benefit by investigating the molecular pathways involved in the cleavage of CD163 from the cell surface, and moreover how the presence of diabetes positions MMPs to actively cleave CD163.

Our results concerning the characteristic differences of CD163+ monocytes in those with and without diabetes, indicated potential mechanisms by which CD163+ monocytes are either decreased in circulation, or may have reduced functional capacities. Future investigations

could further the strength of these findings by confirming the functionality of CD163+ cells using *in vitro* studies, and in animal models.

In Chapter 6 we report various characteristic differences in the function and activation markers of specific white blood cells in individuals with diabetes complications compared to those without. The understanding of these results can be furthered by investigating the relationship between white blood cell subsets in diabetes complications with cytokines, chemokines and MMPs in diabetes complications. This will give a greater understanding of the regulation of other white blood cells in diabetes complications.

8.7 Conclusion

Diabetes and its complications are a modern major health concern, and its increasing global burden is imminent, thus illuminating the need for preventive and therapeutic measures. Inflammation has been implicated in the development of diabetes as well as in the progressive nature of the pathophysiology of the various micro- and macrovascular complications. Furthermore, the anti-inflammatory haemoglobin-haptoglobin scavenger receptor CD163+ monocytes have been found decreased in those with diabetes complications compared to those without (76). Utilising this premise in this thesis, we surveyed the existing literature in a systematic approach to identify the trends in CD163 and sCD163 levels across various diabetes complications and found that serum and plasma sCD163 was found decreased in diabetes complications and implicated in disease progression. Tissue expression of CD163 was increased in DR, but decreased in CVD (153-155), whilst circulating CD163+ monocytes were reported decreased in diabetes complications (76). In the second aim of this thesis, we confirmed the sCD163 trend found in the results of the systemic review, wherein a single cohort study we reported, that plasma sCD163 is found reduced in individuals with DN and also reduced in individuals with advanced liver fibrosis (NASH fibrosis by definition), in

a cohort of individuals with diabetes. Furthermore, we found that despite the mixed cohort of individuals with diabetes complications and NAFLD, sCD163 had clinical utility as a non-invasive biomarker to identify individuals with advanced liver fibrosis, therefore enabling non-invasive monitoring of the NAFLD condition, as well as presenting sCD163 as a potential therapeutic target. In accordance with the third aim and hypothesis of this thesis we demonstrated characteristic differences in the CD163+ monocytes between individuals with and without complications at the gene and protein levels. These differences indicated 1) decreased circulating proportion of CD163+ monocytes in diabetes complications potentially due to cell apoptosis; 2) reduced migration and activation functionality of the CD163+monocytes in diabetes complications; and 3) dysregulated interactions with the adaptive immune system. These three differences in the CD163+ monocytes in diabetes complications may have primed CD163+ monocytes to carry reduced anti-inflammatory functions in diabetes complications, and therefore by this nature contribute to the progressing inflammation in diabetes complications. In conducting the fourth aim of the thesis, we immunophenotyped the other circulating white blood cells in diabetes complications and found potentially decreased ability of certain innate immune cells to transmigrate into local sites of inflammation in those with complications compared to those without. Additionally in diabetes complications the phenotype of the adaptive immune cells indicated the presence of immune dysfunction and suppression, thus also contributing to a state of ongoing pathophysiology in diabetes complications. The final aim of the thesis investigated the levels of circulating cytokines, chemokines and MMPs in individuals with diabetes complications, and found cytokines IL-10 and IL-15 to be increased and thus involved in the inflammatory milieu in diabetes complications, though the role of these proteins in diabetes complications remains to be established. MMP-12 was found to be positively correlated with the sCD163 in individuals with diabetes without complications; and MMP-2, -7 and -10 associated with

sCD163 in diabetes complications. This therefore highlights that MMPs associate with and may contribute to the progression of diabetes complications via the ecto-domain shedding of CD163+ cells, and the formation of sCD163. However, the diabetes associated mechanism that influence the action of MMPs remains to be investigated.

In conclusion, as shown in Figure 8.1 the investigations conducted within this thesis demonstrate that in diabetes complications the circulating CD163+ monocyte levels are decreased, potentially due to up-regulation of centrosome cycle genes, apoptotic genes and MMP mediated cleavage; this leads to a decrease in the circulating CD163+ monocytes, which in turn contributes to the unresolving nature of diabetes complications. The action of MMPs may lead to decreased circulating CD163+ monocytes, and is associated with increased sCD163 levels, which we found to be useful as an indicator of disease severity in diabetes complications and NAFLD. The altered phenotype of the CD163+ monocytes indicated a likely 1) reduced trans-endothelial migration 2) reduced activation; and 3) dysregulated activation of the adaptive immune system; all of which contribute to the ongoing inflammation and progression of diabetes complications. The research presented in this thesis identifies various immune and therapeutic targets concerning the phenotype of the CD163+ monocytes, suggesting a complex interplay between the immune system and inflammatory processes in diabetes complication that can be investigated further in the efforts to counter the implicated contributing inflammatory pathways, in order to help prevent the onset and progression of diabetes complications, and potentially, with progress in research, even aid in diabetes complications reversal.

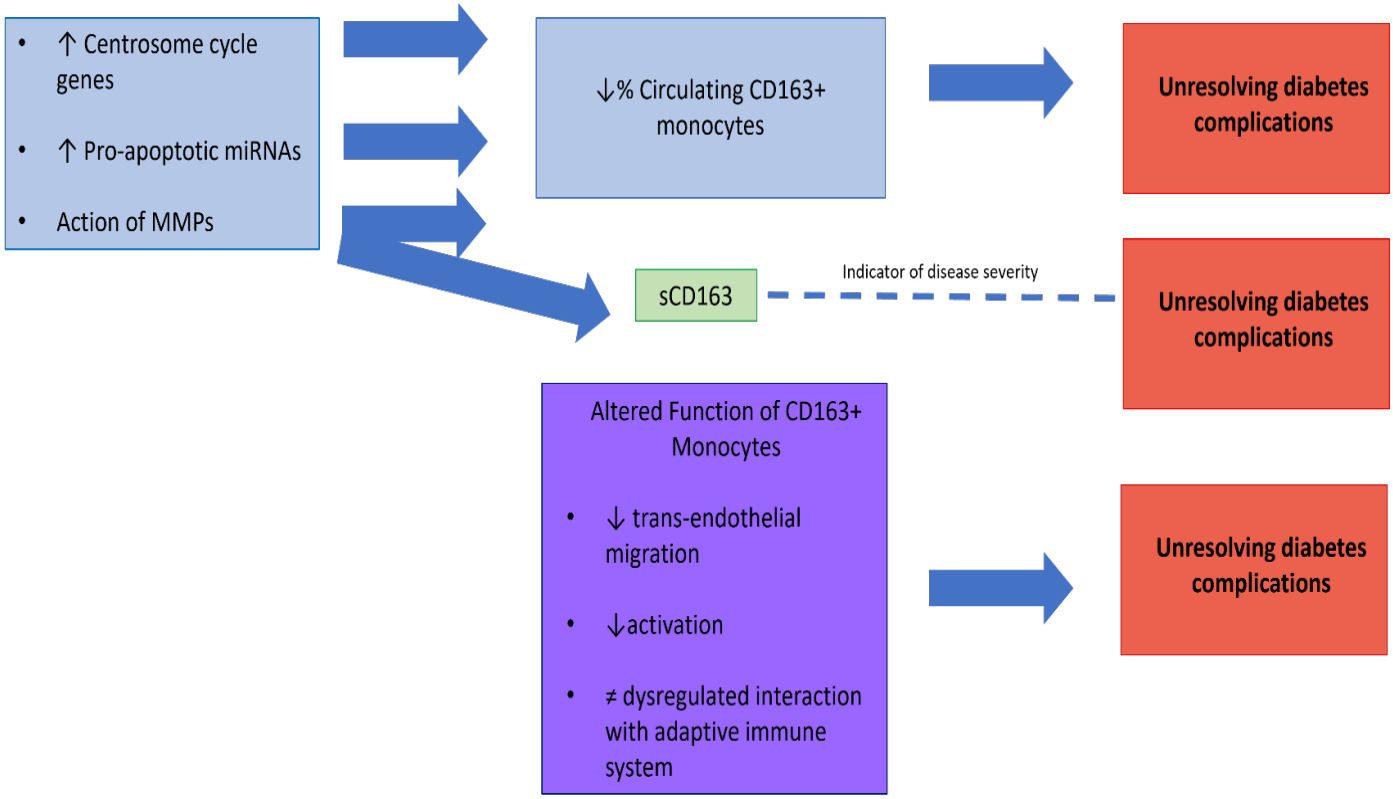


Figure 8.1. The regulation and function of CD163+ monocytes and sCD163 involved in the progressive nature of diabetes complications.

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