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The impact of hyperglycemia on CD163-mediated scavenging of hemoglobin-haptoglobin complexes in human primary monocyte-derived macrophages

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TABLE OF CONTENTS

Page

TABLE INDEX 1

FIGURE INDEX 2

ABBREVIATIONS 3

1 INTRODUCTION 5

1.1 Type 2 diabetes mellitus – a worldwide problem 5

1.1.1 Definition, prevalence and public health burden 5

1.1.2 Hyperglycemia is a key feature of diabetes 6

1.1.3 Subclinical chronic systemic inflammation 7

1.2 Monocytes and macrophages in diabetes and hyperglycemic conditions 9

1.2.1 Monocytes and macrophages are key cells for innate immunity 9

1.2.2 Concepts of macrophage activation 11

1.2.3 Monocytes and macrophages in hyperglycemic conditions 14

1.3 Scavenger receptor CD163: function and role in inflammation 16

1.3.1 Expression and regulation 16

1.3.2 Soluble CD163 17

1.3.3 Functional role 18

1.3.4 CD163 in pathologic conditions 21

1.4 Aims and objectives of this project 23

2 MATERIAL AND METHODS 24

2.1 Chemicals, reagents and kits 24

2.2 Consumables 26

2.3 Equipment 26

2.4 Buffers and solutions 27

2.4.1 Running buffer for agarose gel electrophoresis 27

2.4.2 Solutions for cell fixation 27
2.4.3 Solutions for immunological methods 28
2.4.4 Buffer for monocytes isolation 28
2.5 Molecular biology methods 28
2.5.1 Primers 28
2.5.2 Isolation of RNA 29
2.5.3 cDNA synthesis 30
2.5.4 Polymerase chain reaction (PCR) 31
2.5.5 Agarose gel electrophoresis 31
2.5.6 Real-time quantitative polymerase chain reaction (RT-qPCR) 32
2.6 Cell culture 33
2.6.1 Isolation of $CD14^+$ monocytes from human buffy coats 33
2.6.2 Cell counting using a CASY cell counter 34
2.6.3 Cultivation conditions 35
2.6.4 Harvesting of cells 35
2.7 Protein-related techniques 35
2.7.1 Enzyme-linked immunosorbent assay (ELISA) 35
2.7.2 Inflammatory response protocol 37
2.8 Immunological methods 38
2.8.1 Antibodies 38
2.8.2 Alexa Fluor® 488 labeling of haptoglobin molecules 38
2.8.3 Flow cytometry 41
2.8.4 Endocytosis of hemoglobin-haptoglobin complexes 42
2.8.5 Immunofluorescence and confocal microscopy 43
2.9 Statistical analysis 44

3 RESULTS 45

3.1 Experimental design 45
3.2 Stimulation with IFNγ and IL-4 suppress CD163 gene expression in primary macrophages derived from healthy donor monocytes 46
3.3 RT-qPCR analysis of CD163 mRNA expression in differentially stimulated macrophages in hyperglycemic conditions 47
3.4 Hyperglycemia enhances suppressing effect of IFNγ on CD163 surface expression in differentially stimulated macrophages 49
3.5 Production of soluble CD163 is most efficient in pro-inflammatory conditions 50

3.6 CD163-mediated internalization of Hb-Hp complexes is efficient in normo- and hyperglycemic conditions in M0 and M(IL-4) macrophages..... 52

3.7 Hyperglycemia enhances release of inflammatory cytokines in response to CD163-mediated scavenging of hemoglobin-haptoglobin complexes in M(IFNγ) 59

4 DISCUSSION 68

4.1 Model system to investigate impact of hyperglycemia on diabetic vascular complications..... 68

4.2 Hyperglycemia interferes with cytokine-dependent regulation of CD163 expression in human primary monocyte-derived macrophages 69

4.3 Diabetic vascular complications are promoted by the limited scavenging capacity of CD163 in pro-inflammatory conditions 73

4.4 Diabetes as a disease of less effective anti-inflammatory control of tissue damage 74

5 SUMMARY 78

6 BIBLIOGRAPHY 79

7 CURRICULUM VITAE 94

8 ACKNOWLEDGEMENTS 95

TABLE INDEX

Table 1. Risk factors for the development of type 2 diabetes mellitus. 5
Table 2. Criteria for the diagnosis of diabetes mellitus. 6
Table 3. Selection of populations of tissue-resident macrophages according to their location. 11
Table 4. Regulation of CD163 expression by various stimuli. 16
Table 5. Elevated serum levels of soluble CD163 in various diseases. 21
Table 6. Chemicals and reagents. 24
Table 7. Kits. 25
Table 8. Consumables. 26
Table 9. Equipment. 26
Table 10. Primers and probes. 28
Table 11. Reagents used for DNA digestion. 30
Table 12. Reagents used for cDNA synthesis. 30
Table 13. Reagents used for PCR. 31
Table 14. PCR protocol. 31
Table 15. Size ranges for agarose gel. 31
Table 16. Pipetting scheme for primer optimization. 32
Table 17. Reagents used in RT-qPCR master mix. 33
Table 18. LightCycler 480 amplification protocol. 33
Table 19. ELISA kits used in this project. 35
Table 20. Reagents used in ELISA kits. 36
Table 21. Antibodies and staining reagents used in this project. 38
Table 22. Absorbance of conjugate solutions of haptoglobin1-1 and haptoglobin2-2. 40

FIGURE INDEX

Figure 1. Differentiation and maturation of hematopoietic antigen-presenting cells... 9
Figure 2. Differential macrophage physiologies caused by the stimulation of cytokines and microenvironmental stimuli.. 12
Figure 3. Description framework for macrophage activation based on the M1/M2 concept. 14
Figure 4. Differences in the structure of haptoglobin variants in humans..... 18
Figure 5. Mechanism of scavenging hemoglobin-haptoglobin complexes. 20
Figure 6. ‚Inflammatory Response' planting sketch with stimulations. 37
Figure 7. Column assembly. 39
Figure 8. Planting sketch for endocytosis experiment. 42
Figure 9. Cytospin assembly..... 43
Figure 10. Schematic illustration of the utilised in vitro model system of macrophages.. 45
Figure 11. RT-qPCR and flow cytometry analysis of CD163 expression in differentially stimulated macrophages. 46
Figure 12. CD163 gene expression levels in differentially stimulated macrophages for individual donors..... 47
Figure 13. RT-qPCR analysis of CD163 gene expression levels in differentially stimulated macrophages. 48
Figure 14. CD163 surface expression on differentially stimulated macrophages depicted for individual donors. 49
Figure 15. Flow cytometry analysis of CD163 surface expression on differentially stimulated macrophages. 50
Figure 16. Amount of sCD163 in supernatants secreted by differentially stimulated macrophages of 5 healthy donors. 51
Figure 17. Flow cytometry analysis of Alexa Fluor® 488-labeled acLDL. 52
Figure 18. Confocal microscopy analysis of acLDL endocytosis. 53
Figure 19. Control staining confirming specificity of CD163 antibody used for confocal microscopy analysis of endocytosis..... 54
Figure 20. Confocal microscopy analysis of endocytosis of Hb-Hp-Alexa Fluor® 488 complexes via CD163..... 57
Figure 21. Flow cytometry analysis of endocytosis of Alexa Fluor® 488-labeled hemoglobin-haptoglobin complexes. 58
Figure 22. Experimental design to determine inflammatory reaction of M(IFNγ) after stimulation with hemoglobin-haptoglobin complexes. 59
Figure 23. RT-qPCR analysis of gene expression and flow cytometry analysis of surface expression of CD163 in IFNγ-stimulated macrophages. 60
Figure 24. RT-qPCR analysis of mRNA expression of the cytokines TNFα, IL-1β, IL-6, IL-8 and IL-1RA in IFNγ-stimulated macrophages. 62
Figure 25. Amounts of TNFα, IL-1β, IL-6, IL-8 and IL-1RA detected in the supernatants secreted by IFNγ-stimulated human primary macrophages. 64

ABBREVIATIONS

°C	degrees Celsius
µg	micrograms
µl	microliter
µM	micromolar
Ab	Antibody
acLDL	acetylated low-density lipoprotein
APC	Antigen-presenting cell
bp	base pair(s)
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CLSM	confocal laser scanning microscopy
CO_2	carbon dioxide
ddH2O	double distilled water
ddNTP	dideoxynucleotide triphosphate
DEPC	diethylpyrocarbonate
Dex	dexamethasone
DMEM	Dulbecco's modified Eagle's minimal essential medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
F	forward
FACS	fluorescence-activated cell sorting
FITC	fluorescein-5-isothiocyanate
FSC	forward scatter
g	gram(s)
GAPDH	glutaldehyde phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
h	hour(s)
HDL	high-density lipoprotein
HG	high glucose conditions (25mM)
HRP	horse radish peroxide
IF	immunofluorescence
IFG	impaired fasting glucose
IFNγ	Interferon gamma
IgG	immunoglobulin G
IGT	impaired glucose tolerance
IL-1β	Interleukin-1 beta
IL-1RA	Interleukin-1 receptor antagonist
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
kD	kilodalton
M	molar concentration
M(Control)	non-stimulated macrophages
M(IFNγ)	macrophages stimulated with IFNγ

M(IL-4)	macrophages stimulated with IL-4
M1	classically activated macrophages
M2	alternatively activated macrophages
MACS	magnetic-activated cell sorting
M-CSF	macrophage colony-stimulating-factor
mg	milligram(s)
MgCl2	magnesium chloride
min	minute(s)
ml	milliliter(s)
mM	millimolar
mm	millimeter(s)
mRNA	messenger ribonucleic acid
N	equivalent concentration
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NG	normal glucose conditions (5mM)
ng	nanogram(s)
nm	nanometer(s)
P	probe
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pM	picomolar
R	reverse
rcf	relative centrifugal force
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription real-time polymerase chain reaction
s	second(s)
SSC	side scatter
TAE	tris/Acetate/EDTA-buffer
TNFα	Tumor necrosis factor alpha
x g	centrifugal force/gravity

1 INTRODUCTION

1.1 Type 2 diabetes mellitus – a worldwide problem

1.1.1 Definition, prevalence and public health burden

Diabetes mellitus, being among the WHO's four main types of non-communicable diseases, is a heterogeneous group of metabolic disorders sharing the common ground of chronic hyperglycemia[1, 2]. The pathogenic process leading to hyperglycemia, defined as abnormally high blood glucose levels (fasting blood glucose ≥ 5.5 mM or 100 mg/dl), and including pathological insulin secretion or action and reduced glucose utilization, is used to distinguish between the distinct types of diabetes mellitus[2, 3]. The common classification differentiates between two main categories: type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus is caused by an autoimmune reaction against the insulin-producing beta cells of the pancreas, hereby leading to their destruction and total insulin-deficiency[2]. In contrast to this, the etiological background of type 2 diabetes mellitus is thought to be the result of complex interactions between genetic predisposition and environmental or lifestyle factors (Table 1)[2, 4]. It can range from insulin resistance with a relative insulin deficiency to a predominantly impaired insulin secretion or excessive hepatic glucose production[2]. Notably, the prevalence of type 2 diabetes mellitus is rising faster than the prevalence of type 1 diabetes mellitus, emphasizing the impact of obesity, reduced activity levels and the ageing of the population on the pathogenesis of type 2 diabetes mellitus[2]. An estimation for all age-groups worldwide made by the International Diabetes Federation projected an increase from 425 million affected people in 2017 to 629 million in 2045[5]. Additionally, it is estimated that 50% of individuals with diabetes may be undiagnosed[5]. These numbers mirror the steadily growing public health burden of diabetes mellitus: the International Diabetes Federation states in its 'Global Diabetes Plan 2011-2021' that USD 465 billion were spent worldwide on diabetes in 2011, with a predicted increase in costs to USD 595 billion by 2030[6]. However, 4 years later in 2015, it was estimated that this number had already skyrocketed up to an 'unsustainable' number of USD 673 billion corresponding to a total of 12% of overall health care expenditures worldwide[6].

Table 1. Risk factors for the development of type 2 diabetes mellitus. IFG: impaired fasting glucose; IGT: impaired glucose tolerance. Modified from American Diabetes Association. Classification and diagnosis of diabetes. Sec. 2. In Standards of Medical Care in Diabetes. Diabetes Care 2018;41(Suppl. 1): S13–S27

Risk factors for type 2 diabetes mellitus
family history of diabetes
history of gestational diabetes mellitus, cardiovascular disease
ethnicity (African American, Latino, Native American, Asian American, Pacific Islander)
previously identified with IFG, IGT or a hemoglobin A_{1c} of 5.7 – 6.4 %
overweight or obese (BMI ≥ 25 kg/m^2)
physical inactivity
hypertension (blood pressure ≥ 140/90 mmHg)
HDL cholesterol level < 35 mg/dl and/or a triglyceride level > 250 mg/dl
polycystic ovary syndrome or acanthosis nigricans

Notwithstanding the exact etiology, the early stages of diabetes mellitus are characterized by progressive impairment of glucose homeostasis[2, 3]. Insulin resistance, i. e. the inability of insulin to act efficaciously on the organs aimed at – like muscle, liver and fat – leads via ineffective glucose utilization to a circulus vitiosus by upregulating liver glucose production[2]. Pancreatic beta cells compensate the beginning insulin resistance with elevated insulin secretion, which leads to a hyperinsulinemic state[2]. This situation, however, is hard to maintain by the pancreatic islets, resulting at first in postprandial hyperglycemia and later in fasting hyperglycemia and beta cell failure[2]. An essential aspect of the disease is the fact that even during this first subclinical phase, complications caused by low-grade chronic inflammation, such as atherosclerosis, are already present[7].
Clinically, hyperglycemia is noted by the patients via polyuria (osmotic diuresis), polydipsia, fatigue, weakness and blurry vision[3]. Moreover, frequent superficial infections, e. g. fungal skin infections and impaired healing of wounds after minor trauma can be observed[2]. In order to be able to make the diagnosis of diabetes mellitus, at least one of the depicted criteria in Table 2 has to be met[4].

Table 2. Criteria for the diagnosis of diabetes mellitus. It is recommended to repeat the test or run a different test to confirm the results. Adapted from American Diabetes Association. Classification and diagnosis of diabetes. Sec. 2. In Standards of Medical Care in Diabetes. Diabetes Care 2018;41(Suppl. 1): S13–S27

Diagnosis criteria of diabetes mellitus
symptoms of hyperglycemia or hyperglycemic crisis plus random blood glucose concentration ≥ 200 mg/dl *or*
fasting plasma glucose (no caloric intake for ≥ 8 h) ≥ 126 mg/dl *or*
hemoglobin A_{1c} ≥ 6,5% *or*
plasma glucose ≥ 200 mg/dl after 2 hours during an (75 g) oral glucose tolerance test

1.1.2 Hyperglycemia is a key feature of diabetes

As a key feature for diabetes, hyperglycemia is said to be the cause of long-term damage concerning a number of organs, such as the eyes, nerves, kidneys and blood vessels[8]. The relation of hyperglycemia to both micro- and macroangiopathy is well supported and of crucial importance, with diabetes mellitus being the predominant cause of end-stage renal disease, adult blindness and non-traumatic lower extremity amputations in the US and atherosclerosis-related cardiovascular disease remaining the predominant cause of death in the world[1, 2]. Several big clinical trials, e. g. the DCCT and UKPDS, suggest that the microvascular complications observed, i. e. retinopathy, neuropathy and nephropathy, can directly be linked to chronic hyperglycemia[9, 10]. In the above-mentioned trials, it was found that the duration and degree of hyperglycemia correlates with the extent of microvascular complications and that intensive glycemic control is beneficial in all forms of diabetes mellitus[9, 10]. Each percentage point reduction in HbA_{1c} was associated with a 35% reduction in microvascular complications[9, 10]. In contrast to these findings directly correlated to hyperglycemia, macrovascular complications, like coronary heart disease, peripheral artery disease and cerebrovascular disease, were shown to be strongly influenced by various other factors, such as hypertension and dyslipidemia[11].

However, not all individuals with diabetes mellitus develop the above-mentioned hyperglycemia-related complications or there can be a long asymptomatic period with complications only being detectable after the first decade of the disease[2].
Although being a crucial etiologic factor in the development of diabetes-associated complications, the underlying mechanisms of chronic hyperglycemia causing this variety of pathologic circumstances remain to be elucidated.
Over the last decades, several concepts to explain endothelial damage have been suggested. A commonly accepted theory states that chronic hyperglycemia causes increased levels of reactive oxygen species via an excessive activation of the polyol pathway[12]. Another hypothesis suggests that hyperglycemia results in sped up formation of advanced glycosylation end products (AGEs)[13]. These products, irreversibly cross-linked and senescent, are able to create oxidative stress reactions causing endothelial cell damage through their interaction with their respective receptor (RAGE)[13, 14]. Furthermore, vascular diacylglycerol accumulation and subsequent protein kinase C activation elicits endothelial cell dysfunction and increased permeability as well as synthesis and fibrosis of extracellular matrix[15]. The last two mechanisms are both believed to induce the expression of vascular endothelial growth factor (VEGF), with effects on vascular permeability, neovascularization and the mediation of monocytes recruitment[14, 15].

1.1.3 Subclinical chronic systemic inflammation

Besides the above-mentioned mechanisms, another link between hyperglycemia and the clinical complications of type 2 diabetes mellitus being thoroughly investigated is subclinical chronic systemic inflammation[16].
Typically, in acute inflammation, the inducing agent triggers the release of various immunomodulatory mediators, including chemo- and cytokines secreted by tissue macrophages and mast cells[17-19]. Those cytokines, the ‘classic players’ here being TNFα, IL-β and IL-6, are responsible for the initiation and coordination of an immune response by activating the endothelium as well as leukocytes and facilitating their extravasation[17]. Those local inflammatory processes are most often accompanied by a systemic response, including fever and the triggering of an acute-phase reaction[17]. This reaction is characterized by the release of hepatocyte-produced agents, such as C-reactive protein, which is able to opsonize pathogens and initiate the complement cascade[17, 20]. If this first innate immune response is not able to eliminate the damaging agent, the transition to the adaptive immune system is conducted via antigen presenting cells[17, 21]. Thus, a successful acute inflammatory response enables the organism to reconstitute functionality and homeostasis[17, 18].

In contrast to that, the low-grade chronic inflammation that has strongly been associated with diabetes mellitus, age, smoking and obesity lacks Celsus’ and Galen’s classic cardinal signs of inflammation – *rubor*, *calor*, *dolor*, *tumor* and *functio laesa*[22, 23]. Moreover, it is characterized by an only moderate (2-3 fold) elevation and persistence of systemic serum concentrations of the cytokines designated above (TNFα, IL-1β, IL-6), as well as their respective antagonists, like IL-1RA[24]. Unlike acute inflammation which is induced by exogenous factors like trauma or infection, subclinical chronic inflammation can be caused by a pathologic reaction to a number of endogenous factors[25]. Due to the proximity of metabolic and immune pathways, metabolic factors like advanced glycation end products are reported to have pro-inflammatory features with their circulating indicators correlating with markers of inflammation[26]. Moreover, the interaction with their receptor RAGE is able to promote

inflammatory pathways hence evoking the dismantling of tissues[27]. Modified low-density lipoproteins, whose uptake into macrophages is conducted via scavenger receptors, do not underlie a negative feedback regulation, which contributes to uncontrolled uptake and therefore the formation of foam cells[28]. Additionally, more and more evidence emerges identifying the immune cells of white adipose tissue to play a crucial role in the initiation of low-grade inflammation[29] and obesity-related insulin resistance[30]. Hyperglycemia itself was associated with increased serum levels of C-reactive protein and monocyte chemoattractant protein-1 (MCP-1), both being indicators of inflammation, and worse clinical outcomes in patients with non-ST elevation ACS[31]. It has also been observed that acute hyperglycemia is able to induce an increase in the plasma concentrations of TNFα and IL-6 in healthy controls and an even more pronounced elevation in subjects with impaired glucose tolerance[32].

Whether insulin resistance and inflammation are causally linked remains the subject matter of current investigation. Whereas the role of IL-6 in insulin resistance is still a controversial one[23], the part of TNFα has been meticulously reviewed: in 1993, Hotamisligil et al. were the first to relate the concept of inflammation to metabolic disorders by demonstrating that TNFα expression in adipocytes of obese animals was significantly elevated[33]. Conversely, the neutralization of TNFα in those animals resulted in an increase in insulin sensitivity[33]. Since then, several inflammatory pathways leading to insulin resistance have been discussed. The autophosphorylation of the stimulated insulin receptor and the phosphorylation of members of the insulin receptor substrate family (IRS) are able to initiate a cascade of downstream signaling[34]. TNFα induces inhibitory phosphorylation of IRS, which in term impedes said downstream signaling and therefore insulin action[34]. Various other inflammatory signaling pathways compromising the function of insulin have been identified – inter alia the activation of serine/threonine kinases like c-Jun N-terminal kinase (JNK) and inhibitor of NF-κB kinase (IKKβ) which are able to control the transcription of multiple inflammatory genes by AP-1 and NF-κB[35]. In hyperglycemic conditions an increased activation of NF-κB was found, resulting in an elevated production of pro-inflammatory cytokines[36, 37]. Not only extracellular transmitters, such as cytokines, but also intracellular mediators, such as endoplasmic reticulum (ER) stress caused by a functional overload through hyperlipidemia or an elevated production of reactive oxygen species (ROS) by mitochondria, are capable of provoking said inflammatory pathways[38]. Apart from this, endothelial injury caused by ROS might entice macrophages, hence deteriorating the local inflammation and contributing to endothelial dysfunction which is a predecessor of the micro- and macrovascular complications of type 2 diabetes[39].

1.2 Monocytes and macrophages in diabetes and hyperglycemic conditions

1.2.1 Monocytes and macrophages are key cells for innate immunity

Macrophages and their monocyte precursors form the basis of the mononuclear-phagocyte system and are primary innate immune cells that – in their role as regulators and effectors – exert a multitude of functions within the immune system[40]. Monocytes originate from hematopoietic stem cells located in the bone marrow and share a common myeloid precursor cell with neutrophils (Figure 1)[19].

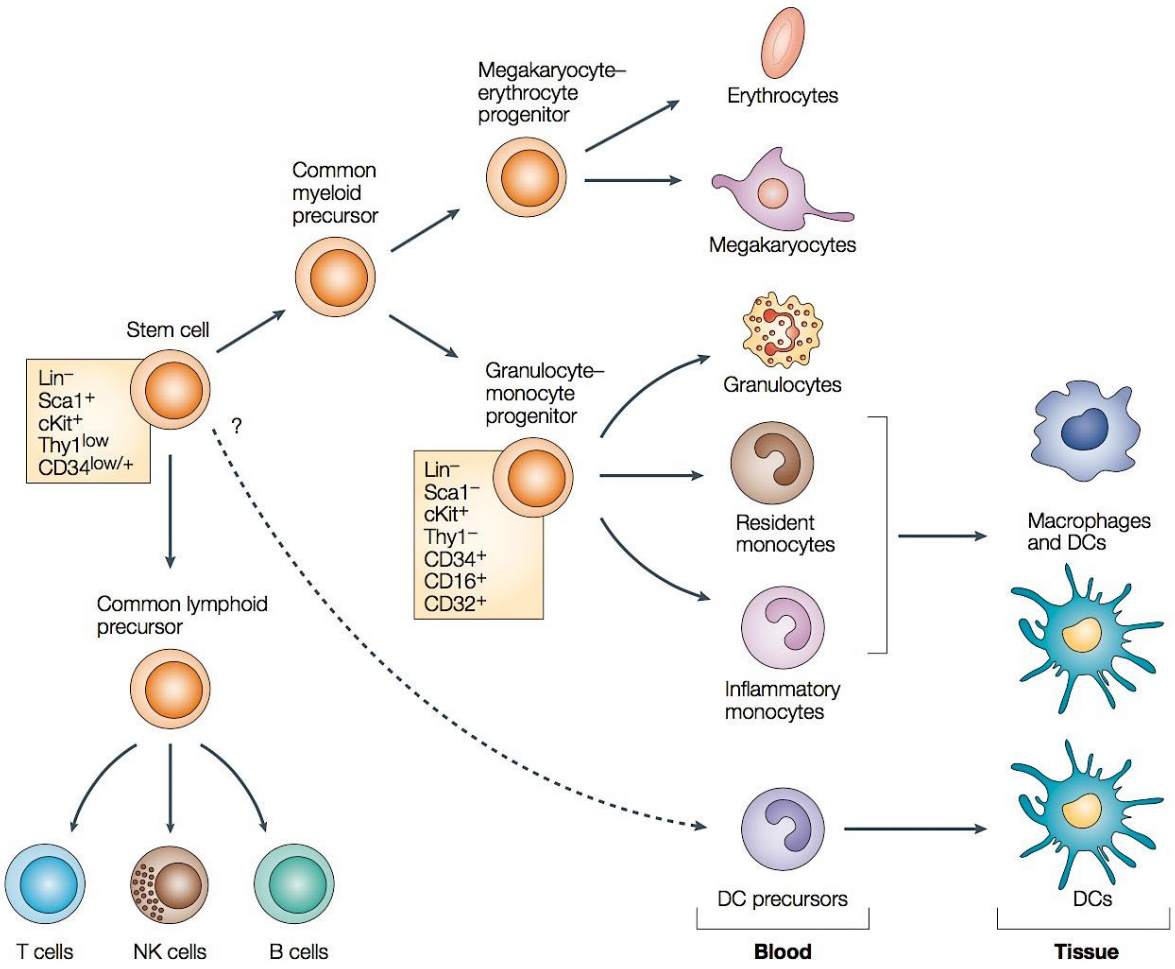


Figure 1. Differentiation and maturation of hematopoietic antigen-presenting cells. Originating from stem cells in the bone marrow, common myeloid precursor cells differentiate into granulocyte-monocyte progenitors. Under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF or CSF-1), those progenitors mature to monocytes which hereafter are able to enter into the blood[41]. Responding to a broad range of local cytokine stimuli, circulating monocytes can be recruited to peripheral tissues, where they perform the differentiation to dendritic cells or macrophages according to the prevailing microenvironment[19]. DC: dendritic cell; NK: natural killer. Extracted from Imhof and Aurrand-Lions. Adhesion mechanisms regulating the migration of monocytes. Nat Rev Immunol[19]. Copyright 2004, with permission from Springer Nature.

They exit from their site of origin in the bone marrow to peripheral blood circulation, where they stay for a few days[42]. Quantitatively, they account for approximately 5-10% of peripheral leukocytes in humans[42]. They form a group with considerate heterogeneity regarding size, surface markers, nuclear morphology and granularity[42]. However, as a common feature and a marker for identification, they all express CD14 which forms part of the lipopolysaccharide receptor[42]. The varying expression of CD14 and CD16 revealed the possible division into subpopulations, with $CD14^{hi}CD16^{-}$ monocytes – first discovered and therefore being called 'classic' monocytes – and $CD14^{+}CD16^{+}$ monocytes which express higher levels of MHC class II molecules[43]. $CD14^{hi}$ monocytes express a variety of genes involved in tissue repair and immune response, demonstrate an elevated production of cytokines in response to LPS and have increased transmigratory activity in response to MCP-1 (high expression of CCR2), that is why they are considered to play a role in chronic inflammation[42, 44, 45]. In contrast to this, $CD14^{+}CD16^{+}$ monocytes elicit limited phagocytic capacity and do not express CCR2[43, 44]. In response to LPS, they generate higher amounts of TNF and IL-6 than $CD14^{hi}$ monocytes, leading to the suggestion that they could be a subpopulation of senescent monocytes with pro-inflammatory capabilities, even supporting pathological conditions such as atherosclerosis and sepsis[46-48]. Since this discovery in 1989, the list of other antigen markers to distinguish between monocyte subpopulations is growing, all differing in phenotypic characteristics such as phagocytic capacity and stimulatory activity[43, 49].

As a response to a broad range of stimuli, be it immunological, metabolic or inflammatory, circulating monocytes can be triggered to migrate into tissues, where the differentiation to specific phenotypes of tissue-resident macrophages or dendritic cells occurs[41]. Depending on the stimulus of the microenvironment, they then contribute to host defense, maintenance of tissue homeostasis, repair and remodeling after inflammation[50]. The above-mentioned heterogeneity is reflected by the spectrum of functions according to the anatomical location and leads to their diverse terminology (Table 3)[21, 42].

The flexibility, strong dynamics and ability of macrophages to respond to both physiological and pathological stimuli stems, inter alia, from the wide range of receptors expressed intracellularly and on their surface[40]. The recognition of pathogens and their subsequent phagocytosis is mediated by integrins and Fc receptors[51]. Additionally, the group of non-Toll-like receptors, including lectins and the family of scavenger receptors contribute to phago- and endocytosis[52]. In contrast to them, Toll-like receptors function as sensing receptors for pathogens[53]. In cooperation with scavenger receptors and lectins, they are able to induce the NF-κB transcription factor family which, in term, regulates the production of pro-inflammatory mediators[40]. This pathway, however, is only one among many other signaling pathways within macrophages contributing to an immune response[21, 41].

Table 3. Selection of populations of tissue-resident macrophages according to their location[21, 42].

Location	Population	Functions
bone	osteoclasts	resorption and remodelling of bone
brain	microglia	antigen presentation and phagocytosis
connective tissue	histiocytes	antigen presentation and phagocytosis
gastrointestinal tract, lamina propria	intestinal macrophages	high phagocytic and bactericidal activity, tolerance of food antigens and microbiota
liver	Kupffer cells	clearance of pathogens and toxins
lung	alveolar macrophages	clearance of microorganisms, allergens, environmental particles
lymph node	subcapsular sinus macrophages and medullary macrophages	antigen capture and presentation to B cells
skin, epidermal layer	Langerhans cells	phagocytosis and antigen presentation
spleen	red pulp macrophages	clearance of senescent red blood cells
	white pulp macrophages, tingible-body macrophages	clearance of apoptotic lymphocytes
	marginal zone macrophages	clearance of blood-borne pathogens
thymus	thymic macrophages	clearance of apoptotic cells

1.2.2 Concepts of macrophage activation

The found diversity of receptors on the surface of macrophages is expression of their exceptional plasticity, i. e. their ability to polarize into different subtypes (Figure 2)[21]. It enables them to preserve tissue homeostasis and organ function, or to fuel disease pathogenesis and maintain inflammatory processes, as can be seen in metabolic inflammation-related diseases, e. g. fatty liver disease, atherosclerosis and diabetes[34].

Over the last years, several terms have been used to describe the state of activation or ‘polarization’ of macrophages as an attempt to classify the distinct patterns of gene and protein expression that result from cytokine-, growth factor-, or pathogen-stimuli[54]. A bisectional nomenclature – namely the use of the dichotomous terms M1 and M2 or ‘classical’ and ‘alternative’ – inspired by the one used for the lymphocyte system, has been proposed in the early 1990s, when the differential effects of IFNγ, LPS and IL-4 on macrophage gene expression were described[54-56]. Inflammatory stimuli, such as LPS and IFNγ induce a pro-inflammatory phenotype (M1 or classically activated) that promotes an adaptive immune response and has microbicidal properties[57]. They release a high amount of pro-inflammatory cytokines, e. g. IL-1, IL-6, or the pro-apoptotic TNFα, and increase the concentrations of oxygen radicals and superoxide

anions[57, 58]. Another characteristic is the high expression of MHC I and MHC II antigens and production of complement factors that facilitate opsonisation and complement-mediated phagocytosis[59]. One main inducer of a M1 phenotype gene expression profile is the transcription factor NF-κB[60].
On the contrary, the diversity of M2, or alternatively activated macrophages, is reflected by their functional spectrum regarding tissue repair, phagocytosis and increased expression of scavenger receptors[55]. They produce important cytokines for the resolution of inflammation, e. g. IL-1RA and IL-10[61]. Additionally, they express a variety of scavenger receptors, e. g. CD163, that enhance their endocytic and phagocytic potential and are essential for the clearance of cellular debris[62].

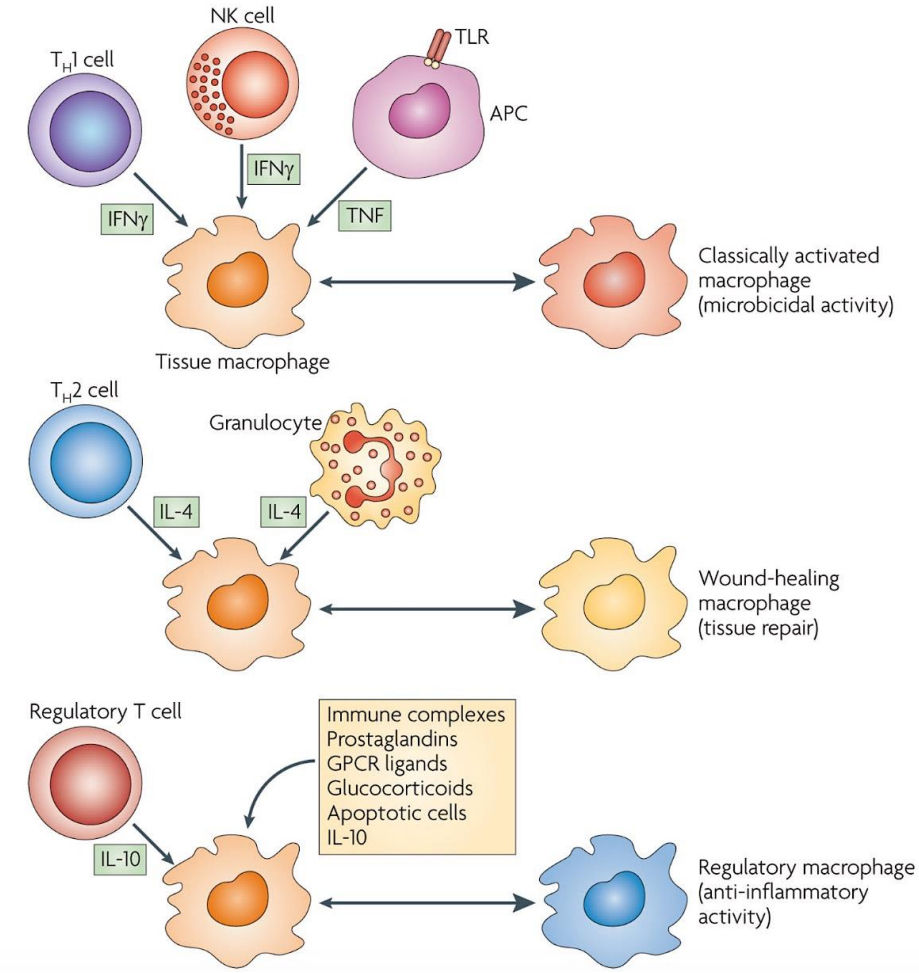


Figure 2. Differential macrophage physiologies caused by the stimulation of cytokines and microenvironmental stimuli. Tissue macrophages receiving IFNγ or TNF stimuli produced by T_H1 cells during an adaptive immune response, NK cells during an innate immune response, or APCs give rise to classically activated macrophages with microbicidal activity. In contrast to that, alternatively activated macrophages responsible for tissue repair develop in response to IL-4 which can be secreted by T_H2 cells during an adaptive immune response or granulocytes during an innate immune response. Regulatory macrophages that are said to have anti-inflammatory activity are created in response to various stimuli, exemplarily depicted in the scheme. APC: antigen-presenting cell; GPCR: G-protein coupled receptor; NK: natural killer; $T_H1/2$: T helper 1/2 cell; TLR: Toll-like receptor. Reproduced from Mosser and Edwards. Exploring the full spectrum of macrophage activation. Nat Rev Immunol[21]. Copyright 2008, with permission from Springer Nature.

The M1/M2 set of nomenclature was soon expanded (M2a, M2b, etc.) by the notion that the two extremes M1 and M2 are not able to do justice to the multitude of activation scenarios and that it should rather be described as a dynamic continuum of functional states[63]. Therefore, it was proposed to use a nomenclature referring to the conditions used for stimulation, i. e. M(IFNγ), M(IL-4) and others[63]. Moreover, in order to unify experimental standards, a collection of markers for functional subdivisions is displayed in Figure 3[25]. Those distinct expression patterns reflect a molecular signature which is alluding to the functional property of the various macrophage populations[25]. Exemplarily, IL-4 was identified to induce a macrophage profile involved in the remodelling of tissues by releasing matrix metalloproteinases and other extracellular components[61], glucocorticoids were demonstrated to be the driving force for tissue homeostasis by promoting endocytic and phagocytic activity towards unwanted-self-products[61], whereas LPS and IFNγ – being M1-driving stimuli – generate subpopulations of macrophages with high inflammatory cytokine production and bactericidal activity[25].

Notwithstanding the specific functional capability of the different subpopulations, the phenotype of macrophages can alter after a second stimulus, e. g. LPS, giving rise to the concept of macrophage plasticity[64]. It is commonly thought that – within a limited continuum – macrophages are capable of changing their activation state in response to a changing microenvironment[64]. It has been shown that adipose tissue macrophages can change their phenotype from alternatively activated macrophages to classically activated macrophages and therefore play a crucial role in adipose tissue remodeling and inflammation[65]. Vice versa, inflammatory macrophages can be converted to healing macrophages by stimulation with cytokines[66, 67].

The extensive heterogeneity of the monocyte/macrophage system still needs clarification for the specific functions of macrophage subsets, both in healthy tissues, as well as in pathology including diabetes and cardiovascular disorders, since macrophages became recently highly attractive as therapeutic targets[68-70].

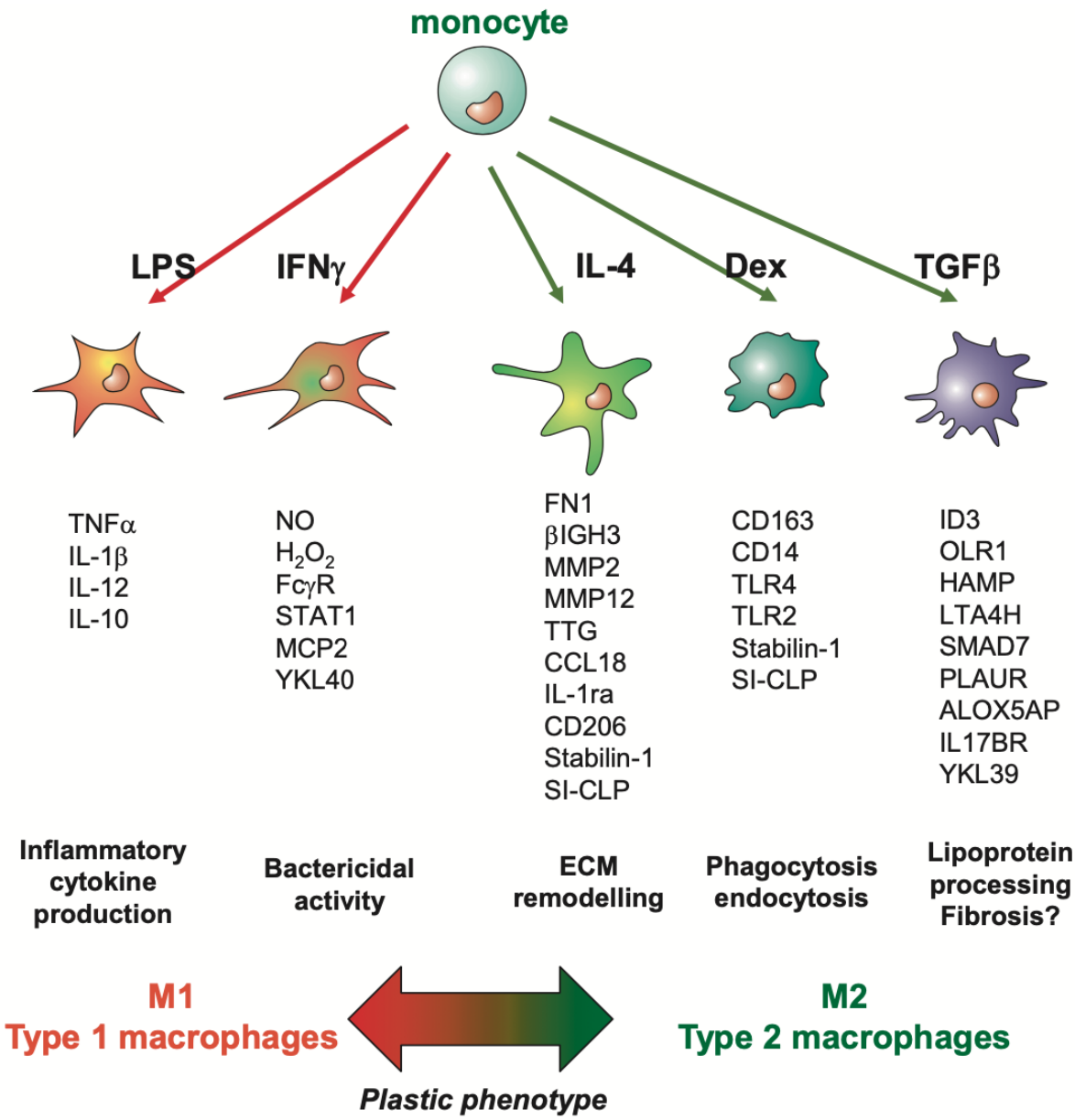


Figure 3. Description framework for macrophage activation based on the M1/M2 concept. The figure shows a suggestion for marker groups and activated genes that can be used to describe the activation state of macrophages and their functional capacity following the existing M1/M2 concept. Stimulating agents are LPS, IFNγ, IL-4, glucocorticoids (dexamethasone; Dex) and transforming growth factor β (TGFβ). Reproduced from Kzhyshkowska et al. Perspectives for Monocyte/Macrophage-Based Diagnostics of Chronic Inflammation. Transfus Med Hemother[25]. Copyright 2016, with permission from Karger Publishers.

1.2.3 Monocytes and macrophages in hyperglycemic conditions

Increasing evidence suggests that hyperglycemia is able to induce intracellular pro-inflammatory pathways, such as NF-κB, and therefore might be a trigger for monocytes and macrophages to be transformed towards a pro-inflammatory state[36, 37]. NF-κB is a transcription factor which administers the expression of a great range of genes

involved in inflammation in response to exo- and endogenous stimuli, such as LPS and TLR4 signaling[71]. In line with this finding is the observation of a significantly higher TLR2 and TLR4 expression on monocytes of newly diagnosed type 2 diabetics than on monocytes of healthy controls, thus adding to an inflammatory state[72]. Moreover, hyperglycemia-mediated activation of the RAGE-NF-κB pathway was shown to mediate enhanced myelopoesis and inflammation[73]. Hyperglycemia also induces activating histone code of the promoters of S100A9 and S100A12 genes in human macrophages resulting in elevated levels of S100A9 and S100A12 gene expression during monocyte to macrophage differentiation[74]. S100A9 and S100A12 are ligands for RAGE receptor on endothelial cells and vascular smooth muscle cells, and induce endothelial inflammation and enhanced permeability, thus contributing to vascular complications[75-77].

It has recently been demonstrated that high glucose concentrations are able to induce a phenotype resembling M1, which is reflected by the overexpression of cell surface markers associated with inflammation, like CD11c, and the increased production of mediators like inducible nitric oxide synthase[78]. This finding is supported by a clinical study showing that pro-inflammatory $CD11c^+$ macrophages are positively associated with systemic insulin resistance in obese patients[79].

More evidence of the shift towards a more pro-inflammatory polarization in macrophage subsets in type 2 diabetes mellitus has emerged from clinical studies with diabetic patients. An increased M1/M2 ratio was observed in diabetic individuals when compared to healthy controls, which could be traced back to a reduced number of M2-like macrophages ($CX3CR1^+CD206^+/CD163^+$)[80]. The imbalanced M1/M2 polarization ratio was directly correlated with waist circumference and HbA_{1c}[80]. Notably, among diabetics, a reduced level of M2-like macrophages and an elevated M1/M2 ratio were associated with microangiopathy, particularly nephropathy and retinopathy[80]. The pro-inflammatory condition of diabetes being in fact attributable to a defect in anti-inflammatory cells goes in line with the hypothesis that diabetes is a disease of 'impaired damage control', in which insufficient repair mechanisms deteriorate injury[81]. Interestingly, this described shift could even be shown in otherwise healthy pre-diabetic subjects[82]. In contrast to diabetic patients, a surplus of pro-inflammatory M1 ($CD68^+CCR2^+$) cells could be detected, reflecting an imbalance of M1/M2 polarization – a potential explanation for the increased cardiovascular risk in pre-diabetic individuals[83]. The expression level of CD68, the oxLDL scavenger receptor driving the cholesterol loading of macrophages and typically expressed on M1 macrophages, could directly be correlated with the level of HbA_{1c}[82]. Inversely, the expression of anti-inflammatory macrophage genes within skeletal muscle probes of obese patients after one year of prescribed exercise correlates with an amelioration of insulin resistance[84].

Considering diabetes as a disease of dysfunctional control of tissue damage, these findings are in accordance with the hypothesis that M2 macrophages are able to control or abrogate inflammatory processes and play a pivotal role in tissue mending and remodeling[25, 61]. Proposed receptors to play an important role in implementing anti-inflammatory actions are some representatives of the vast group of scavenger receptors[85].

1.3 Scavenger receptor CD163: function and role in inflammation

One example is the monocyte- and macrophage-specific scavenger receptor CD163, also known as hemoglobin scavenger receptor (HbSR)[86], M130[87] and RM3/1[88]. It was only in 2001, that its function was identified: the mediation of the internalization and degradation of hemoglobin-haptoglobin complexes which are physiologically built due to intravascular hemolysis[86]. CD163, a 130kDa glycoprotein, belongs to the scavenger receptor cysteine-rich superfamily B, consisting of 9 extracellular scavenger receptor cysteine rich (SRCR) domains, a transmembrane region and a short C-terminal cytoplasmatic tail[87, 89]. Solely the SRCR domains 6 and 7 are linked by a proline-serine-threonine (PST) polypetide, another PST domain links the last SRCR domain with the transmembrane part[87]. Various isoforms have been identified, mostly differing due to alternative splicing of said cytoplasmatic tail[89].

1.3.1 Expression and regulation

High expression of CD163 is described for most mature tissue macrophages, with the most elevated ratio being observed in red pulp macrophages of the spleen, Kupffer cells in the liver, alveolar and interstitial macrophages in the lung, histiocytes of the skin, as well as, in lower rates, in placenta macrophages, meningeal macrophages in the central nervous system and macrophages in lymph nodes and other lymphatic tissues, like the tonsils and the thymus[89-91]. Information regarding CD163 expression on blood monocytes varies from near absence[89] to an approximate percentage of 15-30% of freshly isolated monocytes[92] leading to the suggestion that CD163 might serve as a macrophage marker which indicates their level of maturation[93]. However, more recent studies comparing different antibodies used previously by multiple groups revealed the dependency of the results on the accessibility of epitopes and the presence of extracellular calcium when determining monocytic CD163 expression[94]. By using an antibody which accesses the receptor towards its N-terminal end and therefore minimizing steric hindrance, it can be envisioned that almost all human peripheral blood monocytes express CD163[94, 95].

Table 4. Regulation of CD163 expression by various stimuli. TGF: transforming growth factor; PMA: 12-O-tetradecanoylphorbol-13-acetat

Stimulation	Regulation of CD163	References
glucocorticoids	upregulation	90, 96-98
IL-6	upregulation	96
IL-10	upregulation	90, 96, 99
CXCL4	downregulation	100
GM-CSF	downregulation	96
IFNγ	downregulation	88, 96
IL-4	downregulation/ no regulation	90, 95, 96
PMA	downregulation	101
TGFβ	downregulation	102
TNFα	downregulation	96

A large number of *in vitro*-studies have identified various pro- and anti-inflammatory mediators to tightly regulate the expression of CD163 (Table 4). Administration of acute-phase mediator interleukin-6 and glucocorticoids massively upregulated CD163 expression[90, 96]. The administration of the glucocorticoid prednylidene into the circulation of human test subjects caused an elevation of $CD163^+$ circulating peripheral blood monocytes[98]. A similar glucocorticoid-induced upregulation of CD163 expression on isolated human monocytes could also be shown *in vitro*[97]. The binding of hemoglobin-haptoglobin complexes evoked the secretion of the anti-inflammatory cytokine IL-10, which in turn stimulated CD163 expression in terms of a positive feedback loop[103]. Gene-chip technology revealed that CD163 expression showed the highest upregulation to an IL-10 stimulus among a variety of tested genes[99]. Conversely, a decreased expression of CD163 was found when stimulating with the pro-inflammatory cytokines IFNγ and TNFα[88, 96]. Interestingly, the anti-inflammatory cytokines IL-4 and IL-13 failed to increase its expression[90, 95, 96]. Numerous other cytokines and treatments are reported to suppress the expression of CD163, among which are the granulocyte/macrophage colony stimulating factor (GM-CSF), chemokine ligand 4 (CXCL4), transforming growth factor β (TGFβ) and 12-O-tetradecanoylphorbol-13-acetat (PMA)[96, 100, 102].
Stimulation with lipopolysaccharide (LPS) was long believed to downregulate CD163 expression as well[90]. However, growing evidence suggests that it leads to the shedding of the CD163-ectodomain from the cell surface, thus building the soluble form of CD163[90].

1.3.2 Soluble CD163

Apart from the membrane-bound receptor a soluble form of CD163 (sCD163) exists which is generated by active post-translational ectodomain-shedding from the cell surface[104]. sCD163 is formed by proteolytic cleavage and consists of over 94% of the original receptor suggesting a splitting site near the transmembrane region[105]. Growing evidence alludes that the shedding is mediated by metalloproteinases, with Etzerodt et al. proposing a dissection by a disintegrin and metalloproteinase 17/TNFα-cleaving enzyme (ADAM 17/TACE) as responsible mechanism[106-109]. The shedding seems to be physiological, since relatively high amounts of sCD163 (0.7-3.9 mg/l) are found in plasma and various tissue fluids of healthy subjects[86, 104, 110].
In inflammatory conditions, however, even higher plasma-levels can be detected: *in vitro* studies identified the stimulation with LPS or PMA, as well as oxidative stress, the crosslinking of the Fcγ-receptor and thrombin as factors inducing the shedding of CD163[106, 108, 110-113]. Another mechanism causative of the shedding from monocytic cells is the activation of cell surface Toll-like receptor signaling, which suggests that CD163 plays a role in the acute innate immune response to extracellular pathogens[108]. Moreover, a clinical study observed a rapid increase in soluble CD163 in the plasma of human test subjects in response to intravenously administered LPS[106]. Interestingly, plasma-levels of TNFα, another one of the multiple substrates of ADAM17, are increased by similar conditions[114]. The finding that both TNFα- and sCD163-levels are elevated in inflammatory disease, such as sepsis or rheumatoid arthritis, could indicate a concurrent release of TNFα and CD163[107, 109, 115].

1.3.3 Functional role

To date, several functions of the scavenger receptor CD163 have been identified and thoroughly reviewed[89, 116, 117]. The most comprehensively characterized function is its homeostatic role in the scavenging of hemoglobin-haptoglobin (Hb-Hp) complexes which form due to intravascular hemolysis[86].

Haptoglobin polymorphisms and their clinical significance

Haptoglobin, the protein binding to free extracorpuscular hemoglobin, is a glycoprotein that is physiologically abundantly present in plasma[118]. The observed levels range considerably from 0.3-3.0 mg/ml, but stay reasonably constant in one individual[118]. Thus, the decrease of the individual level can be a clinical indicator of red blood cell destruction[119]. The major share of the degradation of red blood cells happens in the spleen, bone marrow and liver, i. e. the extravascular compartment. Intravascular hemolysis, however, physiologically occurs for 10-20% of erythrocytes and increases considerably in pathological conditions, e. g. hemoglobinopathies or inflammation[120]. In the case of inflammation, the physiological levels of haptoglobin, which is a hepatocyte-produced acute-phase protein, are induced 2-5-fold by the acute-phase mediators IL-1 and IL-6 and can be released locally from storage granules by active neutrophils[121-123]. The two known allelic variants of haptoglobin, Hp1 and Hp2, with Hp2 only found in humans, lead to three possible phenotypes, Hp1-1, Hp1-2 and Hp2-2[118]. Haptoglobin consists of two different polypeptide chains, the α-chain and the β-chain[124]. The polymorphism confirmed by electron microscopy arises from differing α-chains (α^1 and α^2) which create haptoglobin dimers in subjects with Hp1-1, linear polymers in Hp1-2 individuals and cyclic multimers in humans with two Hp2 alleles (Figure 4)[124, 125]. Geographical differences for the various phenotypes have been described with a prevalence of approximately 16% for Hp1-1, 48% for Hp1-2 and 36% for Hp2-2 in northwestern Europe[125].

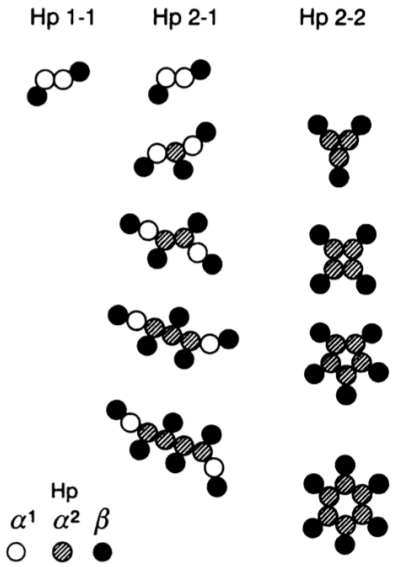


Figure 4. Differences in the structure of haptoglobin variants in humans. The molecular mass ranges from 85 kDa in Hp1-1 to up to 900 kDa in the biggest Hp2-2 molecules. Adapted from Langlois and Delanghe. Biological and clinical significance of haptoglobin polymorphism in humans. Clin Chem[125]. Copyright 1996, with permission from Oxford University Press.

Regarding the functional properties of the different haptoglobin variants, not only differences in function, but also in the involvement in pathological conditions have been observed: for instance, the clearance and antioxidant capacity of haptoglobin, resulting from the binding to hemoglobin and thus mitigating oxidative injury in the form of oxidation of lipid and protein substrates, was reported to be superior in Hp1-1 individuals than in Hp2-2 individuals[126, 127]. Not only antioxidant, but also anti-inflammatory responses have been examined to differ between the haptoglobin variants: the release of IL-6 and IL-10 in response to the binding of Hb-Hp1-1 complexes to CD163 was markedly increased compared to the binding of Hb-Hp2-2 complexes[128]. Regarding the clinical significance of the different haptoglobin variants, it has been shown in several longitudinal prospective studies that Hp2-2 phenotype is an independent risk factor for the development of cardiovascular disease in diabetic individuals in comparison with the homozygous Hp1 variant[118, 129]. As a possible mechanism providing an explanation for the proneness to vascular complications, it has been demonstrated that CD163 is downregulated in macrophages of atherosclerotic plaques of diabetic patients with the Hp2-2 genotype, indicating an compromised hemoglobin clearing capability[130].

Scavenging of hemoglobin-haptoglobin complexes

By the formation of hemoglobin-haptoglobin complexes, the endothelium can be protected from the toxicity of free hemoglobin, i. e. the production of oxygen radicals and the scavenging of nitric oxide (NO)[120]. Moreover, the accumulation of free hemoglobin in the kidneys – potentially leading to renal damage – can be prevented[120]. Hb-Hp complexes form a unique epitope which is recognized by CD163[131]. In the presence of calcium, they bind with high affinity to membrane-bound CD163 of both blood monocytes[132] and tissue macrophages, which leads to their internalization and degradation via heme oxygenase-1[86, 133, 134]. Several groups have demonstrated that this binding process leads to a release of the anti-inflammatory cytokine IL-10, which – apart from heme itself – acts as an inducing factor of the cytoprotective and anti-inflammatory heme oxygenase-1[103, 135] (Figure 5). The emerging heme metabolites promote anti-inflammatory pathways: bilirubin and biliverdin possess antioxidative properties, CO is able to induce antioxidative proteins and Fe^{2+} increases ferritin levels, which serves as cytoprotection against oxidative stress[103, 136, 137].

As macrophage polarization is highly dynamic and dependent on the microenvironment, it has been observed that the exposure to Hb-Hp complexes drives macrophages towards an alternatively activated phenotype termed M(Hb), HA-mac or Mhem[138-140]. This subtype has been located in atherosclerotic plaques, especially on sites of intraplaque hemorrhage[141]. Characteristics are a lower production of pro-inflammatory mediators in comparison to classically activated M1 and fewer cholesterol storage – therefore it is thought to elicit an anti-atherogenic role[142]. Besides these atheroprotective mechanisms, the CD163-mediated Hb-Hp endocytosis may also contribute to an efficient recycling of iron, preventing excessive loss and making it unavailable for pathogens like *Staphylococcus aureus*[108].

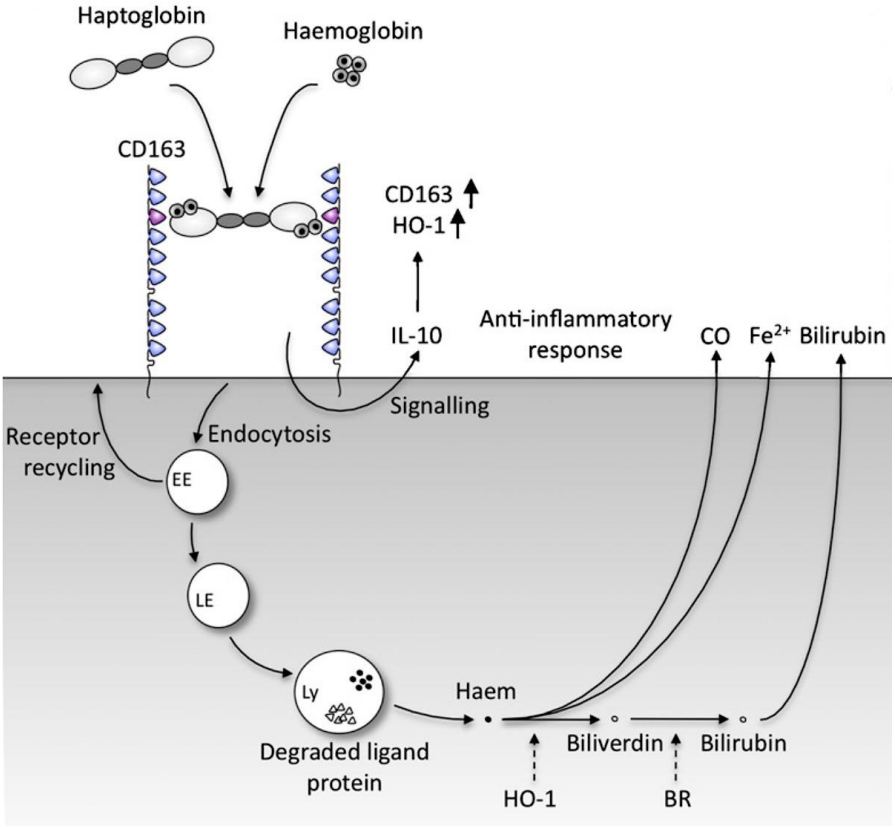


Figure 5. Mechanism of scavenging hemoglobin-haptoglobin complexes. CD163 recognizes Hb-Hp complexes at its SRCR domain 3, which leads to the uptake of the ligand-receptor complex. Whereas the receptor is recycled to the cell surface, Hb-Hp complexes continue the endocytic pathway from early endosomes to lysosomes, where the protein components are dissolved. In the cytosol, heme oxygenase-1 (HO-1) dissects heme into the anti-inflammatory metabolites biliverdin, Fe^{2+} and CO. Biliverdin reductase (BR) converts biliverdin to bilirubin which is released once again into the circulation to be transported to further degradation in the liver. EE: early endosome; LE: late endosome; Ly: lysosome. Adapted from Van Gorp et al. Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. Molecular Immunology[89]. Copyright 2010, with permission from Elsevier.

However, the above-described hypothesis of a $CD163^+$ macrophage population with anti-inflammatory activity is confronted by accumulating evidence questioning this specific role: the analysis of the exact location of M(Hb) in atherosclerotic plaques revealed that they are present in areas with a high rate of neovascularization and microvascular leakage[143]. A recent study showed that Hb-Hp complex ingestion by $CD163^+$ macrophages led to the induction of HIF-1α via Fe^{2+} depletion[143]. In turn, the secretion of VEGF-A mediated angiogenesis, vessel permeability and inflammation[143]. These findings indicate that alternatively activated macrophages, despite their anti-inflammatory profile, could contribute to plaque destabilization as well as progression and consequently could be able to pronounce the detrimental effects of atherosclerosis[138, 143]. Other evidence even suggested a role of CD163 in the pro-inflammatory activation of macrophages: the cross-linking of cell-surface CD163 was shown to trigger protein-kinase C (PKC)- and casein-kinase (CK II)-dependent macrophage activation, which resulted in the release of pro-inflammatory cytokines, such as IL-1β and IL-6[90, 144, 145]. Moreover, it was discovered that – in the case of an exhaustion of haptoglobin – CD163 is able to scavenge uncomplexed free

hemoglobin[146]. Under these circumstances, unbound hemoglobin induces the shedding of the membrane-bound receptor and consequently forms a complex with sCD163[146]. This complex, in turn, was described to recruit IgG and stimulate the release of pro-inflammatory cytokines from endothelial cells[146].
Adding to a possible role of CD163 in pro-inflammatory macrophage activation, alveolar spaces of severely infected COVID-19 lungs contained a large amount of $CD163^+$ macrophages as a sign of altered airway macrophage populations and correlating to diffuse alveolar damage and worse patient outcomes[147]. Moreover, serum levels of sCD163, as a marker of macrophage activation, were enhanced in COVID-19 patients[148, 149].

Other functions of CD163

Similar to other members of the scavenger receptor family, CD163 does not only act as a receptor for hemoglobin, but also possesses binding sites for other molecules: it was identified to play a role as an adhesion receptor in erythropoiesis and to operate as a receptor for TNF-like weak inducer of apoptosis (TWEAK)[150, 151]. Further, it was depicted as a pathogen receptor whose binding to bacteria elicits a pro-inflammatory response[152]. The secretion of pro-inflammatory cytokines like IL-1β, IL-6 or GM-CSF could also be detected after the cross-linking of CD163 with monoclonal antibodies[90].
In summary, there is more and more evidence arising that stands in contrast to the otherwise described homeostatic and anti-inflammatory mechanisms resulting from an activation of CD163, turning CD163 into a double-edged 'immunomodulator'[89].

1.3.4 CD163 in pathologic conditions

Elevated levels of sCD163 can be found in various diseases, and although reflecting different states of inflammation, all of them are accompanied by increased macrophage activity and inflammation[153]. As the enzyme responsible for the cleavage of CD163 from the cell surface – a disintegrin and metalloproteinase 17/TNFα-cleaving enzyme (ADAM 17/TACE) – simultaneously sheds TNFα, sCD163 has been proposed as a more persistent inflammation marker, as TNFα is quickly degraded[109].

Table 5. Elevated serum levels of soluble CD163 in various diseases.

Relative increase (RI, fold change)	Disease	RI	References
Low increase: 1.1 – 1.9 times	Atherosclerosis	1.2 times	154
	Type 2 diabetes mellitus	1.6 times	130, 155
	Rheumatic diseases	1.5 times	107, 156
	Inflammatory bowel disease	1.3 times	157, 158
High increase: > 2 times	Sepsis	3.4 times	115, 159, 160
	Haemophagocytic syndrome	21.7 times	161

This comprises acute inflammatory conditions, as found in sepsis, as well as chronic inflammatory circumstances, e. g. atherosclerosis or rheumatoid arthritis, though with less pronounced elevations[104, 107, 154].
In septic conditions observed in critically ill patients not only a sCD163 elevation in general has been observed, but also a semi quantitative correlation regarding prognosis could be found[159]. The mortality was shown to increase 10-fold when the sCD163 serum level exceeded 9.5 mg/l[159]. Although it was claimed that sCD163 is more exact to classify disease severity than procalcitonin, sCD163 concentrations failed to be able to discriminate between septic patients with and without bacterial infection[115, 160].
The by far highest serum levels of sCD163 have been detected in haemophagocytic syndrome, a condition characterized by an overt activation of macrophages[161]. It has been shown that sCD163 levels are closely linked to disease activity, which led to the suggestion to utilize them as an early diagnosis marker[161]. It can also be associated with other inflammatory disorders like inflammatory bowel disease and rheumatic diseases (Table 5).
Remarkably, elevated levels of sCD163 have also been reported in diseases characterized by low-grade inflammation, as for example rheumatoid arthritis[156], psoriasis[162, 163], inflammatory bowel disease[91, 157], systemic sclerosis[164] and type 2 diabetes mellitus[130, 165]. It has recently been demonstrated in a clinical study – in an attempt to characterize circulating PBMC in obese and diabetic subjects by defining phenotypic markers – that mRNA expression of CD163 was significantly decreased in patients with newly diagnosed type 2 diabetes when compared to PBMC of both lean and obese, but otherwise healthy test persons[166]. Moreover, it was shown that in atherosclerotic plaques and on the surface of PBMC of patients with type 2 diabetes mellitus the percentage of macrophages and PBMC expressing CD163 was significantly reduced when compared to samples from non-diabetic individuals[120, 130]. At the same time, serum sCD163 was found to be increased[130]. It was therefore hypothesized that the clearing capability of free hemoglobin is derogated in those individuals, leading to an elevated incidence of macrovascular complications[130]. Taking these findings into account, sCD163 has been suggested as a risk marker for diabetes, as elevated plasma levels show a positive correlation with indices of the metabolic syndrome, especially central adiposity[165, 167]. In a prospective cohort study it was found that – without being dependent on body mass index and age – the incidence of type 2 diabetes mellitus increased with elevated serum sCD163 percentiles, making sCD163 a suitable marker to distinguish between low- and high-risk groups[165]. Concurrently, another study elucidated the negative correlation of sCD163 plasma levels with insulin sensitivity, supporting the finding that CD163 gene expression in muscle-residing macrophages correlated with better muscle insulin sensitivity[168]. Another study concluded that sCD163 was strongly associated with insulin resistance and not being dependent on other predictors like TNFα or IL-6[169]. In concert with these findings, two independent clinical studies, the first performed with 95 healthy non-diabetic individuals and the second realized with 166 patients with type 2 diabetes mellitus, found that sCD163 was strongly associated with log-transformed HOMA-IR, a surrogate used to detect insulin resistance[167, 170].

1.4 Aims and objectives of this project

Scavenger receptor CD163 is responsible for the internalization and degradation of Hb-Hp complexes which are built due to intravascular haemolysis. This circumstance particularly occurs in inflammation, leading to the development of micro- and macrovascular damage.
Although diabetes mellitus is commonly described as chronic-inflammatory disease and known to cause vascular complications, neither the levels of gene and surface expression of CD163, nor the function of CD163 were examined in hyperglycemia until now. Moreover, it has not been examined whether the scavenging process of Hb-Hp complexes in diabetic conditions is impaired or leads to a negative impact on their microenvironment.
The aim of this project was to examine how hyperglycemia affects the scavenging of Hb-Hp complexes via CD163 in human primary differentially activated macrophages.

The specific aims of this project were:

- Identification of hyperglycemia-induced changes in gene and surface expression of CD163 in human primary non-stimulated, IFNγ-stimulated and IL-4-stimulated macrophages

- Analysis of changes in CD163-shedding from the cell surface in response to hyperglycemia in human primary non-stimulated, IFNγ-stimulated and IL-4-stimulated macrophages

- Analysis of the effect of hyperglycemia on the uptake of Hb-Hp complexes via CD163 in human primary non-stimulated, IFNγ-stimulated and IL-4-stimulated macrophages

- Analysis of the inflammatory response of human primary IFNγ-stimulated macrophages to the stimulation with Hb-Hp complexes in normal and hyperglycemic conditions

2 MATERIAL AND METHODS

This work was written under the operating system macOS Catalina Version 10.15.4 and the word processor Microsoft Office Word 2020. Graphical representations were made using Microsoft Office Excel 2020 and GraphPad Prism 8.

2.1 Chemicals, reagents and kits

Table 6. Chemicals and reagents.

Product	Company
50x Tris-Acetate EDTA (TAE) buffer	Eppendorf
acLDL- Alexa Fluor® 488	Thermo Fisher Scientific
Agarose	Biowhittaker
Anti-Human CD14 FITC	eBioscience
Anti-Human CD163 APC	eBioscience
Anti-Human CD163	Santa Cruz
Anti-Human HLA-DR PE	BD Pharmingen
Biocoll separating solution	Biochrom AG
BSA (Bovine Serum Albumin)	Sigma
β-Mercaptoethanol	Sigma
Casyton Messlösung	OLS
CD14 Microbeads	Miltenyi Biotec
Dako Fluorescent Mounting Medium	Dako Cytomation
Delimiting Pen	Dako
Deoxyribonucleotides (dNTPs) 10 M	Thermo Scientific
DEPC Water	Thermo Scientific
Dexamethasone	Sigma
Distilled water (RNAse free)	Provided in the lab
DMEM	Sigma
DMSO	Sigma
DNAse I	Thermo Scientific
DNAse I buffer with $MgCl_2$ 10x	Thermo Scientific
Donkey Anti-Goat Cy3	Dianova
D-PBS, sterile 1x	Invitrogen
D-PBS (10x)	Biochrom
DRAQ5	New England BioLabs
Earle's Balanced Salt Solution (10x)	Sigma
EDTA (0.5 M, pH=8.0)	Gibco
Ethanol	Carl-Roth
GelRed Nucleic Acid Gel stain	Biotium
GeneRuler DNA ladder	Thermo Scientific
Haptoglobin, Phenotype 1-1	Sigma
Haptoglobin, Phenotype 2-2	Sigma

H_2SO_4 2 N	Carl-Roth
HCl 1 N	Carl-Roth
hTruStain FcX FcR Blocking Reagent	Biolegend
Human hemoglobin A_0	Sigma
IgG1κ Isotype Control APC	eBioscience
IgG1κ Isotype Control FITC	eBioscience
IgG2aκ Isotype Control PE	BD Pharmingen
'Normal goat IgG'	Santa Cruz
Loading Dye 6x	Thermo Scientific
Macrophage-SFM	Life Technologies
$MgCl_2$	Sigma
NaOH 1 N	Sigma
Oligo(dt) primer	Thermo Scientific
PCR primers (designed in the lab)	Eurofins MWG Operon
PCR probes (designed in the lab)	Eurofins MWG Operon
Percoll	GE Healthcare
PFA	Fluka
Reaction buffer (5x)	Thermo Scientific
Recombinant human IFNγ	Preprotech
Recombinant human IL-4	Preprotech
Recombinant human M-CSF	Preprotech
RevertAid H Reverse transcriptase	Thermo Scientific
Ribolock RNAse inhibitor	Thermo Scientific
TaqMan Gene Expression Master Mix	Applied Biosystems
Triton X-100	Sigma
Tween 20	Sigma

Table 7. Kits.

Product	**Company**
Alexa Fluor® 488 Protein Labeling Kit	Molecular Probes
E. Z. N. A. total RNA Kit I	Omega bio-tek
Human CD163 DuoSet ELISA kit	R&D Systems
Human IL-1β DuoSet ELISA kit	R&D Systems
Human IL-1RA DuoSet ELISA kit	R&D Systems
Human IL-6 DuoSet ELISA kit	R&D Systems
Human IL-8 DuoSet ELISA kit	R&D Systems
Human TNFα DuoSet ELISA kit	R&D Systems
MACS separation columns	Miltenyi Biotec
RevertAid H cDNA synthesis kit	Fermentas
SensiMix II probe kit	Bioline
Pierce BCA Protein Assay Kit	Thermo Scientific

2.2 Consumables

Table 8. Consumables.

Product	Company
10 ml syringe	BRAUN
12-well cell culture plates	Greiner bio-one
20G needle	Neoject
22x22 mm coverslips	neolab
Filter cards for Cytospin	Fisher Scientific
6-well cell culture plates	Greiner
Aluminium foil	Roth
Cell scrapers	Greiner bio-one
ELISA plate sealers	R&D systems
ELISA plates	R&D systems
FACS tubes	BD
Glass slides	Servoprax
Kimtech towels	KIMTECH
Parafilm	American National Can
PCR tubes	Star Labs
Pipette tips	Eppendorf, Star Labs
Pipettes	Eppendorf, Gilson
RNAse free Eppendorf tubes	Eppendorf
RT-PCR plate sealers	Axon Labortechnik
RT-PCR plates	Axon Labortechnik
Safe-Lock Eppendorf tubes, 1.5ml	Eppendorf
Scalpel	neolab
Sterile pipette tips	Avantguard, Star Labs, Nerbeplus
T-75 cell culture flask	Greiner bio-one
Falcon tubes: 15ml, 50ml	Greiner bio-one

2.3 Equipment

Table 9. Equipment.

Product	Company
Agarose electrophoresis unit i-Mupid	Erogentec
Autoclave VX-95	Systec
BD FACSCanto II	BD
Benchtop UV Transilluminator	UVP
CASY Cell counter	OLS OMNI Life Science
Centrifuge 5415 R	Eppendorf
Centrifuge Rotina 420	Hettich
Centrifuge Rotina 420R	Hettich

CO_2 Cell Culture Incubator Hera cell 150	Thermo
Cryo freezing container	Nalgene
Cytospin 3 Centrifuge	Shandon
Deep freezer (-80°C)	Sanyo
Electrophoresis comb	Peqlab
Electrophoresis Power Supply	Peqlab
Freezer (-20°C)	Liebherr, Kirsch
Galaxy mini microcentrifuge	VWR
Ice machine AF100	Scotsman
Incubator	Edmund Bühler GmbH
Inverted microscope	Leica
Laminar flow hood	Thermo
LightCycler 480	Roche
Magnetic Stirrer MR3000	Heidolph
Microwave oven	Sharp
Roller	Ortho Diagnostic Systems
Rotator	Neolab
Shaker KS 260 basic	IKA
TCS SP2 laser scanning spectral confocal microscope	Leica
Tecan Infinite 200	Tecan
Thermocycler DNA Engine PTC220 Dyad	MJ Research
Thermomixer 5436	Eppendorf
Vortex Genie 2	Scientific Industries

2.4 Buffers and solutions

2.4.1 Running buffer for agarose gel electrophoresis

50x TAE buffer
242 g of Tris free base and 18.61 g of Disodium EDTA were added to 700 ml ddH_2O and stirred until they dissolved. 57.1 ml Glacial Acetic Acid was added and the volume was adjusted to 1 L.

1x TAE buffer
20 ml of 50x TAE buffer was added to 980 ml of ddH_2O.

2.4.2 Solutions for cell fixation

Fixation solution (4% PFA)
40 g of PFA was weighed out under the fume hood and then dissolved in 700 ml of 1x PBS. A magnetic stirrer was placed inside. The pH was adjusted, with stirring, to 11.4 with 1 N NaOH. After stirring the solution for 1h at RT, the pH was adjusted to 7.4 with 1 N HCl. The volume was adjusted to 1 L with 1x PBS and sterile filtered with a 0.22 µm filter.

2.4.3 Solutions for immunological methods

Wash buffer for ELISA (0.05% Tween 20 in PBS)
500 µl of Tween 20 was pipetted into 1 L of PBS. The solution was stirred on a magnetic stirrer for 30 min and stored at RT.

Blocking solution (3% BSA)
1.5 g of BSA was filled up with 1x PBS to 50 ml and dissolved on the rotator.

FACS buffer (0.4% BSA, 0.02% Sodium Azide)
4 g of BSA and 2 ml of 10% Sodium Azide solution were dissolved in 1 L of PBS. The solution was adjusted to pH 7.4 and sterile filtered with a 0.22 µm filter.

2.4.4 Buffer for monocytes isolation

Percoll gradient
For each 30 ml of Percoll gradient solution 13.5 ml Percoll, 15 ml Minimal Essential Medium Eagle Spinner modification and 1.5 ml of 10x Earle's Balanced Salt Solution were mixed in a 50 ml Falcon tube.

MACS buffer (0.5% BSA, 2 mM EDTA)
2.5 g of BSA was dissolved in 500 ml PBS. 2 ml of 0.5 M EDTA was added and the mixture was filtered to a sterile flask.

2.5 Molecular biology methods

2.5.1 Primers

Table 10. Primers and probes.

Gene	Type	Sequence (5' to 3')	Concentration
hCD163	Forward	TAGTGAGTGTGGGCACAAGG	900 nM
	Reverse	CCGACTGCAATAAAGGATGA	300 nM
	Probe	CACAACAGGTCGCTCATCCCG	250 nM
18sRNA	Forward	CCATTCGAACGTCTGCCCTAT	300 nM
	Reverse	TCACCCGTGGTCACCATG	900 nM
	Probe	ACTTTCGATGGTAGTCGCCGTGCCT	200 nM

All primers and probes (Table 10) were designed in our laboratory with MWG Biotech/GeneScript and ordered from Eurofins Genomics.

2.5.2 Isolation of RNA

The isolation of RNA was performed using the E. Z. N. A. total RNA kit I purchased from Omega bio-tek.

All steps were performed under a laminar flow hood and on ice.

1) 350 µl of TRK Lysis buffer without β-Mercaptoethanol was added to the cells.
2) To homogenize the sample, the cell mass was taken up and down a 20G needle approximately 15-20 times.
3) 70% EtOH was added to the cell samples in a 1:1 ratio, meaning 350 µl if the cells were cultivated in a 6-well plate.
4) The columns provided by the kit were placed on 2 ml Eppendorf tubes. The cell mixture was mixed properly and applied on the columns.
5) The columns were centrifuged at 13200 rpm for 1 min. Afterwards, the fluid in the tube was discarded.
6) 500 µl of RNA wash buffer I was put on the columns and they were centrifuged at 13200 rpm for 1 min. The fluid in the tube was discarded.
7) 500 µl of RNA wash buffer II was put on the columns and centrifuged at 13200 rpm for 1 min. Afterwards, the fluid in the tube was discarded.
8) Once again, 500 µl of RNA wash buffer II was put on the columns and they were centrifuged at 13200 rpm for 1 min. Afterwards, the fluid in the tube was discarded.
9) New 2 ml Eppendorf tubes were taken and centrifuged with the columns at 13200 rpm for 2 min.
10) The columns were placed into new RNAse free Eppendorf tubes.
11) 25 µl + 1 µl (to measure afterwards) of DEPC water were put on the exact center of the columns.
12) After waiting for 5-10 min, the columns (placed into the new RNAse free Eppendorf tubes) were centrifuged at 13200 rpm for 2 min.
13) The tubes were labeled.

Measurement of RNA

The measurement of the amount of RNA was performed via absorbance using a Tecan Infinite 200.

1) To blank the plate, 2 µl of DEPC water was placed in the wells needed for the measurement.
2) Blanking of the plate was performed.
3) The plate was cleaned using a KIMTECH towel.
4) 1 µl of the sample RNA was placed in the wells.
5) The analysis of the measurement was performed using the software provided by the manufacturer.

2.5.3 cDNA synthesis

The synthesis of cDNA was performed using the Revert Aid H cDNA synthesis kit purchased from Fermentas.

1) DNAse digestion

To remove possible contamination of the isolated RNA with fragments of genomic DNA, all RNA templates were digested with DNAse I before the cDNA synthesis. The following reagents (Table 11) were mixed in 1.5 ml Eppendorf tubes and spun down by centrifugation:

Table 11. Reagents used for DNA digestion.

Reagent	Amount
RNA (up to 1 µg)	5 µl
DNAse I buffer with $MgCl_2$ 10x	1 µl
DNAse I	1 µl
DEPC water	3 µl

The tubes were incubated in a thermoblock for 40 min at 37°C. After being spun down by centrifugation, the enzyme was inactivated for 10 min at 70°C.

2) cDNA synthesis

1 µl of Oligo(dt) primer and 1 µl of DEPC water were added to 10 µl of the RNA from above. The mixture was incubated for 5 min at 70°C and then transferred on ice.
A master mix of the following reagents (Table 12) was created:

Table 12. Reagents used for cDNA synthesis.

Reagent	Amount
Reaction buffer (5x)	4 µl
Ribolock RNAse inhibitor	1 µl
dNTP Mix (10 M)	2 µl
Reverse transcriptase	1 µl

8 µl of the master mix was placed into each of the tubes and they were incubated for 60 min at 42°C. After spinning down by centrifugation, enzymatic activity was inactivated for 10 min at 70°C.
The samples were diluted to 1:5 with DEPC water and stored at -20°C.

2.5.4 Polymerase chain reaction (PCR)

To assess the quality of the previously designed primers, a PCR was performed before proceeding to RT-qPCR optimization. Only primers specific to the gene of interest were used to amplify the DNA fragments from cDNA samples.

1) All of the following reagents (Table 13), minus the cDNA template, were added to an Eppendorf tube to make a master mix for the number of samples and mixed by vortexing.

Table 13. Reagents used for PCR.

Reagent	Amount
2x SensiMix	10 µl
Forward primer (10 pM)	1 µl
Reverse primer (10 pM)	1 µl
cDNA template	1 µl
ddH_2O	7 µl

2) The master mix was divided amongst PCR tubes.
3) The cDNA templates were added to the PCR tubes and mixed by pipetting.
4) The tubes were placed in a thermocycler and the following program (Table 14) was used to amplify the target gene:

Table 14. PCR protocol.

Temperature	Time	Repetition
95°C	10 min	1x
95°C	10 s	40x
60°C	1 min	1x
4°C	∞	

Agarose gel electrophoresis was used to visualize PCR products.

2.5.5 Agarose gel electrophoresis

1) Agarose powder and electrophoresis buffer (1x TAE) were used to prepare an agarose solution in a microwave oven. The percentage of agarose was selected according to the size of the DNA fragments (Table 15):

Table 15. Size ranges for agarose gel.

% Agarose	DNA size (bp)
0.5	1000 - 30000
0.7	800 - 12000
1.0	500 - 10000
1.2	400 - 7000
1.5	200 - 3000
2.0	50 - 2000

2) 10 µl of GelRed 10000x nucleic acid gel stain was added to 100 ml of agarose solution and mixed.
3) The solution was cooled down to 55-60°C with magnetic stirring and poured into the previously prepared gel tray equipped with a comb.
4) Once solidified, the comb was removed carefully from the solution.
5) The gel tray was placed in the electrophoresis unit filled with 1x TAE buffer.
6) 2 µl of loading dye was added to 8 µl of the PCR product. The samples were loaded onto the gel.
7) 3 µl of GeneRuler DNA Ladder Mix was added to the first lane to compare the size of the target fragments.
8) Electrophoresis was carried out at constant voltage (100 V) for 1h.

The results were visualized by UV illumination (254 nm) and documented using a gel documentation system.

2.5.6 Real-time quantitative polymerase chain reaction (RT-qPCR)

Primers and probes were obtained from Eurofins Genomics. The probes were dual-labeled with FAM on the 5' and a BHQ1 quencher on the 3' end of sequence. Optimization of both primers and probes was performed using the following pipetting scheme (Table 16):

Table 16. Pipetting scheme for primer optimization. F: Forward Primer; R: Reverse Primer; Pr: Probe

F 900 nM R 900 nM Pr 250 nM	F 300 nM R 900 nM Pr 250 nM	F 50 nM R 900 nM Pr 250 nM
F 900 nM R 300 nM Pr 250 nM	F 300 nM R 300 nM Pr 250 nM	F 50nM R 300nM Pr 250nM
F 900 nM R 50 nM Pr 250 nM	F 300 nM R 50 nM Pr 250 nM	F 50 nM R 50 nM Pr 250 nM

The choice fell on the primer combination displaying the earliest and steepest curves: F 900 nM, R 300 nM. To optimize the probe, concentrations between 50 nM and 250 nM were used. The earliest and steepest curves were obtained with a probe concentration of 250 nM. The final concentrations used in the project are also shown in Table 10.
As housekeeping genes β2M and 18sRNA were chosen as references in RT-qPCR experiments. As 18sRNA primers seemed to interact with hCD163 primers, the two sets of primers were added separately to the samples, with the samples being placed in different wells. Analysis was performed using the LightCycler Software.

First, a master mix containing the components shown in Table 17 was created.

Table 17. Reagents used in RT-qPCR master mix.

Reagent	Amount
SensiMix II Probe kit	5 µl
20x hCD163	0.5 µl
ddH_2O	4 µl

Reagent	Amount
SensiMix II Probe kit	5 µl
20x 18sRNA	0.5 µl
ddH_2O	4 µl

38 µl of the master mix were transferred to Eppendorf tubes, where 2 µl of the cDNA sample was added and mixed by pipetting. The samples were pipetted in triplicates into a 96-well plate. ddH_2O was used as a negative control.

The amplification protocol is shown in Table 18.

Table 18. LightCycler 480 amplification protocol.

Temperature	Time	Repetition
95°C	10 min	1 cycle
95°C	15 s	50 cycles
60°C	1 min	
37°C	1 min	

2.6 Cell culture

2.6.1 Isolation of $CD14^+$ monocytes from human buffy coats

Buffy coats were obtained from healthy individuals who had donated blood to the blood bank of the German Red Cross Blood Service Baden-Württemberg-Hessen in Mannheim.

All steps were performed under a laminar flow hood.

1) A unique donor number was given to each buffy coat. Two 50 ml Falcon tubes were labeled for each donor and filled with 15 ml Biocoll.
2) The blood bag was opened using a sterile scalpel. The whole blood was filled in a T-75 cell culture flask (approximately 30 ml per donor) and PBS was added at a 1:1 ratio. The bottle was rotated to mix.
3) The blood-PBS mixture was added slowly onto the Biocoll without mixing.
4) The tubes were centrifuged in a Hettich Rotina Centrifuge for 30 min at 420 rcf and 20°C without brake.
5) The buffy coat (white ring) was pipetted off with a sterile one-time plastic Pasteur pipet and put into a fresh 50 ml Falcon tube.
6) The Falcon tube was filled up to 50 ml with PBS 1x.
7) The tubes were centrifuged in a Hettich Rotina Centrifuge for 10 min at 420 rcf and 20°C with brake.
8) The supernatant was aspirated. The pellet was resuspended with 5 ml PBS 1x.
9) The Falcon tube was filled up to 50 ml with PBS 1x.

10) The tubes were centrifuged for the second time in a Hettich Rotina Centrifuge for 10 min at 420 rcf and 20°C with brake.
11) 30 ml of Percoll gradient was prepared using 15 ml MEM, 13.5 ml Percoll and 1.5 ml Earle's BSS.
12) The supernatant was aspirated. The pellet was resuspended with 3 ml PBS 1x and covered very slowly on the Percoll gradient.
13) The tubes were centrifuged in a Hettich Rotina Centrifuge for 30 min at 420 rcf and 20°C without brake.
14) The buffy coat (white ring) was pipetted off with a sterile one-time plastic Pasteur pipet and put into a fresh 50 ml Falcon tube.
15) The tubes were centrifuged in a Hettich Rotina Centrifuge for 10 min at 420 rcf and 20°C with brake.
16) The supernatant was aspirated. The pellet was resuspended with 5 ml PBS 1x in a fresh 15 ml Falcon tube.
17) 10µl cell suspension were taken and added into 10 ml CasyTon to count the cells.
18) While counting the cells with a CASY cell counter, the tubes were centrifuged in a Hettich Rotina Centrifuge for 10 min at 420 rcf and 20°C with brake.
19) The supernatant was aspirated. 5 µl $CD14^+$ beads and 95 µl MACS buffer per 10^7 cells were added.
20) The mixture was incubated for 15-20 min on a rotator at 4°C.
21) The samples were filled up to 10 ml with MACS buffer.
22) The tubes were centrifuged in a Hettich Rotina Centrifuge for 10 min at 420 rcf and 20°C with brake.
23) In the meantime, the MACS magnet stand with LS columns was prepared. The LS columns were equilibrated with 3 ml MACS buffer each.
24) The supernatant was aspirated and the centrifuged cells were resuspended in 1 ml of MACS buffer.
25) The resuspension was applied on the LS columns.
26) The columns were washed 3 times with 3 ml of MACS buffer.
27) The LS columns were removed from the magnet and placed on fresh 15 ml tubes. 5 ml MACS buffer was added.
28) The liquid was pressed through the column.
29) A 10 µl aliquot was taken and added into 10 ml of CasyTon to count the cells.
30) The tubes were centrifuged in a Hettich Rotina Centrifuge for 10 min at 420 rcf and 20°C with brake.
31) The supernatant was aspirated and the cells were resuspended and diluted in an appropriate culture medium.

2.6.2 Cell counting using a CASY cell counter

1) The capillary of the cell counter was washed 3 times with fresh CasyTon. The measurement of pure CasyTon was supposed to be less than 50.
2) The appropriate program for PBMC was chosen.
3) 10 µl of cell suspension was added to 10 ml of CasyTon.
4) The sample was placed under the capillary and measured 3 times.
5) Between the different samples the capillary was washed 3 times with fresh CasyTon.
6) Only the number of viable cells was used for further calculations.

2.6.3 Cultivation conditions

- Glucose-free Macrophage-SFM medium was custom-made and purchased from Life Technologies. For the 'normal glucose' (NG) condition, glucose stock was added until a final concentration of 5 mM; for the 'high glucose' (HG) condition, glucose stock was added until a concentration of 25 mM. The concentrations were measured by an Accu-Check glucose monitoring device.
- 10^6 cells per 1 ml of medium
- Macrophage-SFM was supplemented with:
  - 5 ng/ml human M-CSF
  - 10^{-8} M Dexamethasone
- Three subtypes of macrophages were differentiated with the following cytokine stimulations:
  - M(Control): no cytokine stimulation added
  - M(IFNγ): 100 ng/ml IFNγ added
  - M(IL-4): 10 ng/ml IL-4 added
- Cell culture plates were incubated in a Cell Culture Incubator at 37°C and 7.5% CO_2 for 6 days.

2.6.4 Harvesting of cells

Cells were harvested after culturing for 6 days at 37°C and 7.5% CO_2.

1) The cell culture plates were placed directly on ice and covered with a lid for 1h.
2) The cells were scraped using a cell scraper and resuspended in the wells.
3) According to the protocol, the cells were either prepared for RNA isolation, flow cytometry or confocal microscopy.

2.7 Protein-related techniques

2.7.1 Enzyme-linked immunosorbent assay (ELISA)

After 6 days of stimulating the cells in different glucose concentrations and with different cytokines, supernatants were harvested and analyzed by ELISA. In some cases, the supernatants were diluted in Reagent Diluent as indicated in Table 19.

Table 19. ELISA kits used in this project.

ELISA Kit	Dilution	Company
Human CD163 DuoSet ELISA kit	1:2	R&D Systems
Human IL-1β DuoSet ELISA kit	1:2	R&D Systems
Human IL-1RA DuoSet ELISA kit	1:50	R&D Systems
Human IL-6 DuoSet ELISA kit	1:2	R&D Systems
Human IL-8 DuoSet ELISA kit	1:50	R&D Systems
Human TNFα DuoSet ELISA kit	1:2	R&D Systems

ELISA protocol for kits purchased from R&D Systems:

Table 20. Reagents used in ELISA kits.

Reagent	Composition
Wash Buffer	0.05% Tween 20 in PBS pH: 7.2-7.4
Reagent Diluent	1% BSA in PBS pH: 7.2-7.4
Substrate Solution	1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)
Stop Solution	2 N H_2SO_4

All components (Table 20) were brought to room temperature before use.

Plate preparation:

1) The capture antibody was diluted in a 1:120 ratio to the working concentration of 4 µg/ml in PBS.
2) 100 µl of diluted Capture Antibody was added into each well of a 96-well microplate.
3) The plate was sealed and incubated overnight at room temperature.
4) On the next day, the capture antibody was aspirated and the plate was washed 3 times with 200 µl per well of wash buffer (0.05% Tween 20 in PBS). Remaining wash buffer was removed by blotting the plate against clean paper towels.
5) The plate was blocked by adding 100 µl of Reagent Diluent (1% BSA in PBS) to every well and incubating it for 1h at room temperature.
6) The Reagent Diluent was aspirated from the wells.

Assay procedure:

1) The standard was diluted according to the manufacturer's protocol. Samples were thawed and diluted in Reagent Diluent as indicated in Table 19.
2) 100 µl of Reagent Diluent were added to the first two columns of the plate and the standard was pipetted in a serial dilution.
3) 50 µl of Reagent Diluent and 50 µl of the diluted samples were added to the rest of the plate.
4) The plate was covered and incubated for 2h at room temperature.
5) Reagents were aspirated and the plate was washed 3 times with 200 µl per well of wash buffer. Remaining wash buffer was removed by blotting the plate against clean paper towels.
6) 100 µl of detection antibody, diluted in Reagent Diluent, was added per well.
7) The plate was covered and incubated for 2h at room temperature.
8) Reagents were aspirated and the plate was washed 3 times with 200 µl per well of wash buffer. Remaining wash buffer was removed by blotting the plate against clean paper towels.
9) 100 µl of Streptavidin-HRP (working concentration) were added to each well.
10) The plate was covered and incubated for 20 min at room temperature. It was avoided to place the plate in direct light.

11) Reagents were aspirated and the plate was washed 4 times with 200 µl per well of wash buffer. Remaining wash buffer was removed by blotting the plate against clean paper towels.
12) 100 µl of substrate solution (1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)) was added to each well.
13) The plate was covered and incubated for 20 min at room temperature. It was avoided to place the plate in direct light.
14) 50 µl of stop solution (2N H_2SO_4) was added to each well. The plate was tapped gently to ensure thorough mixing.

Measurement:

Immediately after adding the stop solution, the optical density of each well was determined. A TECAN microplate reader set to 450 nm was used. The reference wavelength was set to 570 nm.

2.7.2 Inflammatory response protocol

1) $CD14^+$ monocytes were isolated from buffy coats of 5 healthy donors.
2) The monocytes were stimulated with IFNγ and cultured for 6 days in normal (5mM) and high (25mM) glucose conditions, as indicated in 2.6.3.
3) In every well $4x10^6$ cells were cultured according to the planting sketch (Figure 6), $3x10^6$ cells were later used for RNA isolation and $1x10^6$ cells to perform flow cytometry.

Stimulation	NG, IFNγ	HG, IFNγ	NG, IFNγ	HG, IFNγ
no complexes	$4x10^6$ cells	$4x10^6$ cells		
Hb-Hp1-1	$4x10^6$ cells Hb-Hp1-1: 10µg/ml	$4x10^6$ cells Hb-Hp1-1: 10µg/ml	$4x10^6$ cells Hb-Hp1-1: 1µg/ml	$4x10^6$ cells Hb-Hp1-1: 1µg/ml
Hb-Hp2-2	$4x10^6$ cells Hb-Hp2-2: 10µg/ml	$4x10^6$ cells Hb-Hp2-2: 10µg/ml	$4x10^6$ cells Hb-Hp2-2: 1µg/ml	$4x10^6$ cells Hb-Hp2-2: 1µg/ml

Figure 6. ‚Inflammatory Response' planting sketch with stimulations. NG: normal glucose concentration (5 mM), HG: high glucose concentration (25 mM), Hb-Hp1-1: hemoglobin-haptoglobin1-1 complexes, Hb-Hp2-2: hemoglobin-haptoglobin2-2 complexes

4) After 6 days of culturing, the cells were stimulated with hemoglobin-haptoglobin complexes of the 2 haptoglobin variants (Hb-Hp1-1 and Hb-Hp2-2) in two different concentrations (1 µg/ml and 10 µg/ml).
5) Supernatants were collected at 6h and 24h after stimulation and stored at -80°C to perform ELISAs of sCD163, IL-1β, Il-1RA, IL-6, IL-8 and TNFα.
6) The cells were harvested for RNA isolation and flow cytometry (CD163).
7) The mRNA expression in all 5 donors of the following genes was determined via RT-qPCR: CD163, IL-1β, Il-1RA, IL-6, IL-8 and TNFα.

2.8 Immunological methods

2.8.1 Antibodies

Table 21. Antibodies and staining reagents used in this project.

Antibody	Type	Species/Isotype	Company	Application
anti-human CD14 FITC	primary	mouse IgG1κ	eBioscience	FACS
anti-human CD163 APC	primary	mouse IgG1κ	eBioscience	FACS
anti-human CD163	primary	goat polyclonal	Santa Cruz	IF
anti-human HLA-DR PE	primary	mouse IgG2aκ	Biozol/Biolegend	FACS
IgG1κ APC Control ab	primary	mouse IgG1κ	eBioscience	FACS
IgG1κ Isotype Control FITC	primary	mouse IgG1κ	eBioscience	FACS
IgG2aκ Isotype Control PE	primary	mouse IgG2aκ	Biozol	FACS
'normal goat IgG' Isotype Control	primary	goat IgG	Santa Cruz	IF
anti-goat Cy3	secondary	donkey	Dianova	IF
DRAQ5	nuclear staining	-	Cell Signaling	IF

2.8.2 Alexa Fluor® 488 labeling of haptoglobin molecules

To label haptoglobin1-1 and haptoglobin2-2 with Alexa Fluor® 488 dye, the Alexa Fluor® 488 Protein Labeling kit purchased from Molecular Probes was used.

Preparing the protein:

The haptoglobin isoforms 1-1 and 2-2 were diluted in a 1:100 ratio in PBS as indicated in the manufacturer's protocol.

Labeling the protein:

1) 1 M solution of sodium bicarbonate was created by adding 1 ml of deionized water (ddH_2O) to the provided vial of sodium bicarbonate. It was vortexed until fully dissolved.
2) To 0.5 ml of the 2 mg/ml protein solution, 50 µl of 1 M sodium bicarbonate was added.
3) A vial of reactive dye was allowed to warm to room temperature. The protein solution was transferred to the vial of reactive dye. The vial was inverted a few times to fully dissolve the dye.
4) As the vial contained a magnetic stir bar, the reaction mixture was then stirred for 1h at room temperature.

Purifying the labeled protein

1) The column was assembled according to the manufacturer's protocol: it was positioned upright; a funnel was attached to the top of a column. The column was gently inserted through the X-cut in one of the provided foam holders to avoid damaging the column. Using the foam holder, the column was secured with a clamp to a ringstand. The cap was carefully removed from the bottom of the column.

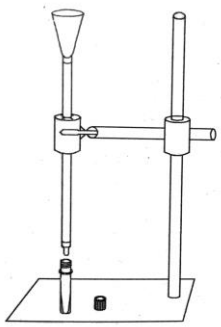


Figure 7. Column assembly. Extracted from the manual provided with the Alexa Fluor® 488 Protein Labeling kit, Molecular Probes.

2) The elution buffer was prepared by diluting the 10x stock 10-fold in ddH_2O at room temperature.
3) Using one of the provided pipets, the purification resin was stirred thoroughly to ensure a homogeneous suspension. The resin was pipetted into the column, allowing excess buffer to drain away into a small beaker. The resin was packed into the column until it was ca. 3 cm from the top of the column.
4) The excess buffer was allowed to drain into the column bed. It was made certain that the buffer eluted through the column with a consistently even flow prior to adding the reaction mixture.
5) The reaction mixture was carefully loaded onto the column and allowed to enter the column resin. The reaction vial was rinsed with 100 µl of elution buffer and applied to the column.
6) Elution buffer was slowly added to not disturb the column bed. The elution buffer was continuously added until the labeled protein had been eluted (duration approximately 30 min).
7) As the column ran, it was periodically illuminated with a handheld UV lamp. Two colored bands could be observed, which represented the separation of labeled protein from unincorporated dye.
8) The first band was collected, as it contained the labeled protein, into one of the provided collection tubes. The 'slower' second band was not collected, as it consisted of unincorporated dye.
9) The labeled protein in PBS was stored at 2-6°C, protected from light.

Determining the degree of labeling:

1) The absorbance of the conjugate solution was measured at 280 nm and 494 nm (A_{280} and A_{494}) in a cuvette with a 1 cm pathlength, results are shown in Table 22.

Table 22. Absorbance of conjugate solutions of haptoglobin1-1 and haptoglobin2-2.

Absorbance at	Haptoglobin1-1	Haptoglobin2-2
280 nm (A_{280})	0.0489	0.0179
494 nm (A_{494})	0.0333	0.0286

2) The concentration of the protein in the sample was calculated according to the following formula:

$$\text{protein concentration (M)} = \frac{[A_{280} - (A_{494} \text{ x } 0.11)] \text{ x dilution factor}}{\text{molar extinction coefficient}}$$

With 0.11 being the correction factor to account for absorption of the dye at 280 nm.
Molar extinction coefficient for haptoglobin: 102,000 cm^{-1} M^{-1}

Haptoglobin1-1:

$$\frac{[0.0489 - (0.0333 \text{ x } 0.11)] \text{ x } 100}{102{,}000} = \frac{4.5237}{102{,}000} = 4.435 \text{ x } 10^{-5} \text{ M}$$

Haptoglobin2-2:

$$\frac{[0.0179 - (0.0286 \text{ x } 0.11)] \text{ x } 100}{102{,}000} = \frac{1.475}{102{,}000} = 1.446 \text{ x } 10^{-5} \text{ M}$$

3) The degree of labeling was calculated according to the following formula:

$$\text{moles dye per mole protein} = \frac{A_{494} \text{ x dilution factor}}{71{,}000 \text{ x protein concentration (M)}}$$

With 71,000 cm^{-1} M^{-1} being the approximate molar extinction coefficient of the Alexa Fluor® 488 dye at 494 nm.

Haptoglobin1-1:

$$\frac{0.0333 \text{ x } 100}{71{,}000 \text{ x } 4.435 \text{ x } 10^{-5}} = \frac{3.33}{3.14885} \approx 1.058 \text{ moles dye per mole protein}$$

Haptoglobin2-2:

$$\frac{0.0286 \text{ x } 100}{71{,}000 \text{ x } 1.446 \text{ x } 10^{-5}} = \frac{2.86}{1.02666} \approx 2.787 \text{ moles dye per mole protein}$$

4) Measurement of protein concentration by Pierce BCA Protein Assay Kit purchased from Thermo Scientific:

   1. A diluted albumin (BSA) standard and the working reagent (BCA Reagent A and BCA Reagent B) were prepared in a 50:1 ratio according to the manufacturer's protocol.
   2. 0.1 ml of each standard and unknown sample was pipetted in a test tube.
   3. 2 ml of the working reagent were added to each tube and mixed well.
   4. The tubes were incubated for 30 min at 37°C.
   5. Before continuing, the tubes were cooled down to room temperature.
   6. The spectrometer was set to 562 nm and the absorbances of a blank and all samples were measured within 10 min.
   7. A standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard against its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

      Haptoglobin1-1: ≈ 1.3 mg/ml

      Haptoglobin2-2: ≈ 1.1 mg/ml

2.8.3 Flow cytometry

Flow cytometry was used to determine the amount of CD163 scavenger receptor on the cell surface of $CD14^+$ monocytes.

All procedures were performed in FACS tubes.

1) After isolating $CD14^+$ monocytes from buffy coats, 1.5x 10^6 cells were taken to stain them for flow cytometry.
2) 2 ml of FACS buffer was added to each FACS tube.
3) The tubes were centrifuged for 4 min at 1200 rpm and 4°C.
4) After centrifuging, the supernatant was aspirated and the cells were resuspended in 750 µl of FACS buffer, before being split into 3 sections.
5) 10 µl of FcR blocking Reagent (hTruStain FcX) was added into every tube and incubated for 10 min at room temperature.
6) The tubes were replaced on ice and antibodies (Table 21) were added according to the manufacturer's instructions.
7) The cells were incubated for 30 min on ice.
8) After incubation, the cells were washed with 2 ml of FACS buffer and centrifuged at 1200 rpm and 4°C for 4 min after every washing step.
9) The cells were resuspended in 200 µl of FACS buffer before measurement.
10) The analysis of the staining was performed using a BD FACS Canto II, the BD FACSDiva software and the FlowJo software.

2.8.4 Endocytosis of hemoglobin-haptoglobin complexes

To measure the uptake of hemoglobin-haptoglobin complexes (Hb-Hp1-1 or Hb-Hp2-2) mediated by CD163, we determined the mean fluorescence intensity of the previously Alexa Fluor® 488-labeled haptoglobin molecules by using flow cytometry. As a positive control, Alexa Fluor® 488-labeled acLDL was chosen. As a negative control ('no ligand'), MFI of M(Control), M(IFNγ) and M(IL-4) without stimulation with Hb-Hp complexes was determined.

1) $CD14^+$ monocytes isolated from buffy coats were cultured in normal (5 mM) and high (25 mM) glucose conditions and stimulated with IFNγ (M(IFNγ)), IL-4 (M(IL-4)) or not stimulated at all (M(Control)) as indicated in 2.6.3.

2) Building of hemoglobin-haptoglobin complexes:

    1. Human hemoglobin A_0 was added in a 1:1 ratio to previously Alexa Fluor® 488-labeled haptoglobin1-1 or haptoglobin2-2 molecules.
    2. The mixture was incubated for 10 min at 37°C on a rotator.

3) Adding of ligands to the cell culture:

    10 µg/ml of Hb-Hp1-1 complexes, 10 µg/ml of Hb-Hp2-2 complexes or 5 µg/ml Alexa Fluor® 488-acLDL was added to the cells according to the following scheme (Figure 8):

Stimulation	NG			HG		
no ligand	ns	IFNγ	IL-4	ns	IFNγ	IL-4
acLDL	ns	IFNγ	IL-4	ns	IFNγ	IL-4
Hb-Hp1-1	ns	IFNγ	IL-4	ns	IFNγ	IL-4
Hb-Hp2-2	ns	IFNγ	IL-4	ns	IFNγ	IL-4

Figure 8. Planting sketch for endocytosis experiment. NG: normal glucose concentration (5 mM), HG: high glucose concentration (25 mM), Hb-Hp: hemoglobin-haptoglobin complexes, ns: non-stimulated

After adding the complexes, the plates were incubated for 30 min at 37°C.

4) Preparation of the cells for flow cytometry:

    1. The cell culture plates were covered from light and put on ice for 30 min.
    2. The cells were harvested by scraping and resuspending them in their wells.
    3. They were replaced in FACS tubes and spun down for 4 min at 1200 rpm and 4°C.
    4. The supernatant was aspirated and the cells were resuspended in 1ml of PBS, followed by another centrifugation for 4 min at 1200 rpm and 4°C. This washing step was repeated for 4 times in total.
    5. Before measuring the fluorescence intensity, the cells were resuspended in 200 µl of cold FACS buffer.

5) Analysis of uptake:

The analysis of the staining was performed using a BD FACS Canto II, the BD FACSDiva software and the FlowJo software. MFI values of Hb-Hp complex uptake were calculated by subtracting MFI values of 'no ligand' controls.

2.8.5 Immunofluorescence and confocal microscopy

Cytospin preparation

1) Cell culture with $CD14^+$ monocytes and stimulation with hemoglobin-haptoglobin complexes after 6 days was performed under the same conditions as described in 2.8.4.
2) The cell culture plates were placed on ice and the cells were harvested by scraping and resuspending them in the wells.
3) The cells were transferred to FACS tubes and washed 3 times by adding 500 µl of PBS and centrifuging them for 4 min at 1200 rpm and 4°C.
4) The glass slides for cytospin were cleaned with 70% ethanol.
5) The glass slides, filter cards, reusable chambers and metal clips were assembled and placed into the cytospin centrifuge.

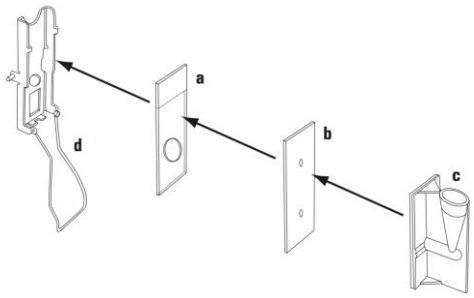


Figure 9. Cytospin assembly. a: glass slide, b: filter card, c: reusable sample chamber, d: slide clip. Extracted on 21th June 2021 from: https://static.thermoscientific.com/images/D21681~.pdf

6) 150 µl of cell suspension were pipetted into the funnels of the cytospin chambers.
7) The cells were sedimented onto the glass slide by centrifugation for 4 min at 700 rpm and room temperature.
8) After centrifugation, the cells were air-dried at room temperature.
9) Cell density was checked by microscopy.

Fixation of cells

The dry cells sedimented onto the glass slides were put in racks and fixed according to the following protocol at RT:

1) Cells were fixed with 2% PFA in PBS for 10 min.
2) Next, the cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min.

3) Afterwards, intracellular structures were fixed with 4% PFA in PBS for 10 min.
4) The fixed cells were washed in PBS for 5 min on the shaker for 3 times in total.
5) The glass slides were dried on paper towels.
6) The plates were sealed and frozen at -80°C.

Immunofluorescent staining procedure

The staining for confocal microscopy was performed at room temperature according to the following protocol:

1) The glass slides with fixed cells were thawed and dried on paper towels.
2) The cells were cycled with a hydrophobic Dako pen.
3) The cells were rehydrated in PBS for 3 min and then briefly washed with 0.1% Tween 20 in PBS.
4) To block the cells, 100 µl of 3% BSA was added to each slide and incubated for 1h at room temperature.
5) After blocking, the cells were washed in 0.1% Tween 20 in PBS for 1 min.
6) 100 µl of antibody or isotype control diluted in 1% BSA were added onto the slides and incubated for 1h at room temperature.
7) The cells were washed for 5 min in PBS for 3 times and once in 0.1% Tween 20 in PBS for 5 min.
8) 100 µl of the secondary antibody and the nuclear staining reagent DRAQ5 diluted in 1% BSA were added to the slides and incubated for 45 min at room temperature.
9) The cells were washed for 5 min in PBS for 3 times.
10) A drop of Dako fluorescent mounting medium was placed onto the glass slide and a coverslip was carefully put on top of it, avoiding air bubbles.
11) The samples were wrapped in aluminium foil and stored at 4°C.

Confocal laser scanning microscopy (CLSM)

CLSM was performed using a Leica TCS SP2 laser scanning spectral confocal microscope equipped with a 63x1.32 objective. An argon laser with an emission wavelength of 488 nm, a krypton laser with an emission wavelength of 568 nm and a helium/neon laser with an emission wavelength of 633 nm were used to perform excitation. Data were acquired and analyzed with the Leica Confocal software. Two- and three-color images were acquired using the sequential scan mode.

2.9 Statistical analysis

The significance of difference between two groups of experimental data was determined by using Wilcoxon matched-pairs rank test or a paired t test. A p-value less than 0.05 was considered statistically significant. All analyses were performed using the software GraphPad Prism 8.

3 RESULTS

3.1 Experimental design

Inflammation is an essential pathological process leading to the development of micro- and macrovascular diabetic complications[16, 36, 37, 39]. To control inflammatory reactions, the clearance of both exogenous components and unwanted-self-products is executed by scavenger receptors on monocytes and macrophages[85]. To examine how hyperglycemia affects the scavenging function via CD163, we established a model system based on primary human monocyte-derived macrophages.
In this system, $CD14^+$ primary human monocytes were isolated from buffy coats of anonymous healthy donors. They were cultured in serum-free medium (SFM) with two different glucose concentrations: 5 mM, complying with normal blood glucose concentrations (NG) and 25 mM, determined as 'high glucose conditions' (HG). To mimic physiological conditions and to increase the viability of monocytes and macrophages in culture conditions, M-CSF at a concentration of 5 ng/ml and 10^{-8} M Dexamethasone were added. A simulation of the prototype activation states of macrophages (M1 and M2) was achieved by stimulating monocytes with IFNγ and IL-4. Supernatants and RNA were collected after 6 days of culture. The experimental design of cell culture and stimulations is illustrated in Figure 10.
The effects of hyperglycemia on CD163 gene and surface expression were quantified by RT-qPCR and flow cytometry. The function of CD163 was analysed by flow cytometry using fluorescently labeled ligands (human hemoglobin complexed to haptoglobin1-1 and haptoglobin2-2) and uptake was visualized by confocal microscopy. The effect of hyperglycemia on the release of soluble CD163 and of the pro- and anti-inflammatory cytokines TNFα, IL-1β, IL-6, IL-8 and IL-1RA was detected by ELISA.

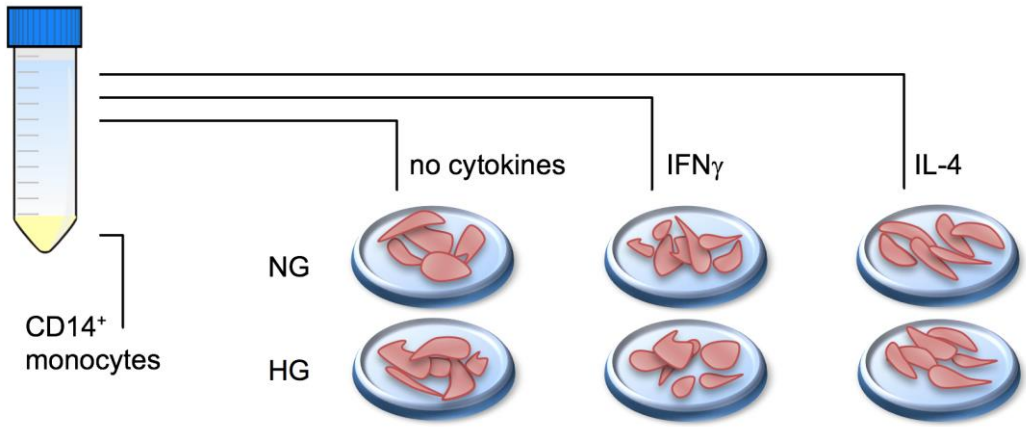


Figure 10. Schematic illustration of the utilised *in vitro* model system of macrophages. Isolation of monocytes from buffy coats was performed using density gradient centrifugation followed by $CD14^+$ magnetic separation. The serum-free medium used for culture with either normal (5 mM; NG) or high (25 mM; HG) glucose concentration was supplemented with M-CSF (5 ng/ml) and Dexamethasone (10^{-8} M). For further stimulation either no additional cytokines (M(Control)), IFNγ (M(IFNγ)), or IL-4 (M(IL-4)) were added. CD163 gene and surface expression, as well as sCD163 secretion, were analyzed on day 6 of cultivation by RT-qPCR, flow cytometry and ELISA.

3.2 Stimulation with IFNγ and IL-4 suppress CD163 gene expression in primary macrophages derived from healthy donor monocytes

Various pro- and anti-inflammatory mediators were found to tightly regulate the expression of CD163: Buechler et al. observed a decreased CD163 mRNA and cell surface expression when stimulating peripheral blood monocytes from healthy volunteers with the pro-inflammatory cytokine IFNγ[96], whereas the anti-inflammatory cytokine IL-4 alone failed to affect its expression[90, 95, 96].
To examine the effect of the aforementioned cytokines, we determined CD163 gene expression via RT-qPCR and CD163 surface expression by flow cytometry. $CD14^+$ monocytes were cultured for 6 days in serum-free medium in normal glucose conditions (5 mM) and stimulated with either IFNγ or IL-4.

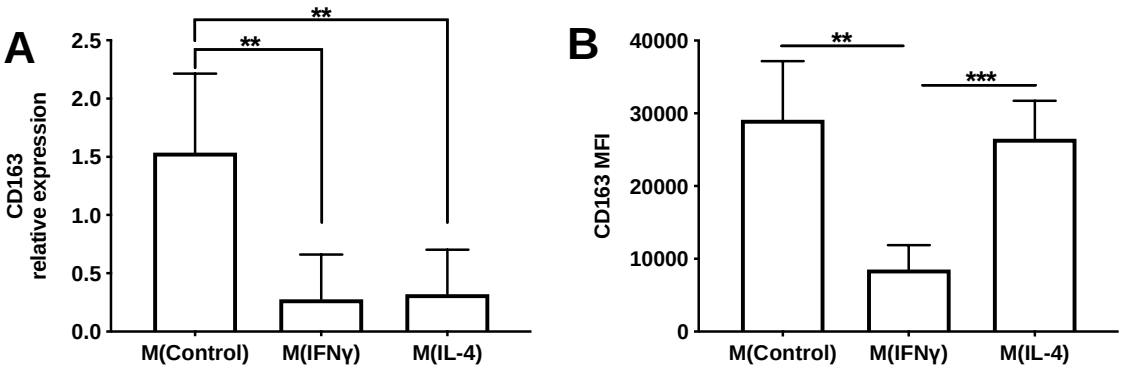


Figure 11. RT-qPCR and flow cytometry analysis of CD163 expression in differentially stimulated macrophages. Human monocyte-derived macrophages were cultured for 6 days and stimulated with either IFNγ or IL-4 or without further cytokines (M(Control)). **A:** relative expression levels of CD163 gene expression in normal glucose (5 mM) conditions detected by RT-qPCR. The graph represents mean values of 8 donors with standard deviations. **B:** mean fluorescence intensity of surface CD163 expression in normal glucose (5 mM) conditions detected by flow cytometry. The graph represents mean values of 6 donors with standard deviations. For statistical analysis of mRNA data, Wilcoxon matched-pairs rank test was used. For statistical analysis of flow cytometry data, a paired t test was used. * denotes statistical significance between different cytokine stimulations. (**p < 0.01; ***p < 0.001)

Both the stimulation with IFNγ and IL-4 had a statistically significant inhibitory effect on CD163 mRNA expression in comparison to non-stimulated macrophages (M(IFNγ): 5.5 times decrease, p-value < 0.01; M(IL-4): 4.8 times decrease, p-value < 0.01). Although no statistically significant difference in the mRNA expression level between IFNγ- and IL-4-stimulated macrophages could be detected, CD163 mRNA expression tended to be suppressed in M(IFNγ) (1.2 times) (Figure 11A). To determine whether the same expression patterns could be observed on cell surface level, surface expression of CD163 was determined using flow cytometry. In accordance to the observations on mRNA expression level, the level of fluorescence intensity of M(Control) was 3.4 times higher than the level of fluorescence intensity of M(IFNγ) (p-value < 0.01). In contrast to mRNA expression, CD163 surface expression on non-stimulated and IL-4-stimulated macrophages did not differ in a statistically significant way (Figure 11B).
In summary, IFNγ and IL-4 effectively suppressed CD163 gene expression in differentially stimulated macrophages.

3.3 RT-qPCR analysis of CD163 mRNA expression in differentially stimulated macrophages in hyperglycemic conditions

In a clinical study, Al Dubayee et al. demonstrated a significantly suppressed CD163 mRNA expression in patients with newly diagnosed type 2 diabetes when compared to PBMC of healthy subjects[166].
To examine the effect of hyperglycemia on CD163 gene expression in different macrophage subpopulations, we analyzed M(Control), M(IFNγ) and M(IL-4) of 8 healthy donors after culturing for 6 days in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. CD163 mRNA expression was detected by RT-qPCR.

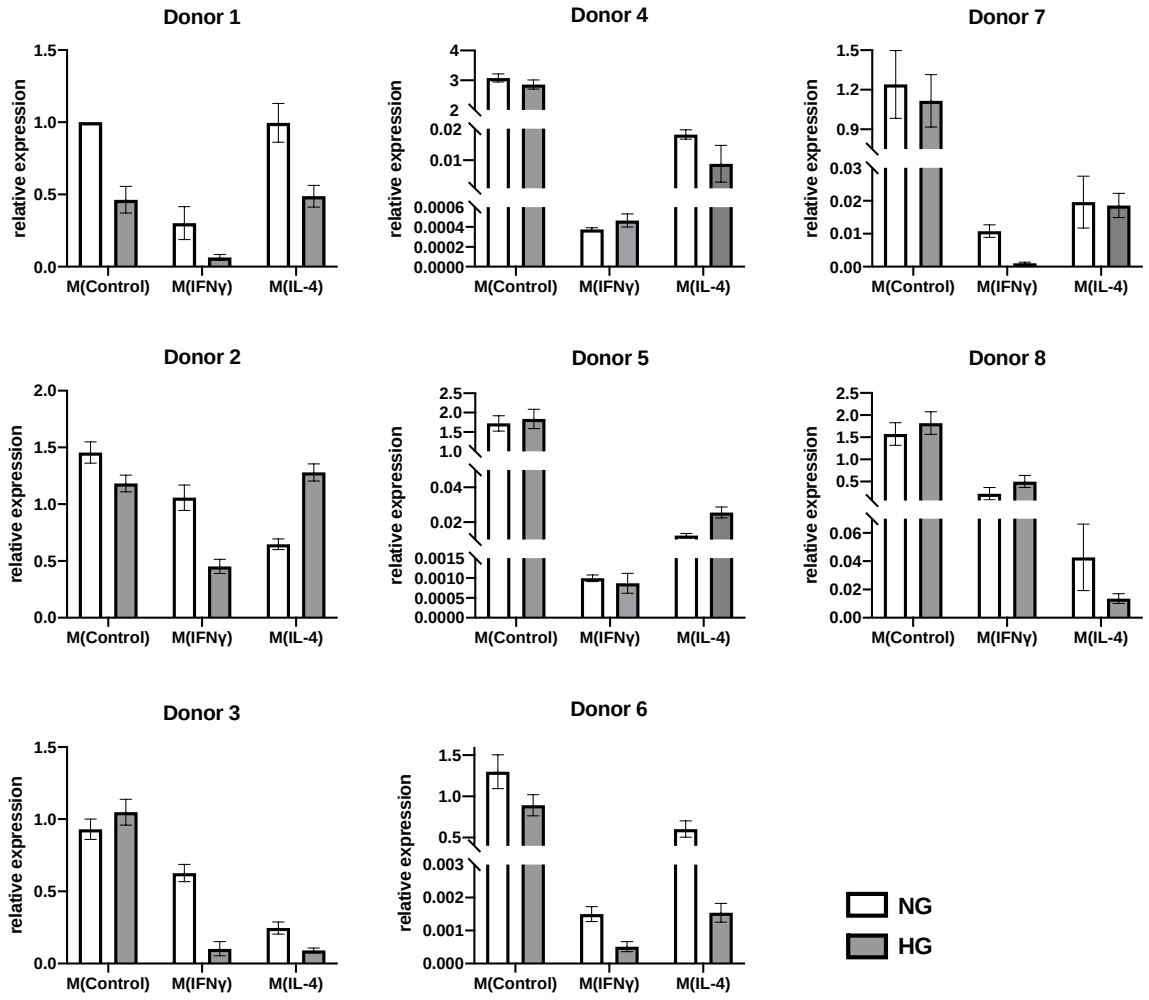


Figure 12. CD163 gene expression levels in differentially stimulated macrophages for individual donors. Relative expression of CD163 mRNA in normal glucose (5 mM, NG, white bars) and high glucose (25 mM, HG, grey bars) conditions for 8 individual healthy donors. The cells were cultured for 6 days in SFM and stimulated with either IFNγ or IL-4 or without further cytokines (M(Control)). CD163 mRNA expression levels were detected by RT-qPCR.

Figure 12 shows CD163 gene expression for 8 individual donors. In hyperglycemia, CD163 mRNA expression was decreased in 5 out of 8 donors in M(Control), in 6 out of 8 donors in M(IFNγ) and in 6 out of 8 donors in M(IL-4). Notwithstanding this tendency towards CD163 gene suppression in hyperglycemia, a high variability of the effect and extent of suppression was detected.

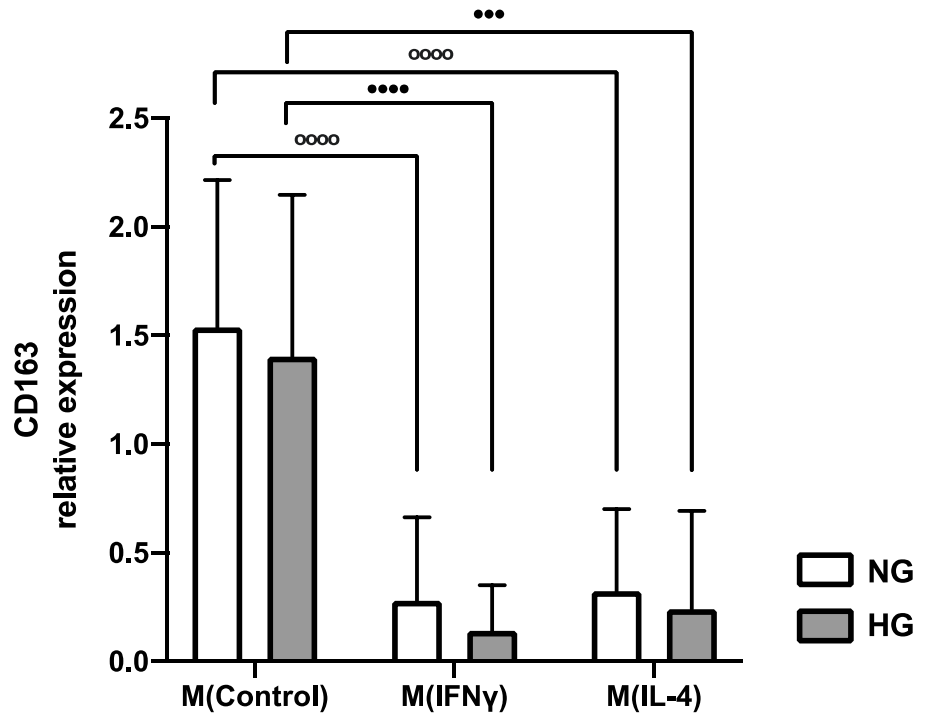


Figure 13. RT-qPCR analysis of CD163 gene expression levels in differentially stimulated macrophages. Relative expression levels of CD163 mRNA expression in normal glucose (5 mM, NG) and high glucose (25 mM, HG) conditions. Monocyte-derived macrophages were cultured for 6 days and stimulated with either IFNγ or IL-4 or without further cytokines (M(Control)). mRNA expression was detected by RT-qPCR. The graph represents mean values of 8 donors with standard deviations. For statistical analysis, Wilcoxon matched-pairs rank test was used. º denotes statistical significance between different cytokine stimulations in NG conditions (ººººp < 0.0001). • denotes statistical significance between different cytokine stimulations in HG conditions (•••p < 0.001, ••••p < 0.0001)

Figure 13 illustrates that both IFNγ and IL-4 induced a statistically significant downregulation of CD163 gene expression in normoglycemic conditions compared to M(Control) (IFNγ: 5.5 times (p-value < 0.0001); IL-4: 4.8 times (p-value < 0.0001)). Hyperglycemia enhanced this effect 1.99 times in M(IFNγ), however, statistical significance was not reached due to individual donor responses (Figure 12). Downregulation of CD163 mRNA expression in hyperglycemia could also be observed for M(IL-4), but did not reach the suppressive level of IFNγ (1.34 times).

In summary, hyperglycemia emphasizes the suppressing effect of IFNγ on CD163 gene expression, however this effect failed to reach statistical significance due to donor-specific responses.

3.4 Hyperglycemia enhances suppressing effect of IFNγ on CD163 surface expression in differentially stimulated macrophages

In accordance to Al Dubayee's clinical findings regarding CD163 mRNA expression, Levy et al. showed that PBMCs acquired from diabetic patients expressed significantly less CD163 on their cell surface than those collected from healthy controls[130, 166].
To examine the effect of hyperglycemia on CD163 surface expression on differentially stimulated primary macrophages *in vitro*, flow cytometry was used to detect CD163 surface expression of 6 healthy donors cultured in normal (5 mM) and high (25 mM) glucose conditions.

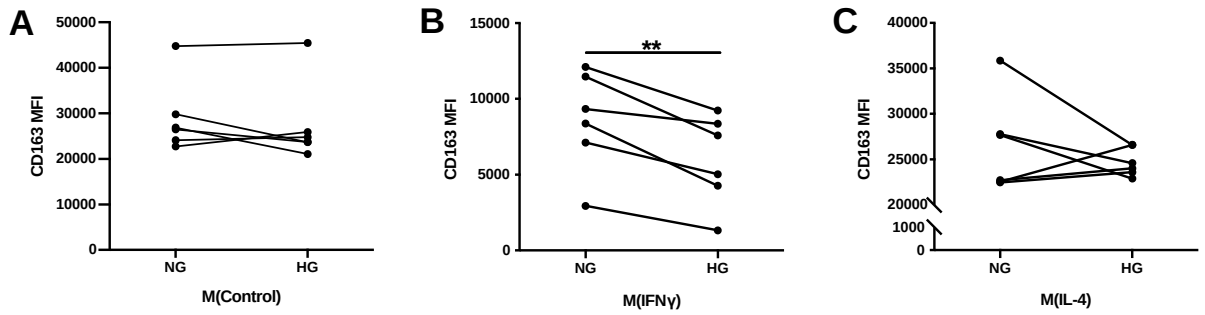


Figure 14. CD163 surface expression on differentially stimulated macrophages depicted for individual donors. CD163 MFI values of 6 healthy donors after 6 days of culture in normal (5 mM, NG) and high (25 mM, HG) glucose conditions detected by flow cytometry. Each line represents one donor, comparing the values for normal and high glucose conditions. The donors used for determining CD163 surface expression differ from the donors used for CD163 mRNA expression analysis. For statistical analysis, a paired t test was used. * denotes statistical significance between normal and high glucose conditions (**p < 0.01).

Whereas our data suggests that hyperglycemia has a rather donor-dependent effect on CD163 mRNA expression, Figure 14B depicts that hyperglycemic conditions elicited a statistically significant decline of CD163 surface expression in M(IFNγ) in all 6 donors (1.43 times, p-value < 0.01). Differing from this observation, hyperglycemia provoked mixed responses in M(Control) and M(IL-4): 3 out of 6 donors showed an increase, whereas the other 3 donors showed a decrease of CD163 surface expression.

Similar to our findings concerning CD163 gene expression, IFNγ induced a significant suppression of CD163 surface expression regardless of glucose level compared to M(Control) (NG: 3.4 times, p-value < 0.01; HG: 4.6 times, p-value < 0.01). Whereas no statistically significant difference of CD163 gene expression between M(IFNγ) and M(IL-4) was detected (Figure 13), the surface expression of CD163 was significantly suppressed in M(IFNγ) compared to M(IL-4) (NG: 3.1 times, p-value < 0.001; HG: 4.2 times, p-value < 0.001, Figure 15), suggesting that an additional mechanism in healing macrophages can support sufficient levels of surface CD163 to ensure the control over inflammatory responses.

In summary, hyperglycemia enhances the suppressing effect of IFNγ on CD163 surface expression.

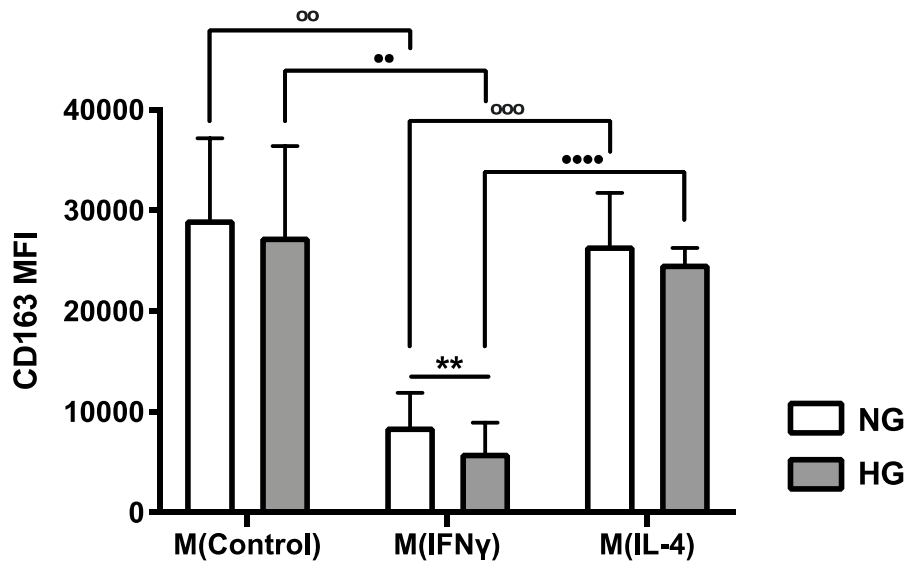


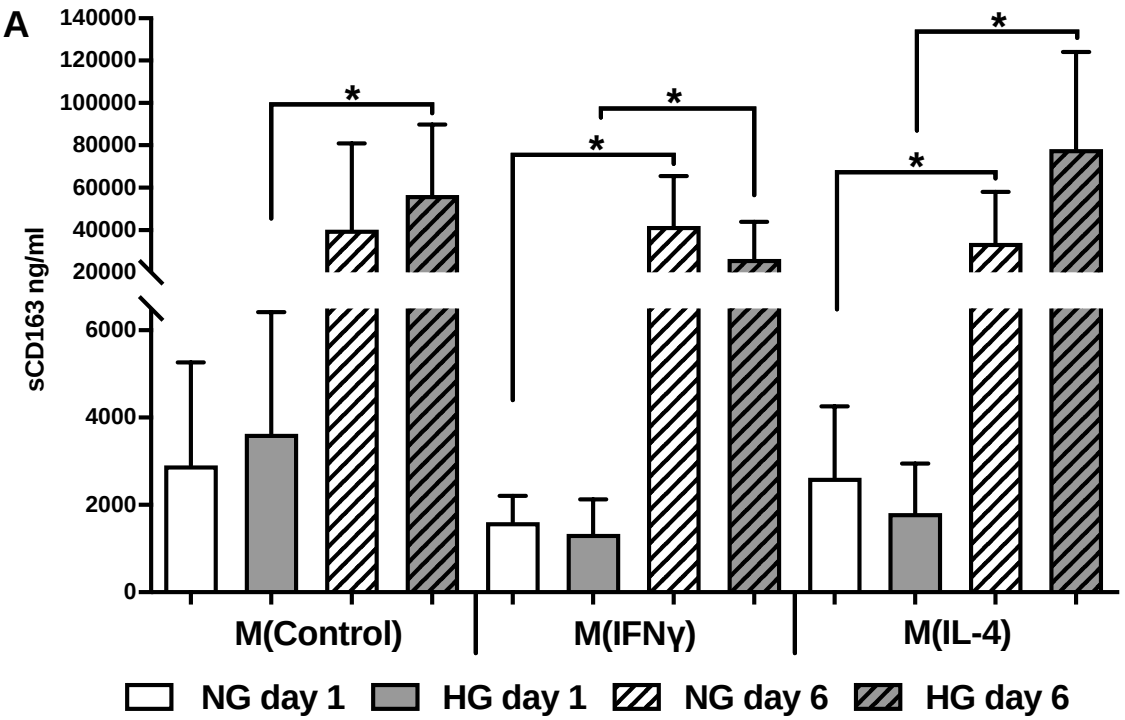
Figure 15. Flow cytometry analysis of CD163 surface expression on differentially stimulated macrophages. Monocyte-derived macrophages were stimulated with either IFNγ or IL-4 or without further cytokines (M(Control)) and cultured for 6 days in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. The graph represents mean values of 6 donors with standard deviations. For statistical analysis, a paired t test was used. * denotes statistical significance between normal and high glucose conditions (**p < 0.01). º denotes statistical significance between different cytokine stimulations in NG conditions (ººp < 0.01, ºººp < 0.001). • denotes statistical significance between different cytokine stimulations in HG conditions (••p < 0.01, ••••p < 0.0001)

3.5 Production of soluble CD163 is most efficient in pro-inflammatory conditions

The soluble form of CD163 (sCD163) is created by ectodomain-shedding from the cell surface, this mechanism being enhanced in inflammatory conditions[104]. Elevated serum levels of sCD163 have been described for type 2 diabetes mellitus, substantiating the negative correlation of sCD163 levels with insulin sensitivity reported by Fink et al.[84, 130].
To elucidate the question whether hyperglycemia itself, as key factor for the low-grade chronic inflammation in diabetic patients[26, 27, 31, 32], contributes to the shedding of membrane-bound CD163, we quantitatively determined the levels of soluble CD163 in the supernatants of differentially stimulated primary macrophages by ELISA. Human monocytes were differentiated for 6 days in SFM complemented with either IFNγ, IL-4 or without cytokines in normal (5 mM) and high (25 mM) glucose conditions. Supernatants were obtained on day 1 and day 6 of cell culture and the amount of sCD163 was detected by ELISA.

In general, sCD163 levels were significantly increased on day 6 compared to day 1 in all three subpopulations M(Control), M(IFNγ) and M(IL-4) in both normo- and hyperglycemia (Figure 16). The highest rate of sCD163 increase over 6 days-time was detected in M(IL-4) in hyperglycemia (43.1 times, p-value < 0.05, Figure 16A and 16C), with an increase of only 13.0 times in normoglycemic conditions (Figure 16A and 16B). In M(Control), a 13.8-fold increase of sCD163 was detected on day 6 than on day 1 in NG and a 15.6-fold increase in HG (p-value < 0.05).

A



B

	NG – day 1	**NG – day 6**
M(Control)	2905.7 ng/ml ± 2107.5 ng/ml	40224.7 ng/ml ± 36364.6 ng/ml
M(IFNγ)	1608.3 ng/ml ± 540.9 ng/ml	41994.4 ng/ml ± 21065.9 ng/ml
M(IL-4)	2620.3 ng/ml ± 1466.3 ng/ml	33989.4 ng/ml ± 21477.3 ng/ml

C

	HG – day 1	**HG – day 6**
M(Control)	3634.3 ng/ml ± 2486.4 ng/ml	56582.1 ng/ml ± 29731.0 ng/ml
M(IFNγ)	1336.5 ng/ml ± 712.5 ng/ml	26476.0 ng/ml ± 15679.2 ng/ml
M(IL-4)	1816.0 ng/ml ± 1014.8 ng/ml	78295.6 ng/ml ± 40959.2 ng/ml

Figure 16. Amount of sCD163 in supernatants secreted by differentially stimulated macrophages of 5 healthy donors. Monocyte-derived macrophages were stimulated with IFNγ, IL-4 or without further cytokines and cultured in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. sCD163 in supernatants was quantified by ELISA on day 1 and day 6 of cell culture. **A:** depiction of mean values of sCD163 with standard deviations of all stimulations; **B:** sCD163 mean values of 5 donors with standard deviations in NG; **C:** sCD163 mean values of 5 donors with standard deviations in HG. For statistical analysis, a paired t test was used. * denotes statistical significance between days 1 and 6 (*$p < 0.05$)

Contrasting to M(Control) and M(IL-4), in M(IFNγ), the increase of sCD163 was more pronounced in normoglycemia. Whereas in normoglycemic conditions sCD163 secretion on day 6 was 26.1 times higher than on day 1 (p-value < 0.05), it was only 19.8 times higher (p-value < 0.05) in hyperglycemic conditions.
Comparing M(Control), M(IFNγ) and M(IL-4), in normoglycemic conditions M(IFNγ) showed the highest relative increase of sCD163 over the time course of 6 days, whereas in hyperglycemic conditions, M(IL-4) showed the highest relative increase of sCD163.
There was no statistically significant difference of sCD163 secretion between normoglycemic and hyperglycemic conditions, neither on day 1, nor on day 6.

Although clinical studies reported elevated sCD163 serum levels in pro-inflammatory conditions, our data showed similar sCD163 levels for M(Control), M(IFNγ) and M(IL-4). However, taking into account that CD163 surface expression was significantly suppressed in M(IFNγ) compared to M(Control) and M(IL-4), this indicates that the shedding process is most efficient in pro-inflammatory conditions.

3.6 CD163-mediated internalization of Hb-Hp complexes is efficient in normo- and hyperglycemic conditions in M0 and M(IL-4) macrophages

The most comprehensively characterized function of CD163 is its homeostatic role in the scavenging of hemoglobin-haptoglobin (Hb-Hp) complexes which form due to intravascular hemolysis[86]. The two known allelic variants of human haptoglobin, Hp1 and Hp2, lead to three possible phenotypes, Hp1-1, Hp1-2 and Hp2-2[124]. The Hp1 allele creates dimeric variants of haptoglobin (Hp1-1), whereas the Hp2 allele was found to generate haptoglobin multimers (Hp2-2)[124, 171].
To study the functional consequences of the observed receptor regulation, we examined the effect of hyperglycemia on the scavenging function of CD163. Therefore, we quantitatively evaluated the uptake of fluorescently labeled hemoglobin-haptoglobin complexes (Hp1-1 and Hp2-2 variants) by differentially stimulated macrophages via flow cytometry. Human $CD14^+$ monocytes were differentiated for 6 days in serum-free medium complemented with either IFNγ, IL-4 or without cytokines in normal (5 mM) and high (25 mM) glucose conditions. On the sixth day of culturing, the cells were incubated for 30 min with Hb-Hp1-1 complexes (10 µg/ml), Hb-Hp2-2 complexes (10 µg/ml) or acetylated low-density lipoprotein (acLDL) (5 µg/ml), which served as positive endocytosis control. The two haptoglobin variants and acLDL were previously labeled with Alexa Fluor® 488 to determine the fluorescence intensity by flow cytometry. To visualize the endocytosis of Hb-Hp complexes and the co-localization of CD163, confocal microscopy was performed.

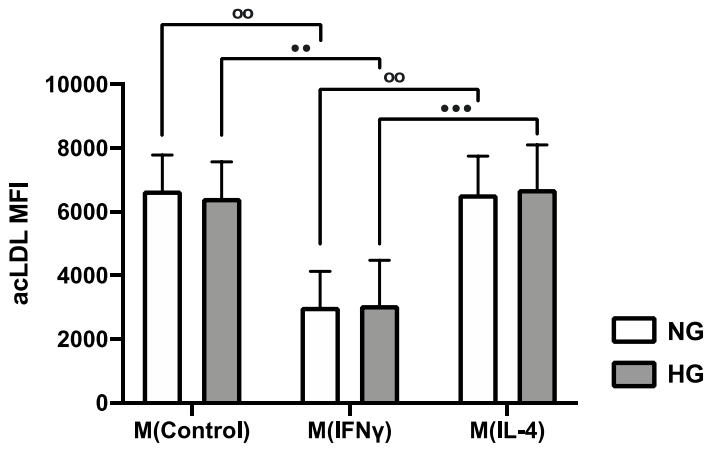


Figure 17. Flow cytometry analysis of Alexa Fluor® 488-labeled acLDL. Uptake of Alexa Fluor® 488-labeled acLDL was detected by flow cytometry in M(Control), M(IFNγ) and M(IL-4) after 6 days of culture in normal (5 mM, NG) and high (25 mM, HG) glucose conditions and incubation with acLDL for 30 min. Indicated is the mean fluorescence intensity with standard deviations of 5 donors. For statistical analysis, a paired t test was used. º denotes statistical significance between different cytokine stimulations in NG conditions ($^{oo}p < 0.01$). • denotes statistical significance between different cytokine stimulations in HG conditions ($^{••}p < 0.01$, $^{•••}p < 0.001$).

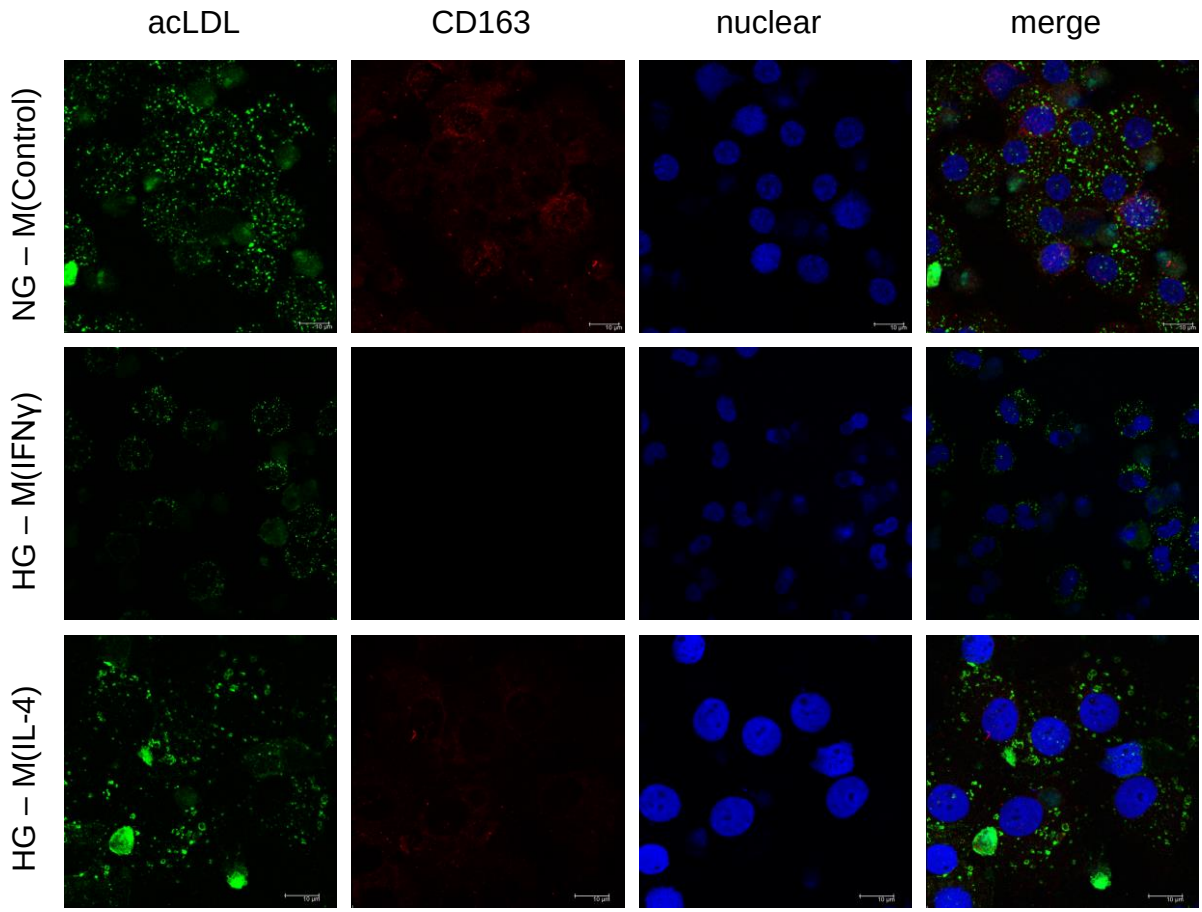


Figure 18. Confocal microscopy analysis of acLDL endocytosis. Monocyte-derived macrophages were cultured for 6 days in 5 mM (NG) and 25 mM (HG) glucose conditions and incubated for 30 min with Alexa Fluor® 488-labeled acLDL (shown in green). Macrophages were fixed with PFA. CD163 was detected with goat polyclonal anti-human CD163 antibody and Cy3-labeled donkey anti-goat secondary antibody (shown in red). Blue color visualizes nuclei stained with DRAQ5. The scale bar indicates 10 µm.

Acetylated low-density lipoprotein (acLDL) was used as a positive endocytosis control in M(Control), M(IFNγ) and M(IL-4). Figure 17 shows the mean fluorescence intensity of acLDL in all conditions used in this experimental design. The pattern of acLDL endocytosis closely resembled the pattern observed for the endocytosis of Hb-Hp-complexes, illustrating the limited endocytosis capacity of pro-inflammatory M(IFNγ) in comparison to M(Control) and M(IL-4). Figure 18 exemplarily depicts that acLDL uptake worked in both normal and high glucose conditions in M(Control), M(IFNγ) and M(IL-4).
The isotype control performed with ‘normal goat IgG’ antibody confirmed that there was no background signal (Figure 19).

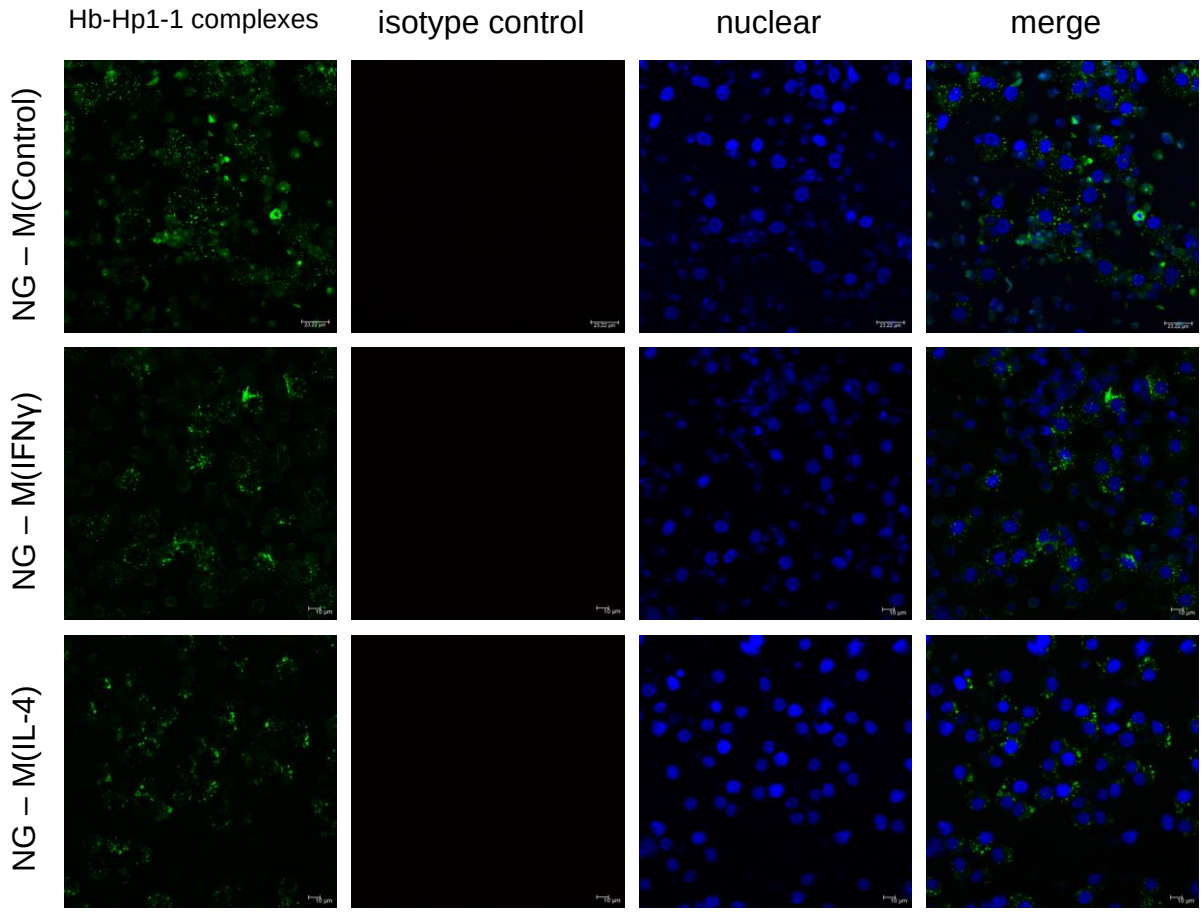
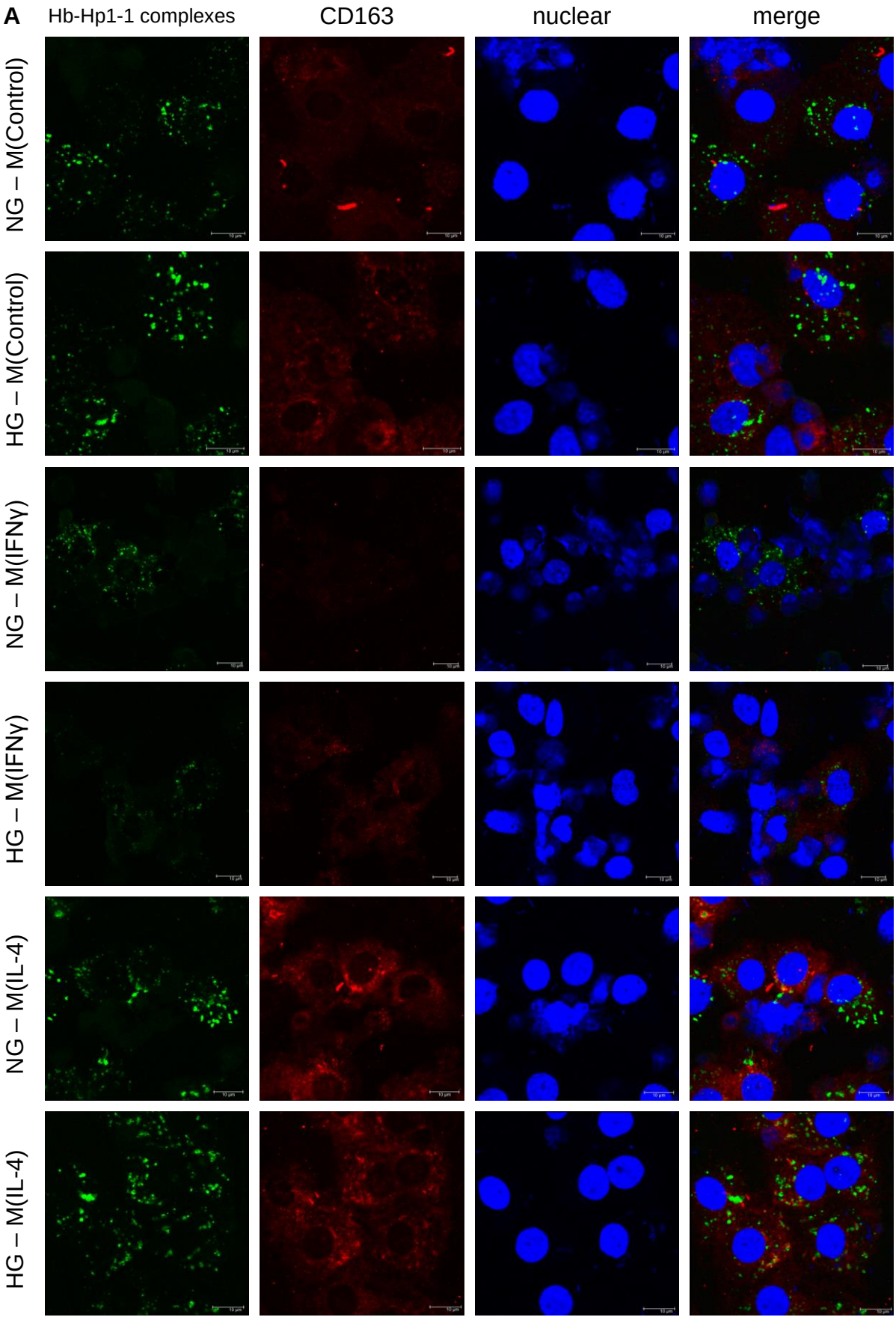


Figure 19. Control staining confirming specificity of CD163 antibody used for confocal microscopy analysis of endocytosis. Monocyte-derived macrophages were cultured for 6 days in serum-free medium and incubated for 30 min with Hb-Hp1-1 complexes. Cells were fixed with PFA. Hb-Hp complexes were previously labeled with Alexa Fluor® 488 (shown in green). The cells were stained with control IgG primary antibody and Cy3-labeled donkey anti-goat secondary antibody, and no signal in red was detected. Nuclei were stained with DRAQ5 (shown in blue). The scale bars indicate 10 µm and 23.22 µm (M(Control)).


A
Hb-Hp1-1 complexes
CD163
nuclear
merge
NG – M(Control)
HG – M(Control)
NG – M(IFNγ)
HG – M(IFNγ)
NG – M(IL-4)
HG – M(IL-4)

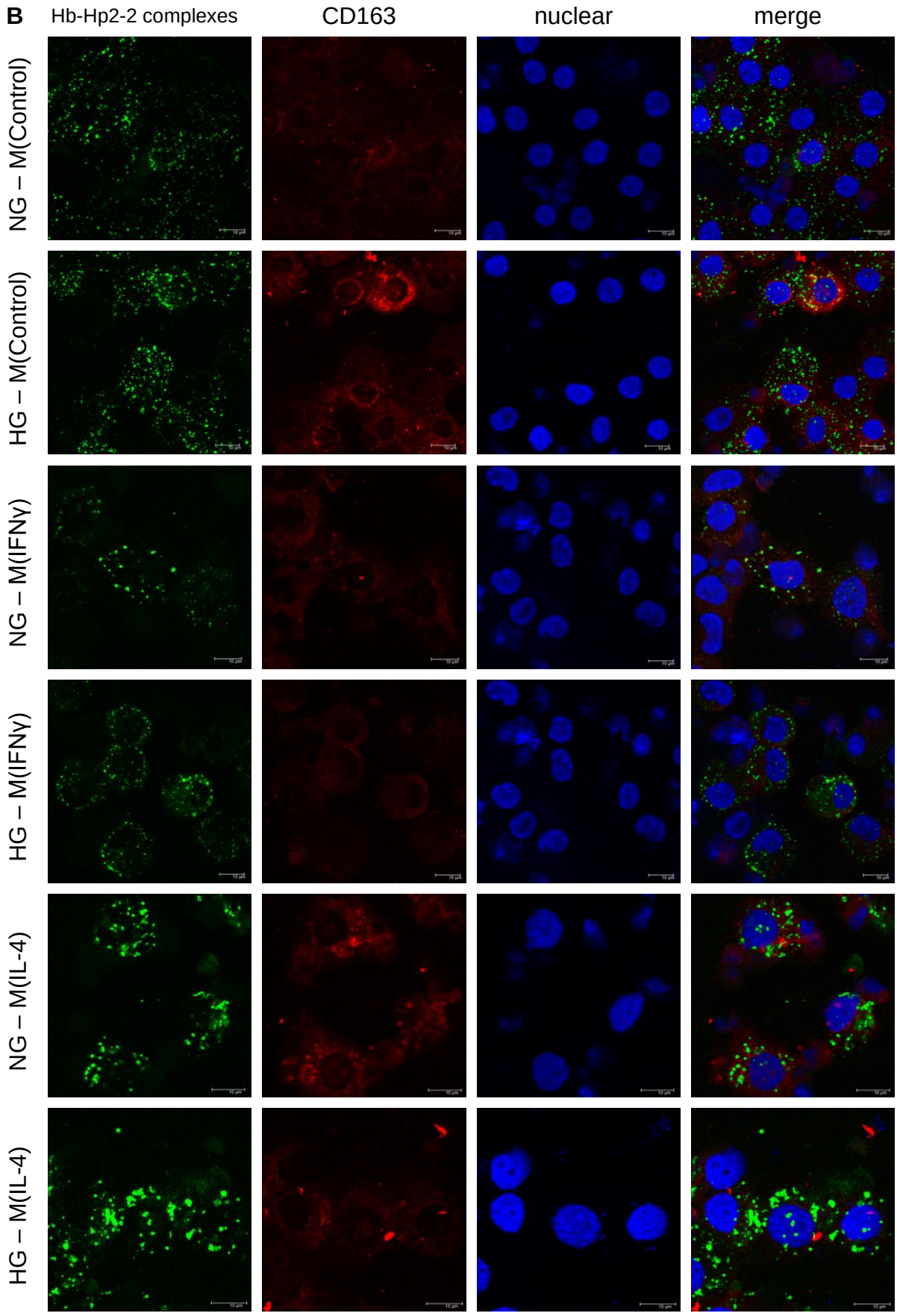

B
Hb-Hp2-2 complexes
CD163
nuclear
merge
NG – M(Control)
HG – M(Control)
NG – M(IFNγ)
HG – M(IFNγ)
NG – M(IL-4)
HG – M(IL-4)

Figure 20. Confocal microscopy analysis of endocytosis of Hb-Hp-Alexa Fluor® 488 complexes via CD163. Monocyte-derived macrophages were cultured for 6 days in serum-free medium in normal (5 mM, NG) and high (25 mM, HG) glucose conditions and incubated for 30 min with Hb-Hp1-1 and Hb-Hp2-2 complexes. Cells were fixed with PFA. Hb-Hp complexes were previously labeled with Alexa Fluor® 488 (shown in green), CD163 was detected with goat polyclonal anti-human CD163 antibody and Cy3-labeled donkey anti-goat secondary antibody (shown in red). Nuclei were stained with DRAQ5 (shown in blue). Yellow-orange color indicates co-localization of Hb-Hp complexes and CD163. **A:** endocytosis of Hb-Hp1-1 complexes; **B:** endocytosis of Hb-Hp2-2 complexes. The scale bar indicates 10 µm.

The internalization of Hb-Hp1-1 and Hb-Hp2-2 complexes was visualized by confocal microscopy (Figure 20) which confirmed efficient endocytosis of complexes in M(Control) and M(IL-4), and only residual endocytosis in M(IFNγ), correlating to the levels of CD163 surface expression (Figure 15). Confocal microscopy showed that CD163 is distributed throughout the cytoplasm and organized in vesicles. No visually detectable difference between normal and high glucose conditions within the respective subpopulations could be observed. When comparing the two haptoglobin variants, a stronger Alexa Fluor® 488 signal of Hb-Hp2-2 than Hb-Hp1-1 complexes could be noted.

Figure 21 shows the varying capacity of Hb-Hp complex uptake of the different macrophage subpopulations M(Control), M(IFNγ) and M(IL-4) cultured in either normal (5 mM) or high (25 mM) glucose conditions.
To begin with – as a confirmation of immunofluorescence analysis – the mean fluorescence intensity level of Hb-Hp2-2 complexes detected via flow cytometry was generally higher than the MFI level of Hb-Hp1-1 complexes (Figure 21A vs. 21B).
Even though the haptoglobin variants Hp1-1 and Hp2-2 differ considerably in their molecular structure[118], we observed the same uptake pattern for both complexes.
In comparison to M(IFNγ), M(Control) and M(IL-4) efficiently internalized both Hb-Hp1-1 (M(IL-4) compared to M(IFNγ): fold change 3.7, p-value <0.05, Figure 21A) and Hb-Hp2-2 complexes (M(IL-4) compared to M(IFNγ): fold change 4.7, p-value <0.05, Figure 21B) in normoglycemia. In hyperglycemic conditions, too, uptake of Hb-Hp1-1 and Hb-Hp2-2 was significantly impaired in M(IFNγ) compared to M(Control) and M(IL-4) (Hb-Hp1-1: M(IL-4) compared to M(IFNγ): fold change 3.6, p-value <0.01, Figure 21A; Hb-Hp2-2: M(IL-4) compared to M(IFNγ): fold change 4.2, p-value <0.01, Figure 21B), which correlated with significantly suppressed surface levels of CD163 in M(IFNγ) (Figure 15). There was no difference in endocytosis efficiency of Hb-Hp1-1 and Hb-Hp2-2 in hyperglycemic conditions.

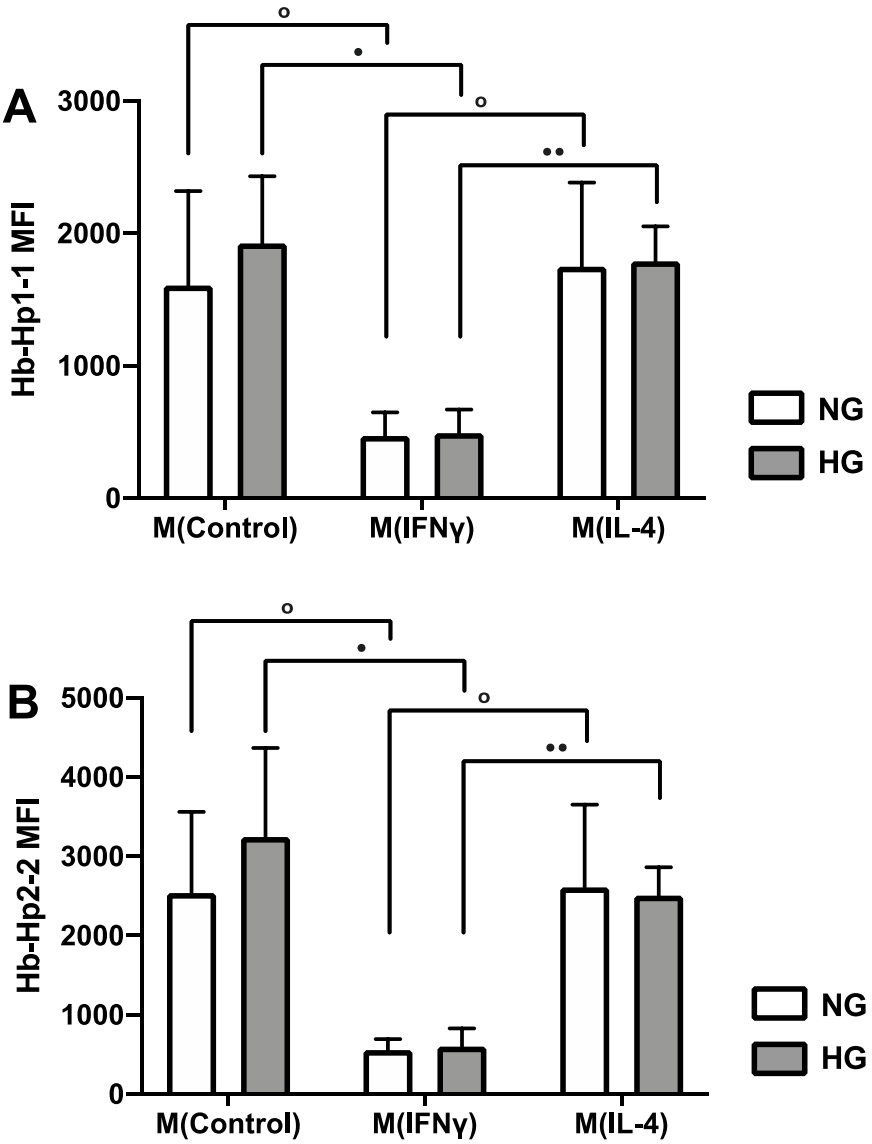


Figure 21. Flow cytometry analysis of endocytosis of Alexa Fluor® 488-labeled hemoglobin-haptoglobin complexes. Uptake of Alexa Fluor® 488-labeled Hb-Hp1-1 and Hb-Hp2-2 complexes was measured by flow cytometry in non-stimulated (M(Control)), IFNγ-stimulated and IL-4-stimulated monocyte-derived macrophages. After cultivation for 6 days in normal (5 mM, NG) and high (25 mM, HG) glucose conditions, the macrophages were incubated with Hb-Hp1-1 or Hb-Hp2-2 complexes for 30 min. **A:** stimulation with Hb-Hp1-1 complexes; **B:** stimulation with Hb-Hp2-2 complexes. Graphs depict the mean fluorescence intensity of 5 donors with standard deviations. To obtain the values for MFI shown on the graphs, MFI values of 'no ligand' controls were subtracted from the MFI values after Hb-Hp complex uptake to exclude background signal. For statistical analysis, a paired t test was used. º denotes statistical significance between different cytokine stimulations in NG conditions (º$p < 0.05$). • denotes statistical significance between different cytokine stimulations in HG conditions (•$p < 0.05$, ••$p < 0.01$)

Altogether, the uptake efficiency was dependent on macrophage polarization which defines the levels of CD163 surface exposure. There was no functional impairment of endocytosis of Hb-Hp1-1 and Hb-Hp2-2 complexes via CD163 in hyperglycemia.

3.7 Hyperglycemia enhances release of inflammatory cytokines in response to CD163-mediated scavenging of hemoglobin-haptoglobin complexes in M(IFNγ)

It has not been elucidated yet whether CD163 expression on macrophages is related to an anti-inflammatory activation profile or rather promoting a pro-inflammatory setting: on the one hand, it was suggested that cross-linking of cell-surface CD163 triggered protein-kinase C- and casein-kinase-dependent macrophage activation resulting in the release of pro-inflammatory cytokines, such as IL-1β and IL-6[90, 144, 145]. On the other hand, Schaer et al. stated that the contact to hemoglobin is able to provoke an anti-inflammatory response in macrophages, indicated by an upregulation of the cytoprotective heme oxygenase (HO-1)[135].
Additionally, the haptoglobin variants were found to have different functional properties: Hp1-1 was reported to elicit a faster clearance of hemoglobin and to have a superior antioxidative capacity compared to Hp2-2[126, 127].
Therefore, we analyzed whether the uptake of Hb-Hp complexes is able to affect the inflammatory activation of macrophages. $CD14^+$ monocytes of 5 healthy donors were differentiated for 6 days in serum-free medium complemented with IFNγ in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. On day 6, macrophages were stimulated with Hb-Hp1-1 complexes or Hb-Hp2-2 complexes in the concentrations 1 µg/ml or 10 µg/ml, as the haptoglobin level varies individually from 0.3-3 mg/ml and – as an acute-phase protein – its physiological plasma levels increase 2-5-fold in inflammation [118, 121-123]. We have selected a set of inflammatory cytokines (TNFα, IL-1β, IL-6, IL-8 and IL-1 receptor antagonist (IL-1RA)) as a read-out. The concentration of these cytokines was identified 6h and 24h after stimulation with Hb-Hp1-1 or Hb-Hp2-2 complexes (Figure 22). After collecting supernatants, we harvested the cells for RT-qPCR analysis of mRNA expression of the following genes: CD163, TNFα, IL-1β, IL-6, IL-8 and IL-1RA. Moreover, we performed an analysis of CD163 surface expression via flow cytometry in order to correlate the determined cytokine levels to the overall scavenging capacity of the individual donors.

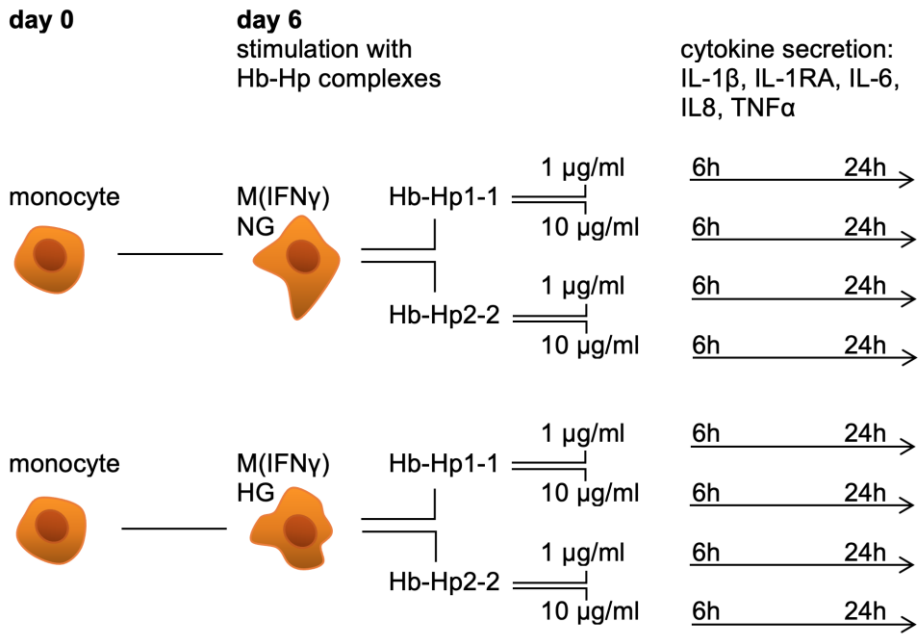


Figure 22. Experimental design to determine inflammatory reaction of M(IFNγ) after stimulation with hemoglobin-haptoglobin complexes.

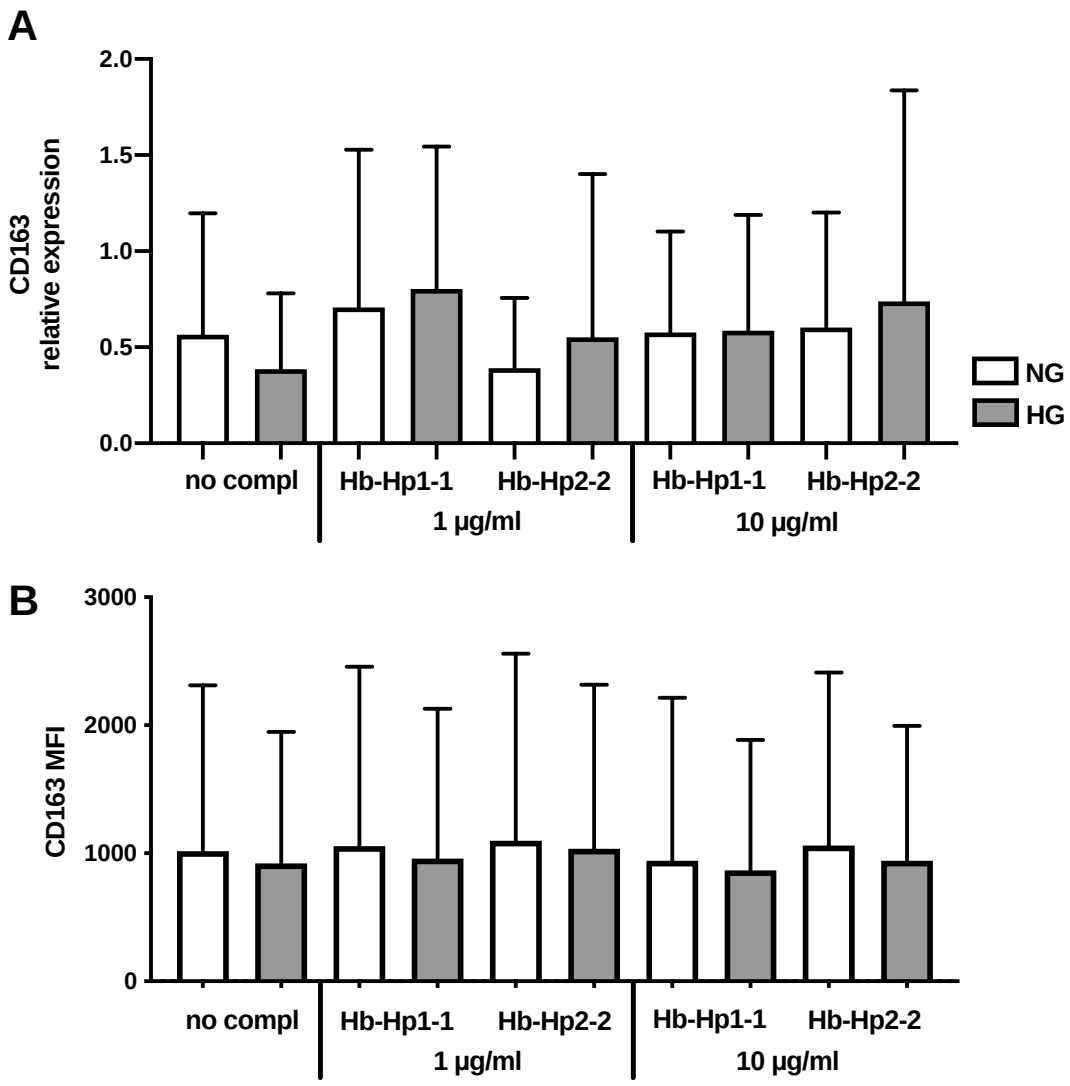


Figure 23. RT-qPCR analysis of gene expression and flow cytometry analysis of surface expression of CD163 in IFNγ-stimulated macrophages. Human monocyte-derived macrophages were stimulated with IFNγ and cultured for 6 days in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. On day 6, Hb-Hp1-1 and Hb-Hp2-2 complexes in 2 different concentrations (1 µg/ml or 10 µg/ml) were added to M(IFNγ) or, serving as a negative control, no complexes ('no compl') were added. Macrophages were harvested 24h after stimulation with the complexes. **A:** CD163 gene expression level analyzed by RT-qPCR; **B:** surface expression of CD163 analyzed by flow cytometry. All graphs represent mean values of 5 donors with standard deviations. For statistical analysis, a paired t test was performed.

To begin with, CD163 gene expression levels in M(IFNγ) were analyzed. They remained relatively stable, neither being significantly influenced by high glucose conditions, nor by stimulation with the two differing haptoglobin variants (Figure 23A). CD163 gene expression in M(IFNγ) without complex stimulation was decreased 1.5 times in hyperglycemia (Figure 23A), mirroring the results obtained in earlier analysis (Figure 13). CD163 gene expression levels corresponded to CD163 surface expression, also lacking a regulation caused by glucose concentration or hemoglobin-haptoglobin complex stimulation (Figure 23B).

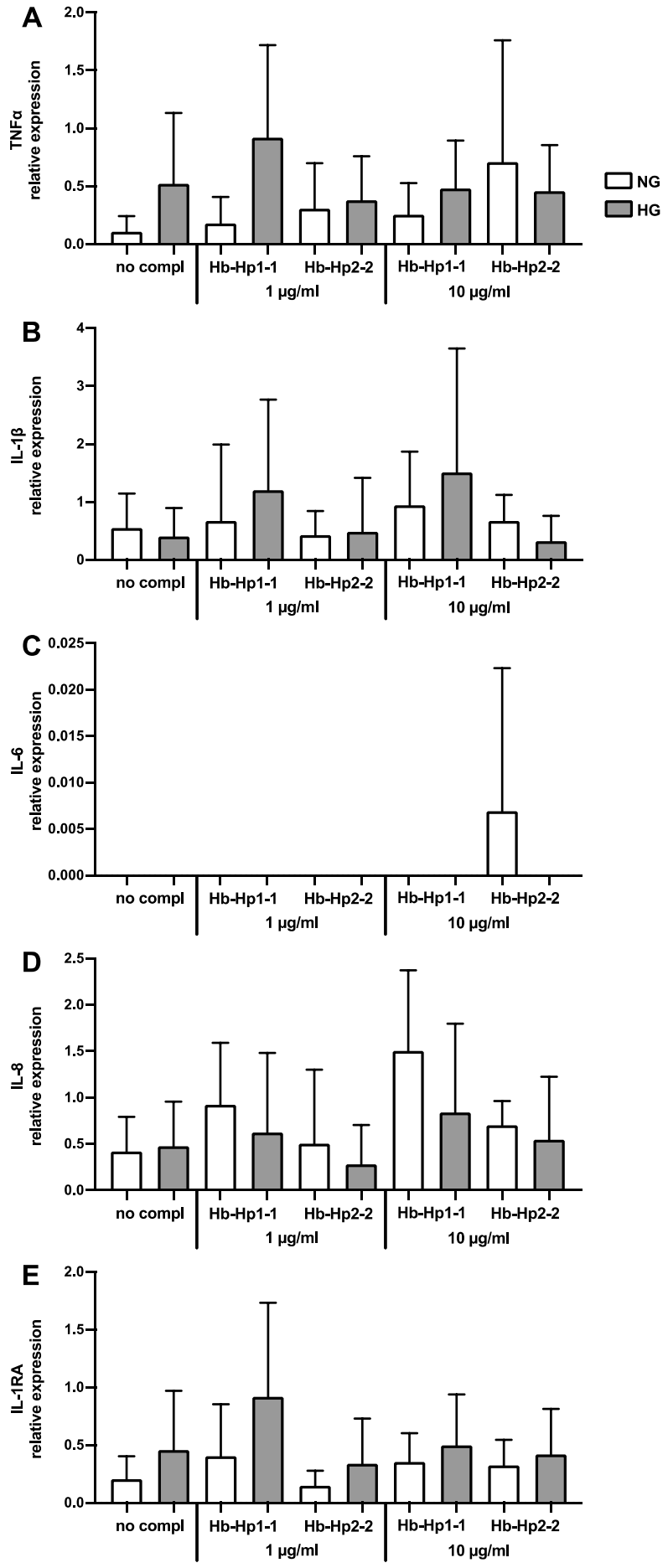

A
TNFα
relative expression
2.0
1.5
1.0
0.5
0.0
NG
HG
no compl
Hb-Hp1-1
Hb-Hp2-2
Hb-Hp1-1
Hb-Hp2-2
1 µg/ml
10 µg/ml
B
IL-1β
relative expression
4
3
2
1
0
C
IL-6
relative expression
0.025
0.020
0.015
0.010
0.005
0.000
D
IL-8
relative expression
2.5
2.0
1.5
1.0
0.5
0.0
E
IL-1RA
relative expression

Figure 24. RT-qPCR analysis of mRNA expression of the cytokines TNFα, IL-1β, IL-6, IL-8 and IL-1RA in IFNγ-stimulated macrophages. Human monocyte-derived macrophages were stimulated with IFNγ and cultured for 6 days in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. On day 6, Hb-Hp1-1 and Hb-Hp2-2 complexes in 2 different concentrations (1 µg/ml and 10 µg/ml) were added to M(IFNγ) or, serving as a negative control, no complexes ('no compl') were added. Gene expression levels of the above-mentioned cytokines were analyzed by RT-qPCR 24h after stimulation with the complexes. **A:** TNFα expression; **B:** IL-1β expression; **C:** IL-6 expression; **D:** IL-8 expression; **E:** IL-1RA expression. All graphs represent mean values of 5 donors with standard deviations. For statistical analysis, a paired t test was performed.

Gene expression levels of the selected cytokines TNFα, IL-1β, IL-6, IL-8 and IL-1RA are shown in Figure 24.

The gene expression pattern of TNFα showed an upregulation in HG after stimulation with Hb-Hp1-1 complexes (1 µg/ml: 5.1 times; 10 µg/ml: 1.9 times) and Hb-Hp2-2 complexes (1 µg/ml: 1.2 times). Contrarily, after stimulation with 10 µg/ml Hb-Hp2-2 complexes, a 1.6-times suppression was detected. Hyperglycemia itself elicited an upregulation of 4.8 times ('no compl'). However, due to individual donor-responses, statistical significance was not reached (Figure 24A).

Figure 24B illustrates that expression levels of IL-1β did not change in a statistically significant way in hyperglycemia. However, a tendency towards an increased expression was observed after Hb-Hp complex uptake. Here, the increase was more pronounced upon the stimulation with Hb-Hp1-1 complexes (1 µg/ml: 1.8 times; 10 µg/ml: 1.6 times) compared to Hb-Hp2-2 complexes (1 µg/ml: 1.1 times increase). After stimulation with 10µg/ml of Hb-Hp2-2 complexes, a 2.1-fold decrease was detected.

Figure 24C depicts that IL-6 mRNA expression was very low (Ct > 40) and could only be detected in normal glucose conditions upon the stimulation with Hb-Hp2-2 complexes.

For IL-8, decreased gene expression levels were found in hyperglycemic conditions after stimulation with Hb-Hp1-1 complexes (1 µg/ml: 1.5 times; 10 µg/ml: 1.8 times) and Hb-Hp2-2 complexes (1 µg/ml: 1.8 times; 10 µg/ml: 1.3 times) (Figure 24D).

IL-1RA gene expression (Figure 24E) showed a tendency to higher expression levels in hyperglycemia in M(IFNγ) without complex stimulation (2.2 times), stimulation with Hb-Hp1-1 complexes (1 µg/ml: 2.3 times; 10 µg/ml: 1.4 times) and Hb-Hp2-2 complexes (1 µg/ml: 2.3 times; 10 µg/ml: 1.3 times). However, the observed regulation failed to be statistically significant due to donor-dependent responses.

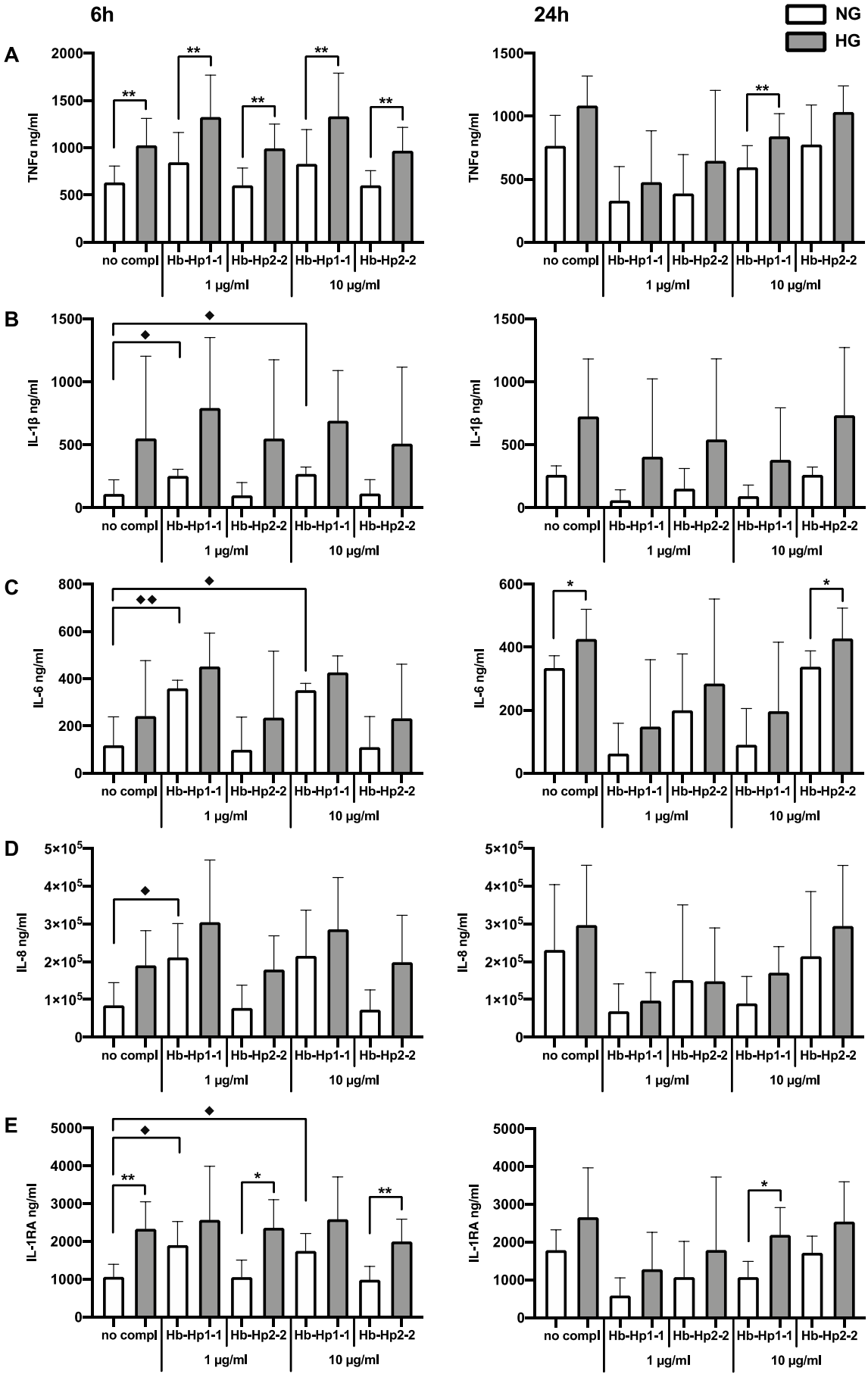

6h
24h
NG
HG
A
B
C
D
E
TNFα ng/ml
IL-1β ng/ml
IL-6 ng/ml
IL-8 ng/ml
IL-1RA ng/ml
no compl
Hb-Hp1-1
Hb-Hp2-2
Hb-Hp1-1
Hb-Hp2-2
1 µg/ml
10 µg/ml

Figure 25. Amounts of TNFα, IL-1β, IL-6, IL-8 and IL-1RA detected in the supernatants secreted by IFNγ-stimulated human primary macrophages. Monocyte-derived macrophages were stimulated with IFNγ and cultured for 6 days in normal (5 mM, NG) and high (25 mM, HG) glucose conditions. On day 6, Hb-Hp1-1 and Hb-Hp2-2 complexes in 2 different concentrations (1 µg/ml or 10 µg/ml) were added to M(IFNγ) or, serving as a negative control, no complexes ('no compl') were added. The amounts of TNFα, IL-1β, IL-6, IL-8 and IL-1RA were detected in the supernatants by the according ELISAs 6h and 24h after stimulation with Hb-Hp complexes. **A:** TNFα secretion; **B:** IL-1β secretion; **C:** IL-6 secretion; **D:** IL-8 secretion; **E:** IL-1RA secretion (mean values of 5 donors with standard deviations). For statistical analysis, a paired t test was used. * denotes statistical significance between normal and high glucose conditions (*$p < 0.05$, **$p < 0.01$) ◆ denotes statistical significance between no stimulation and stimulation with Hb-Hp1-1 complexes after 6h (◆$p < 0.05$, ◆◆$p < 0.01$)

To determine whether the observed regulation of cytokine expression could also be observed on protein level, the secretion of the same cytokines was analyzed by ELISA (Figure 25).
Hyperglycemia enhanced the release of all tested cytokines in the absence and also in the presence (after 6h and 24h) of Hb-Hp complex stimulation (Figures 25A-E).

Release of acute-phase inflammatory cytokine TNFα was stimulated by Hb-Hp1-1 complexes after 6h in NG, this effect being significantly enhanced by hyperglycemia (Figure 25A). The increase in hyperglycemic conditions was similar for Hb-Hp1-1 complex concentrations 1 µg/ml (1.57 times, p-value < 0.01) and 10 µg/ml (1.61 times, p-value < 0.01). However, 24h after the stimulation with Hb-Hp1-1 complexes, a suppression of TNFα secretion was detected in both normo- and hyperglycemic conditions, with a statistically significant effect in hyperglycemia 24h after stimulation with 10 µg/ml Hb-Hp1-1 complexes (1.29 times suppression, p-value < 0.05).
The tendency to a decreased TNFα secretion after 24h was also detected in normoglycemia after stimulation with 1 µg/ml Hb-Hp1-1 complexes (2.32 times) and after stimulation with 10 µg/ml Hb-Hp1-1 complexes (1.29 times). In hyperglycemia, a similar decrease of TNFα release after 24h was observed (1 µg/ml: 2.27 times).
In contrast to that, hyperglycemia significantly enhanced TNFα release 6h after stimulation with Hb-Hp2-2 complexes (1 µg/ml: 1.65 times, p-value < 0.01; 10 µg/ml: 1.61 times, p-value < 0.01), similar to the effect on non-stimulated M(IFNγ) (1.62 times enhancement, p-value < 0.01). The stimulation with Hb-Hp2-2 complexes per se had no statistically significant effect on TNFα release compared to non-stimulated macrophages.
6h after the stimulation with Hb-Hp2-2 complexes, TNFα secretion level was not altered in comparison to non-stimulated macrophages in both normo- and hyperglycemic conditions. 24h after the stimulation with Hb-Hp2-2 complexes, a suppression of TNFα secretion was measured in both normo- and hyperglycemia, with the strongest suppression detected in normoglycemia after stimulation with 1 µg/ml Hb-Hp2-2 complexes (1.98 times). In hyperglycemia, the suppression of TNFα release was not as pronounced as in normoglycemia (1 µg/ml: 1.68 times). However, the effect failed to be statistically significant due to donor-dependent responses.
A statistically significant increase in TNFα secretion could only be detected 6h after the stimulation with Hb-Hp complexes in hyperglycemia, regardless of the present haptoglobin variant.

The release of pro-inflammatory IL-1β was significantly increased 6h after the stimulation with Hb-Hp1-1 complexes in normoglycemic conditions for both complex concentrations (1 µg/ml: 2.29 times, p-value < 0.05; 10 µg/ml: 2.43 times, p-value < 0.05, Figure 25B).

Hyperglycemia elicited a strong enhancement of IL-1β release after stimulation with Hb-Hp complexes regardless of Hp variant – the strongest effect being detected 24h after stimulation with 1 µg/ml Hb-Hp1-1 complexes (6.75 times). A higher concentration of Hb-Hp1-1 complexes (10 µg/ml) elicited a 4.09-fold increase. However, statistical significance was not reached due to individual donor responses. The enhancement after 6h was more pronounced for the stimulation with 1 µg/ml Hb-Hp1-1 complexes (3.13 times) than 10 µg/ml Hb-Hp1-1 complexes (2.57 times), but was not as strong as after 24h.
Hyperglycemia enhanced IL-1β secretion 5.56 times 6h after stimulation with 1 µg/ml Hb-Hp2-2 complexes and 4.52 times after stimulation with 10 µg/ml Hb-Hp2-2 complexes. 24h after the stimulation with 1 µg/ml Hb-Hp2-2 complexes, IL-1β release was enhanced 3.58 times, and after stimulation with 10 µg/ml Hb-Hp2-2 complexes, IL-1β release was enhanced 2.81 times.
Regarding the dynamics of IL-1β secretion, 24h after the stimulation with Hb-Hp1-1 complexes, IL-1β secretion was suppressed in both normo- and hyperglycemia, with the strongest suppression detected in normoglycemia after stimulation with 1 µg/ml Hb-Hp1-1 complexes (4.21 times, p-value < 0.01) and 2.89 times after stimulation with 10 µg/ml Hb-Hp1-1 complexes (p-value < 0.01). In hyperglycemia, the suppression of IL-1β release was not as pronounced as in normoglycemia (1 µg/ml: 1.95 times, p-value < 0.01; 10 µg/ml: 1.81 times, p-value < 0.05).
The stimulation with 1 µg/ml or 10 µg/ml Hb-Hp2-2 complexes did not elicit any effect on IL-1β release compared to non-stimulated M(IFNγ), neither in normoglycemic, nor in hyperglycemic conditions. Enhanced IL-1β secretion was detected 24h compared to 6h after stimulation with 10 µg/ml Hb-Hp2-2 complexes (NG: 2.32 times, p-value < 0.01; HG: 1.44 times, p-value < 0.01).
Overall, the enhancing effect of hyperglycemia on the secretion of IL-1β was strongest after 24h after the stimulation with 1 µg/ml of Hb-Hp1-1 complexes (6.75 times).

In normoglycemic conditions, the release of pro-inflammatory cytokine IL-6 was significantly enhanced 6h after the stimulation with Hb-Hp1-1 complexes (1 µg/ml: 3.03 times, p-value < 0.01; 10 µg/ml: 2.96 times, p-value < 0.05 times, Figure 25C). In hyperglycemic conditions, the release of IL-6 tended to be enhanced, however, a statistically significant effect could only be detected 24h after stimulation with 10 µg/ml Hb-Hp2-2 complexes (1.26 times enhancement, p-value < 0.05).
Hyperglycemia tended to increase IL-6 release 6h after stimulating M(IFNγ) with Hb-Hp1-1 complexes compared to normoglycemia (1 µg/ml: 1.26 times; 10 µg/ml: 1.21 times). 24h after the stimulation with Hb-Hp1-1 complexes, a clear, however not statistically significant, enhancement of IL-6 release in hyperglycemia was detected (1 µg/ml: 2.38 times; 10 µg/ml: 2.18 times).
After stimulation with Hb-Hp2-2, hyperglycemia elicited a tendency to an enhanced IL-6 secretion, with an enhancement of 2.37 times 6h after stimulation with 1 µg/ml Hb-Hp2-2 complexes and 2.1 times after stimulation with 10 µg/ml Hb-Hp2-2 complexes. 24h after the stimulation with 1 µg/ml Hb-Hp2-2 complexes, IL-6 release was enhanced 1.43 times, and after stimulation with 10 µg/ml Hb-Hp2-2 complexes, a 1.26-times enhancement of IL-6 secretion was detected (p-value < 0.05).
Regarding the dynamics of IL-6 release over time, 24h after the stimulation with Hb-Hp1-1 complexes, a suppression of IL-6 secretion was measured in both normo- and hyperglycemia, with the strongest suppression detected in normoglycemia after stimulation with 1 µg/ml Hb-Hp1-1 complexes (5.78 times, p-value < 0.001) and 3.9 times suppression after stimulation with 10 µg/ml Hb-Hp1-1 complexes (p-value < 0.01). In hyperglycemia, the suppression of IL-6 release was not as pronounced as in

normoglycemia (1 µg/ml: only 3.05 times decrease, p-value < 0.001; 10 µg/ml: 2.17 times decrease, p-value < 0.05).
The stimulation with 1 µg/ml or 10 µg/ml Hb-Hp2-2 complexes did not elicit any statistically significant effect on IL-6 release compared to non-stimulated M(IFNγ), neither in normoglycemic, nor in hyperglycemic conditions. Enhanced IL-6 secretion was detected 24h compared to 6h after stimulation with 10 µg/ml Hb-Hp2-2 complexes in normoglycemia (3.06 times, p-value < 0.01).
Overall, 24h after Hb-Hp1-1 complex uptake, the initially detected cytokine release was suppressed in normoglycemic conditions, the strongest effect being detected for IL-6 (5.78 times; p-value < 0.001). Hyperglycemia interfered with this Hb-Hp1-1-mediated suppression (only 3.05 times suppression of IL-6 secretion in HG).
In contrast to Hb-Hp1-1, Hb-Hp2-2 complex uptake increased the secretion of all read-out cytokines after 24h, the release of IL-6 being stimulated the most (3.06 times, p-value < 0.01).

The release of IL-8 was significantly enhanced 6h after the stimulation with 1 µg/ml Hb-Hp1-1 complexes in normoglycemic conditions (2.51 times, p-value < 0.05, Figure 25D). The increase was similar for both complex concentrations in normoglycemia (1 µg/ml: 2.51 times, p-value < 0.05; 10 µg/ml: 2.55 times) and hyperglycemia (1 µg/ml: 1.6 times; 10 µg/ml: 1.5 times). 24h after the stimulation with Hb-Hp1-1 complexes, a suppression of IL-8 secretion was measured in both normo- and hyperglycemia, with the strongest suppression detected in normoglycemia after stimulation with 1 µg/ml Hb-Hp1-1 complexes (3.06 times, p-value < 0.05) and 2.4 times suppression after stimulation with 10 µg/ml Hb-Hp1-1 complexes (p-value < 0.05). In hyperglycemia, only stimulation with 10 µg/ml Hb-Hp1-1 complexes suppressed IL-8 release in a statistically significant way (1.67 times, p-value < 0.05).
The stimulation with Hb-Hp2-2 complexes did not elicit a statistically significant change in IL-8 release.
Only 24h after the stimulation with Hb-Hp2-2 complexes, IL-8 secretion tended to be suppressed in normo- and hyperglycemia, with the strongest suppression detected in hyperglycemia after stimulation with 1 µg/ml Hb-Hp2-2 complexes (2 times). In normoglycemia, the suppression of IL-8 release was not as pronounced as in hyperglycemia (1.53 times) after stimulation with 1 µg/ml Hb-Hp2-2 complexes.
Hyperglycemia had no statistically effect on IL-8 release 6h and 24h after stimulating M(IFNγ) with Hb-Hp1-1 and Hb-Hp2-2 complexes. However, a tendency to enhanced IL-8 release after stimulation with Hb-Hp complexes was detected. The enhancement was similar for the complex concentrations 1 µg/ml (1.44 times) and 10 µg/ml (1.33 times). 24h after the stimulation with Hb-Hp1-1 complexes, a slightly increased IL-8 release in hyperglycemia was measured (1 µg/ml: 1.41 times; 10 µg/ml: 1.9 times, not statistically significant).
Hyperglycemia enhanced IL-8 secretion 2.3 times 6h after stimulation with 1 µg/ml Hb-Hp2-2 complexes and 2.72 times after stimulation with 10 µg/ml Hb-Hp2-2 complexes. After 24h after the stimulation with 1 µg/ml Hb-Hp2-2 complexes, no effect on IL-8 release was detected; after stimulation with 10 µg/ml Hb-Hp2-2 complexes, a 1.37 times enhancement of IL-8 secretion was measured. Enhanced IL-8 secretion was detected 24h compared to 6h after stimulation with 10 µg/ml Hb-Hp2-2 complexes in hyperglycemia (1.48 times, p-value < 0.05).
Overall, the enhancing effect of hyperglycemia on the secretion of IL-8 was strongest after 24h after the stimulation with 10 µg/ml of Hb-Hp2-2 complexes (2.72 times).

The secretion of anti-inflammatory cytokine IL-1RA was enhanced 6h after the stimulation with Hb-Hp1-1 complexes in both normo- and hyperglycemia, however, the enhancement was only statistically significant in normoglycemia (1 µg/ml: 1.8 times, p-value < 0.05; 10 µg/ml: 1.66 times, p-value < 0.05, Figure 25E). 24h after the stimulation with Hb-Hp1-1 complexes, a suppression of IL-1RA secretion was measured in both normo- and hyperglycemia, with the strongest suppression detected in normoglycemia after stimulation with 1 µg/ml Hb-Hp1-1 complexes (3.24 times, p-value < 0.05) and 1.62 times after stimulation with 10 µg/ml Hb-Hp1-1 complexes (p-value < 0.01). In hyperglycemia, the suppression of IL-1RA release was not as pronounced as in normoglycemia (1 µg/ml: 2 times; 10 µg/ml: 1.18 times).
The stimulation with 1 µg/ml and 10 µg/ml Hb-Hp2-2 complexes had no effect on the release of IL-1RA in normoglycemia and hyperglycemia after 6h.
24h after the stimulation with Hb-Hp2-2 complexes, a similar suppression of IL-1RA secretion was detected in normo- and hyperglycemia, with the strongest suppression detected after stimulation with 10 µg/ml Hb-Hp2-2 complexes (NG: 1.66 times; HG: 1.49 times).
Hyperglycemia enhanced IL-1RA secretion in all stimulations, however, the effect was only statistically significant 6h after stimulation with Hb-Hp2-2 complexes (1 µg/ml: 2.24 times, p-value < 0.05; 10 µg/ml: 2.03 times, p-value < 0.01) and 24h after stimulation with 10 µg/ml Hb-Hp1-1 complexes (2.04 times, p-value < 0.05).
Overall, the enhancing effect of hyperglycemia on the secretion of IL-1RA was strongest after 6h after the stimulation with 1 µg/ml of Hb-Hp2-2 complexes (2.24 times).

Hb-Hp1-1 complex uptake was the strongest stimulus for M(IFNγ) for the acute (6h) release of both pro-inflammatory TNFα, IL-1β, IL-6, IL-8 and anti-inflammatory IL-1RA, with IL-6 secretion being enhanced the most (3.03 times, p-value < 0.01). However, we found differences between Hp1-1 and Hp2-2 effects in hyperglycemic conditions in terms of dynamics and spectrum of inflammatory response. 24h after Hb-Hp1-1 complex uptake (1 µg/ml), the initially detected cytokine release was suppressed in normoglycemic conditions, the strongest effect being detected for IL-6 (5.78 times; p-value < 0.001, Figure 25C) and IL-1β (4.21 times; p-value < 0.01, Figure 25B). Hyperglycemia weakened this suppression for all cytokines, resulting in higher levels of cytokine release (e. g. IL-6: only 3.05 times suppression in HG, Figure 25C; IL-1β: only 1.95 times suppression in HG, Figure 25B).
Contrarily, Hb-Hp2-2 complex uptake did not affect cytokine release in a significant way after 6h, but increased the secretion of all read-out cytokines after 24h, the release of IL-6 being stimulated the most (3.06 times, p-value < 0.01).
Overall, the strongest enhancing effect of hyperglycemia was detected for IL-1β release 24h after M(IFNγ) were stimulated with 1 µg/ml Hb-Hp1-1 complexes (6.75 times).

4 DISCUSSION

4.1 Model system to investigate impact of hyperglycemia on diabetic vascular complications

Diabetes mellitus, as a heterogeneous group of metabolic disorders, is more and more advancing to become a worldwide public health problem[6]. An alarming increase in the prevalence of type 2 diabetes mellitus over the last decades suggests the importance of a sedentary lifestyle, obesity and the ageing of the population[2, 5]. The continuously growing diabetes-related health care expenditures particularly result from the costly treatment of its long-term vascular complications, e. g. non-traumatic lower extremity amputations and end stage renal disease, as well as atherosclerosis-related cardiovascular disease[1, 2, 6].
In order to sustainably treat the skyrocketing number of affected patients, it is not only important to establish prevention programs, but to better understand the pathogenic mechanisms of above-mentioned micro- and macrovascular complications.
Supporting the idea of the proximity of metabolic and inflammatory pathways, there is increasing evidence of hyperglycemia and subclinical chronic inflammation being linked[26, 29, 30]. This state of inflammation is characterized by an only moderate elevation of the cytokines promoting and coordinating immune responses, e. g. TNFα, IL-1β and IL-6, and the fact that it might not only be caused by exogenous, but also endogenous factors[24]. The pro-inflammatory pathways believed to be triggered by hyperglycemia, such as NF-κB, are orchestrated by monocytes and macrophages – key cells of the immune system and inflammation[25, 71-73, 81]. In order to develop new strategies to detect diabetes-related micro- and macrovascular complications at an early stage, and to treat them more effectively, it is crucial to understand the immunological mechanisms leading to a derogated control of vascular damage[80, 81]. A receptor playing an important role in this balance of controlling inflammation is CD163 – being responsible for the internalization and degradation of hemoglobin-haptoglobin complexes[86]. Those complexes are built due to intravascular hemolysis which particularly occurs in inflammatory conditions and contributes to micro- and macrovascular damage[120, 130].

In this study, we demonstrate that hyperglycemia is able to impair the hemoglobin-haptoglobin scavenging process via CD163 on human primary differentiated macrophages in various ways. So far, a number of studies have tried to elucidate the role and regulation of CD163 in pathological conditions like diabetes mellitus or inflammation altogether[130, 153, 165, 166]. In recent studies, mainly samples of already differentiated tissue macrophages[84, 130], undifferentiated peripheral blood mononuclear cells[166], or the plasma concentration of the shed receptor, soluble CD163[154, 167] were used. Compared to them, our group used human primary peripheral blood macrophages derived from circulating monocytes. In order to do justice to the phenotypic plasticity of macrophages dependent on their microenvironment, the cells were stimulated with IFNγ and IL-4 in order to simulate the two most extreme macrophage phenotypes regarding their homeostatic role[25, 34, 64]. IFNγ was used to induce a pro-inflammatory phenotype (M(IFNγ)) that promotes the release of pro-inflammatory cytokines (IL-6, IL-1β)[57, 58], whereas IL-4 was used to create a macrophage profile (M(IL-4)) promoting tissue repair and release of anti-inflammatory cytokines (IL-1RA, IL-10)[61, 172-174]. Additionally, a phenotype without further stimulation (M(Control)) was used as a ‘baseline’ phenotype.

To get a more differentiated view on the regulation and function of CD163 in hyperglycemic conditions, we took into account the two genetic variants of its ligand haptoglobin (Hp1-1 and Hp2-2) displaying the most distinct functional properties[118, 126-128], particularly with regard to the role of Hp2-2 having been identified as a risk factor for cardiovascular disease in patients with type 2 diabetes mellitus[118, 129]. Moreover, to simulate acute and chronic inflammatory response, the data were assessed at several time points (6h and 24h).

4.2 Hyperglycemia interferes with cytokine-dependent regulation of CD163 expression in human primary monocyte-derived macrophages

We sought to determine CD163 regulation on differentially stimulated macrophages, as they play a key role in the mediation of inflammatory reactions[40, 50]. IFNγ and IL-4 were chosen to represent two contrasting macrophage activation states, as they embody the pro-inflammatory M(IFNγ) with their secretory and microbicidal activity[57, 58], as well as the tissue remodeling, phagocytic and anti-inflammatory M(IL-4)[55, 61, 172]. Previous studies showed that a multitude of pro- and anti-inflammatory mediators, e. g. cytokines[88, 90, 96], or glucocorticoids[90, 96-98], as well as growth factors like GM-CSF[96], were able to regulate the expression of CD163.
In human primary macrophages isolated from healthy donors and cultured for 6 days, the stimulation with IFNγ alone downregulated both CD163 gene and CD163 surface expression compared to non-stimulated macrophages. These results, although being observed after a longer duration of cultivation (6 days), correlate with findings of other studies in which CD163 mRNA and surface expression were found to be decreased after the stimulation with IFNγ of freshly isolated[88] or one-day old[96] peripheral blood monocytes from healthy volunteers.
Collectively, these results indicate that lFNγ alone is an effective suppressor of CD163 expression on human primary macrophages, thus impairing the scavenging capacity of pro-inflammatory macrophages.

Apart from IFNγ- and IL-4 dependent regulation of CD163, a recent *in vitro* study showed that hyperglycemia is able to directly affect the polarization of primary human macrophages towards an M1-like phenotype[78].
To examine whether cytokine stimulation and diabetes, characterized by a low-grade inflammatory state[16], have synergistic effects on CD163 expression, we analyzed the CD163 gene expression in a hyperglycemic cell culture model with differentially stimulated macrophages. After 6 days of cultivation in either normal (5 mM) or high (25 mM) glucose conditions, CD163 gene expression of non-stimulated, IFNγ-stimulated and IL-4-stimulated macrophages was analyzed by RT-qPCR.
Hyperglycemia elicited an additional suppression of CD163 mRNA in M(IFNγ) compared to normoglycemia. This finding goes in line with the observation that gene expression of CD163 was significantly decreased in patients with newly diagnosed type 2 diabetes compared to PBMC of healthy subjects[166]. It could be shown in clinical studies that pre-diabetic subjects displayed a significant increase in pro-inflammatory M1 peripheral blood monocytes[82]. A subsequent report revealed that diabetic patients showed an elevated percentage of M1 in blood monocytes and bone marrow[80]. This elevated M1/M2 ratio was correlated with a higher prevalence of microangiopathy[80]. Simultaneously, the expression of CD163 in muscle tissue positively correlated with normoglycemia and a high glucose-disposal rate[84].

Regarding CD163 gene expression levels in individual donors, the majority displayed a downregulated expression in hyperglycemia in IFNγ-stimulated, as well as in IL-4 stimulated macrophages. However, the individual donor analysis showed, especially in M(IL-4), a donor-specific effect of downregulation and a highly variable extent of the reaction. Moreover, the effect in M(IL-4) was not as pronounced as seen in IFNγ-stimulated macrophages. These observations indicate an inhomogeneous influence of hyperglycemia on macrophage subpopulations, thus creating a distinguished macrophage activation profile for each donor, correlating to the clinically observed heterogeneity of manifestation and onset of vascular diabetic complications. In this study, we show that cytokines like IFNγ and IL-4 define the direction of macrophage response, whereas hyperglycemia interferes by enhancing or annulating this cytokine effect.

As CD163 is a scavenger receptor exercising its function on the surface of monocytes and macrophages, we then sought to correlate the gene expression to the surface expression in hyperglycemia. Flow cytometry was used to detect CD163 surface expression of monocyte-derived macrophages isolated from healthy donors cultured for 6 days in normal (5 mM) and high (25 mM) glucose conditions.
It could be shown that hyperglycemia significantly suppressed CD163 surface expression in M(IFNγ). This decrease in surface expression in hyperglycemic conditions is congruent to the results of Levy et al. who showed that PBMCs acquired from individuals diagnosed with diabetes mellitus expressed significantly less CD163 on their cell surface than those collected from healthy donors[130]. Concerning tissue macrophages, the same study reported that in aortic plaques of diabetic patients, while an increase in macrophage infiltration could be observed, the amount of macrophages expressing CD163 was lower[130]. Moreover, it was found that – in patients with confirmed type 2 diabetes mellitus – monocyte surface CD163 expression inversely correlated with insulin resistance, indicating a possibility to use CD163 as diagnostic marker[170]. On top of that, it was shown that surface CD163 expression could even distinguish between the presence and absence of vascular complications: lower surface levels of CD163 expression were detected on monocytes isolated from diabetic patients expressing vascular complications compared to a control group of diabetics without vascular complications[175]. This reduced scavenging capacity might lead to an elevated heme toxicity contributing to endothelial damage and indicates the susceptibility of the diabetic patient to vascular complications by a dysfunctional control of tissue damage [81, 135].

Remarkably, we found a discrepancy between CD163 gene and surface expression in M(IL-4) compared to non-stimulated macrophages. Whereas CD163 mRNA expression was significantly downregulated in M(IL-4) compared to M(Control), the surface expression of CD163 was not affected by stimulation with IL-4. In agreement to this observation, Staples et al. described a downregulation of CD163 gene expression upon the stimulation with IL-4[176] and both Sulahian et al. and van den Heuvel et al. demonstrated that stimulation with IL-4 did not alter CD163 surface expression compared to M(Control)[95, 177]. This indicates that healing macrophages possess a compensatory mechanism to ensure sufficient levels of surface CD163 to ensure the control over the inflammatory response.

Another possible explanation for the found difference between CD163 gene and surface expression in M(IFNγ) and M(IL-4) with significantly lower CD163 surface

expression in M(IFNγ) could be the higher shedding rate of the surface receptor in pro-inflammatory conditions.
As a next step, we therefore looked into the production of soluble CD163 via ELISA, using the described culture conditions with normo- and hyperglycemia over a duration of 6 days on IFNγ-stimulated, as well as IL-4-stimulated macrophages – the likely assumption being an elevated soluble form production in hyperglycemia.
A statistically significant increase of sCD163 was detected on day 6 in comparison to day 1 in all subpopulations regardless of glucose level. This indicates a constant and physiological shedding over time, mirrored by the relatively high amounts of sCD163 (0.7-3.9 mg/l) that can be found in plasma and various tissue fluids of healthy subjects[86, 104, 110]. Additionally, in accordance with the presence of sCD163 in plasma of healthy test persons, a constitutive proteolytic shedding of CD163 has been observed in cultured human monocytes[104, 110].
In this study, we showed that the shedding mechanism of membrane-bound CD163 proved to be most effective in M(IFNγ). This finding goes in line with a previously reported increased CD163-shedding rate in inflammatory conditions: to begin with, it was shown *in vitro* that PMA (phorbol 12-myristate 13-acetate, an activator of protein kinase C) triggered shedding of CD163 from the cell surface[110]. In accordance to the finding that protein kinase C – as an essential part of activating immune responses – is involved in the shedding, it was observed that LPS, as a natural activator of monocytes, is able to induce shedding of CD163 *in vitro* on PBMC, as well as increased sCD163 plasma levels of healthy volunteers after intravenous injection of LPS[106, 178].
In addition to that, two *in vitro* studies found that the release of surface CD163 into the supernatants of freshly isolated PBMC can be caused by pathways contributing to acute innate immune responses to pathogen infections, namely FcγR cross-linking and in response to TLR signaling, which activates a metalloproteinase responsible for the cleavage of the surface receptor[108, 112]. As possible candidate ADAM 17/TACE was identified, an enzyme also mediating the shedding of TNFα, hereby indicating why elevated sCD163 plasma levels can empirically be found in inflammatory conditions with increased macrophage activity[109].
However, our model system of differentially stimulated primary macrophages – being able to simulate the phenotypic plasticity already given by the microenvironment of macrophages – differed from the cell culture conditions of the presented studies, limiting direct comparability. In most of the above-mentioned studies, PBMC – freshly isolated[112], or cultured up to two days without further differentiation[106, 108] – were used. One study even used CD163 cDNA-transfected HEK293 cells[109]. Moreover, none of these studies addressed the low-grade inflammation conditions found in diabetes mellitus[16].
Concerning the possibility of a direct relation between hyperglycemia and shedding of CD163 from the cell surface, a clinical study found that less CD163 was expressed on the cell surface of PBMC isolated from diabetic patients and elevated sCD163 plasma levels could be detected compared to a healthy control group[130].
Notwithstanding our finding of the highest relative increase of sCD163 over the culturation time of 6 days in normoglycemia in M(IFNγ), we found that in hyperglycemic conditions, however, M(IFNγ) showed the lowest relative increase over 6 days of cell culture compared to the other two subpopulations.
As a result of this observation, three ideas emerge: firstly, the observation of the shedding of surface CD163 being least effective in M(IFNγ) in hyperglycemia leads to the consideration that hyperglycemia might impair the function of the enzymes eliciting the cleavage. This, however, seems to be improbable, as several studies describe an activation or upregulation of the designated enzyme ADAM 17/TACE in hyperglycemia

and therefore playing a role in the development of insulin resistance[179-181]. Or, vice versa, as the proportional increase of sCD163 over time in hyperglycemic conditions is most pronounced in M(IL-4), hyperglycemia itself might be the stimulus for shedding surface CD163 expressed on otherwise anti-inflammatory macrophages and thus impairing the scavenging function of those macrophages. This consideration is encouraged by the concept of diabetes mellitus being a disease of disabled damage control[81]. For example, it was found that the hypoxia-inducible factor 1α (HIF-1α) pathway is downregulated in hyperglycemic conditions, thus causing a reduced angiogenic activity as a reply to tissue ischemia, along with an impaired wound healing in diabetic patients[182]. This pathologic condition might be caused, inter alia, by a reduced ingestion of hemoglobin-haptoglobin complexes because of lesser CD163 surface expression and therefore a reduced rate of angiogenesis via a reduced activation of HIF-1α[143]. Seen in a broader context, the observations in this present study go in line with the finding that hyperglycemia not only promotes the differentiation of macrophage subpopulations towards pro-inflammatory phenotypes[78], but also alters the expression profile of M2 subpopulations in a way that can promote insulin resistance and diabetes-related inflammation[183].

The third idea emerging is that hyperglycemia itself does not add to a more pronounced shedding rate of surface CD163 in an already inflammatory microenvironment. This stands in contrast to the numerous reports of elevated sCD163 plasma levels in diabetic patients[120, 130, 153, 165], the reports on an elevated M1/M2 ratio in diabetic and even pre-diabetic individuals[80, 82] and the observation that hyperglycemia is able to induce a pro-inflammatory polarization of macrophages[78, 184]. However, the simple difference between those clinical studies and this present *in vitro* study might be an elevated total amount of circulating monocytes and tissue macrophages in diabetic conditions compared to healthy individuals[82, 130], so that not the rate of CD163 shedding – as can be detected in controlled laboratory conditions – but rather the increased total amount of monocytes and macrophages is responsible for elevated sCD163 plasma levels. Furthermore, clinical studies are more susceptible to confounding variables, such as the fact that obesity is more prevalent in patients with type 2 diabetes mellitus than in healthy subjects[2], thus involving an increased infiltration of fat tissue with pro-inflammatory macrophages[185]. Supporting this observation, it has been reported that an increase in adipose tissue is associated with elevated sCD163 levels[186].

Last but not least, the limitations of our study, possibly restricting the comparability to other studies, have to be taken into consideration. Foremost, the duration of cell culture in this study was longer than in the studies to which the results were compared[106, 108, 112]. Additionally, we already differentiated the macrophages into subpopulations. Secondly, the donors used for the analysis of sCD163 via ELISA and the donors used for the analysis of surface CD163 via flow cytometry were not the same. Considering the high inter-donor variability of CD163 expression, which was also shown regarding CD163 mRNA levels, a comparison between the different test modalities can only be made under the premise of donor-dependency. Thirdly, a peak of sCD163 protein concentrations after a pro-inflammatory stimulus has been described after 1.5 hours after exposure to said stimulus and being only at 25% of the initially measured amount after a period of 24 hours[109], whereas in the present study, sCD163 concentrations were determined after one and six days after starting the cell culture. However, in the study quoted, sCD163 concentrations were measured in human plasma and not in cell culture supernatants[109].

To summarize these results, it has to be stated that firstly, macrophages respond in donor-dependent patterns to hyperglycemia correlating with the clinically observed

heterogeneity of the manifestation and onset of diabetic complications. We show that cytokines like IFNγ and IL-4 define the direction of macrophage response, whereas hyperglycemia interferes by enhancing or annulating this cytokine effect.
Despite individual donor responses to hyperglycemia, certain patterns can be observed: hyperglycemia is able to emphasize the suppressing effect that IFNγ has on the gene as well as the surface expression of CD163. The shedding mechanism of surface CD163 was most effective in pro-inflammatory conditions in normoglycemic conditions – however, in hyperglycemia the shedding proved to be most pronounced in IL-4-stimulated healing macrophages, hereby indicating the susceptibility of the diabetic patient to vascular complications by a dysfunctional control of tissue damage.

4.3 Diabetic vascular complications are promoted by the limited scavenging capacity of CD163 in pro-inflammatory conditions

Suppression of CD163 expression in M(IFNγ) in hyperglycemia raised the question whether high glucose conditions additionally have a direct impact on the scavenging function of CD163.
There are various functions of CD163 described in literature[89, 150-152], with its most comprehensively characterized function being its homeostatic role in the scavenging of hemoglobin-haptoglobin (Hb-Hp) complexes which form due to intravascular hemolysis[86]. CD163's ligand haptoglobin is known to be expressed as one of three possible phenotypes, Hp1-1, Hp1-2 and Hp2-2, resulting from two allelic variants[124]. The Hp1 allele creates dimeric variants of haptoglobin (Hp1-1), whereas the Hp2 allele was found to generate haptoglobin multimers (Hp2-2)[124, 171].
To study the functional consequences of the observed receptor regulation, we sought to determine whether hyperglycemia has an impact on the scavenging function of CD163 – first, by visualization of the complex-ligand-interaction by dual confocal microscopy and further by quantitatively evaluating the uptake of fluorescently labeled Hb-Hp complexes (Hp1-1 and Hp2-2 variants) by differentially stimulated macrophages via flow cytometry.
As described in literature[132, 135, 187], the visualization of the complexes via confocal microscopy confirmed a nearly homogeneous distribution of the complexes throughout the cytoplasm organized in vesicles, hereby displaying a large intracellular receptor pool. In an *in vitro* study on untreated PBMC and HEK293 cells, a co-localization of CD163 and transferrin, a marker protein of early endosomes, could be visualized, whereas no signal in LAMP-1 positive lysosomes could be detected – these, in turn, containing Hb-Hp complexes[135]. Furthermore, treatment with monensin, an endosomal receptor recycling inhibitor, reduced CD163 surface expression and caused an accumulation of the receptor in perinuclear vesicles, thus indicating a constant endocytosis and recycling of CD163 to and from the cell surface[132, 135].
The weakest signal of Hb-Hp complexes was detected in M(IFNγ). This finding complies with the results obtained from the examination of CD163 gene and surface expression on differentially stimulated macrophages. A visual difference between normoglycemic and hyperglycemic conditions in the respective subpopulations could not be detected.
Other than that, a generally stronger signal of Hb-Hp2-2 complexes compared to the one of Hb-Hp1-1 complexes could be noted. This visual perception was supported by the measurement of higher MFI values for Hb-Hp2-2 complexes in comparison with the complexes formed with the Hp1-1 variant in all three macrophage subpopulations. This tendency supports the finding of the receptor's structural analysis, describing a

higher affinity of the haptoglobin 2-2 variant for the CD163 binding site located in SRCR domain 3[86, 171]. However, one limitation of the detected differences is the fact that the ligation of the two tested haptoglobin variants to Alexa Fluor® 488 particles was performed in our laboratory, and therefore is not immune to intensity differences.
Although the two variants Hp1-1 and Hp2-2 differ considerably in their molecular structure – with polymorphisms arising from two different polypeptide chains, thus creating haptoglobin dimers in subjects with Hp1-1, linear polymers in Hp1-2 individuals and cyclic multimers in humans with two Hp2 alleles[118, 124] – the uptake patterns of Hb-Hp complexes matched CD163 mRNA and surface expression patterns. The notion deriving from this observation – that the structure of haptoglobin itself is not the crucial factor in the process of internalizing hemoglobin-haptoglobin complexes – is controversially discussed in recent literature. On the one hand, an *in vitro* study performed on monocytes showed that the uptake of Hb-Hp complexes is competitively inhibited by free hemoglobin, thus indicating a common binding-site of free as well as complexed hemoglobin and demonstrating that CD163-hemoglobin interactions are not affected by changes in structure resulting from the binding process of hemoglobin to haptoglobin[187]. This seems to be in contradiction to various other studies describing CD163 as the specific receptor for hemoglobin complexed to haptoglobin, but not the free hemoglobin molecule, making a low-affinity reversible binding of free hemoglobin to CD163 in scenarios of haptoglobin exhaustion a probable suggestion[86, 127, 187].
As to our best knowledge, there have not been any studies reporting a qualitative derogation of CD163 scavenging function. However, the clinically observed proneness to vascular complications in diabetic patients is enhanced by the limited availability – and therefore limited capacity to mitigate vessel damage – of CD163 in pro-inflammatory conditions[135].

4.4 Diabetes as a disease of less effective anti-inflammatory control of tissue damage

As no functional impairment of Hb-Hp complex uptake via CD163 was detected, we analyzed whether the uptake itself can provoke an inflammatory activation of macrophages.
It has not yet been elucidated whether hemoglobin-scavenging by CD163-expressing macrophages is related to an anti-inflammatory response or whether it rather promotes a pro-inflammatory setting. An indication for a pro-inflammatory reaction of hemoglobin-scavenging by $CD163^+$ macrophages is the activation of protein-kinase C- and casein-kinase-dependent macrophage pathways by cross-linking of cell surface CD163, triggering the release of pro-inflammatory cytokines, such as IL-1β and IL-6[90, 144, 145]. Studies performed on human atherosclerotic plaques reported that the intake of hemoglobin-haptoglobin complexes via CD163 leads to a particular macrophage phenotype, named M(Hb) or Mhem[138, 143]. This phenotype is characterized by an abundant expression of surface CD163, a downregulated cytokine production and the lack of lipid withholding[140-142]. As these macrophages are particularly present in areas of hemorrhage and neoangiogenesis, a role in plaque vascularization, microvessel leakage and inflammation of the surrounding endothelium has been suggested[143], thus questioning the long-established notion that $CD163^+$ macrophages are involved in the resolution of inflammation[88]. This concept of an anti-atherogenic role of hemoglobin-scavenging via CD163 is backed by various studies, beginning with the fact that CD163 expression can be highly enhanced by anti-inflammatory mediators, e. g. glucocorticoids or IL-10[95, 188, 189], or the clinical finding that neither systemic hemolysis

nor local release of hemoglobin induces an exaggerated inflammatory reaction[190], culminating in the observation that the contact to hemoglobin is able to provoke an anti-inflammatory response in macrophages, indicated by an upregulation of the cytoprotective heme oxygenase (HO-1)[135].
As these stated concepts differ considerably in their suggestions whether CD163-mediated hemoglobin-scavenging elicits a pro- or anti-inflammatory reaction, it might be possible that another factor is responsible for diverging results. As stated above, the haptoglobin variants were found to have not only differences in function, but also in the involvement in pathological conditions. For instance, the clearance and antioxidant capacity of haptoglobin1-1 by binding hemoglobin and thus mitigating oxidative injury in the form of oxidation of lipid and protein substrates, has been observed to be superior than the clearance of haptoglobin2-2[126, 127]. Not only antioxidant, but also anti-inflammatory responses have been examined to differ between the haptoglobin variants: the release of interleukin-10 in response to the binding of hemoglobin-haptoglobin1-1 complexes to CD163 was markedly increased compared to hemoglobin-haptoglobin2-2 complexes[128]. Regarding the clinical significance of the different haptoglobin variants, it has been shown in several longitudinal prospective studies that the Hp2-2 phenotype is an independent risk factor for the development of cardiovascular disease in diabetic individuals in comparison with the homozygous Hp1 variant[118, 129]. As a possible mechanism providing an explanation for the increased proneness to vascular complications, it has been demonstrated that CD163 is downregulated in macrophages of atherosclerotic plaques of diabetic patients with the Hp2-2 genotype, indicating a constrained hemoglobin clearing capacity[130]. In healthy subjects however, the concentration of sCD163 – as an inversely correlated surrogate parameter for surface CD163 expression – was not linked to the prevalent haptoglobin phenotype: the median level for individuals with haptoglobin1-1 was 1.8 mg/l and 1.9 mg/l for persons with haptoglobin1-2 as well as haptoglobin2-2[104].
To measure the inflammatory – i. e. cytokine – response of hemoglobin-haptoglobin scavenging in hyperglycemic conditions, we selected a number of read-out cytokines displaying the complex interaction and the different stages of an inflammatory reaction. In healthy individuals, the process of inflammation serves a homeostatic purpose, containing pro- and anti-inflammatory phases[191]. In individuals suffering from type 2 diabetes mellitus however, the balance is tilted towards the pro-inflammatory side, manifested by an upregulation of pro-inflammatory intracellular pathways[35, 36], or an elevation in circulating inflammatory markers, such as C-reactive protein, IL-6 or TNFα[16, 32, 192, 193].
TNFα, being often described as a ‘classical player’ of the acute-phase immune response involved in the production of acute-phase proteins and as a protective agent against pathogens, also plays a role in the development of chronic inflammatory diseases[20, 194]. Elevated TNFα-levels have been detected in newly-diagnosed diabetic patients compared to a healthy cohort[195-197], and a role in the induction of insulin resistance, inter alia by preventing insulin production in pancreatic beta cells, has been described[33, 193, 198]. Moreover, the production of TNFα strongly links with the induction of microvascular and cardiovascular complications associated with diabetes[199, 200]. Recently, it was shown that especially blood monocytes are able to cause elevated TNFα-serum levels in diabetics[183].
Another pro-inflammatory cytokine involved in innate immune response and in chronic inflammatory diseases is IL-1β, its signaling occurring through NF-κB activation[191, 201, 202]. Hyperglycemic conditions were found to increase IL-1β mRNA expression, as well as secretion from IFNγ-stimulated macrophages, hereby supporting chronic

inflammation in endothelial and adipose tissue, leading to an induction of beta cell demise and to impaired insulin secretion in obesity[183, 203]. Additionally, it was found in a prospective population-based clinical study that an elevated IL-1β level increases the risk of developing type 2 diabetes mellitus[204].
The influence of IL-1β on inflammatory and metabolic pathways can be diminished by the competitive inhibition by IL-1RA[205]. Interestingly, the basal serum values of IL-1RA of individuals suffering from type 2 diabetes mellitus tend to be higher than those of healthy counterparts and are linked to insulin resistance in obese patients [191, 206, 207]. In two cohort studies, increased IL-1RA levels were identified to be associated with the existing diagnosis of type 2 diabetes mellitus and furthermore could be detected 6 years prior to the clinical onset of type 2 diabetes[206, 208]. These findings led to a new therapeutic approach: application of human recombinant IL-1RA resulted in lower levels of the inflammation markers C-reactive protein and IL-6, as well as an improved beta cell function and a normalization of blood glucose levels[209].
IL-6, being produced mainly by cells of the immune system, is a cytokine directly affecting a variety of cells and tissues and is thus exerting tissue-dependent reactions, such as cell differentiation and proliferation[191, 210, 211]. Macrophages were found to secrete increased amounts of IL-6 after stimulation with pro-inflammatory agents, such as LPS, IFNγ, IL-1α, or TNFα[201, 212]. It is therefore not surprising that IL-6 plays a role in the pathophysiology of chronic inflammatory diseases, such as arthritis, systemic lupus erythematodes, or Crohn's disease[213]. Moreover, IL-6 plays a key role in the regulation of acute-phase protein production by hepatocytes, upregulating the secretion of haptoglobin, C-reactive protein and others[214]. In diabetic patients, IL-6 levels are increased in comparison to healthy non-diabetic subjects and an *in vitro* study reported the induction of cellular insulin resistance of hepatocytes by IL-6-triggered impairment of insulin receptor signal transduction[192, 215].
IL-8, or CXCL8, is a chemokine/cytokine involved in the activation of neutrophils, the process of angiogenesis as well as macrophage infiltration and activation of adipose tissue of insulin-resistant patients, therefore possibly playing a role in the pathogenesis of type 2 diabetes mellitus[216-218]. An elevated circulating IL-8 level was found to be able to identify diabetics with a more pernicious profile of circulating pro-inflammatory cytokines, lipids and glycometabolic factors among other diabetic patients and healthy subjects[219]. Furthermore, increased Toll-like receptor 9 expression was shown to be associated with an upregulation of IL-8 levels in diabetic wound tissue samples, thus contributing to the formation of chronic ulcers by restricted wound healing[220].
To address the question whether Hb-Hp complex uptake results in an inflammatory activation of macrophages in hyperglycemic conditions, we selected M(IFNγ) as read-out macrophage population, taking into account that hyperglycemia might be responsible for a M1-like polarization of macrophages and to investigate the characteristics of an impaired scavenging via CD163 caused by the downregulation of both CD163 and surface expression[78].

We showed that hyperglycemia enhanced the pro-inflammatory response of M(IFNγ) to Hb-Hp complex uptake by stimulating the production of TNFα, IL-1β, IL-6, IL-8 and IL-1RA, supporting the observation that hyperglycemia itself can induce the secretion of pro-inflammatory cytokines[183].
A statistically significant increase in TNFα secretion, the 'classical player' of the acute-phase immune response[20], can only be detected 6h after the stimulation with Hb-Hp complexes in hyperglycemia, regardless of the present haptoglobin variant. This finding is backed by the clinical observation of elevated TNFα-levels being detected in newly-diagnosed diabetic patients compared to a healthy cohort[195, 197].

We showed that in response to the uptake of Hb-Hp1-1 complexes, the release of IL-6 was enhanced the most among our read-out cytokines. IL-6 plays a key role in the regulation of acute-phase protein production by hepatocytes, upregulating – among others – the secretion of haptoglobin and C-reactive protein[214]. Elevated IL-6 release might contribute to diabetes progression, as an *in vitro* study reported the induction of cellular insulin resistance of hepatocytes by IL-6-triggered impairment of insulin receptor signal transduction[215]. Another *in vitro* study showed that lower concentrations (20 µg/ml) of Hb-Hp1-1 complexes induced the secretion of pro-inflammatory IL-6, higher concentrations (100 µg/ml) of Hb-Hp1-1 complexes, however, led to an increased CD163 surface expression on monocytes, in terms of a positive anti-inflammatory feedback loop[207].

We found that hyperglycemia increased the secretion of both IL-1β and IL-1RA, hereby confirming an enhanced IL-1RA release in response to hyperglycemia by pro-inflammatory M(IFNγ)[183]. Increased IL-1RA secretion could be a possible compensation mechanism or negative feedback for the enhanced release of IL-1β, whose serum level was found to be elevated in diabetic patients, contributing to insulin resistance and progression of atherosclerotic lesions in obesity[203]. A compensatory role of IL-1RA might also be an explanation for the controversial observations concerning IL-1RA levels in hyperglycemia: on the one hand, elevated IL-1RA levels could be correlated to insulin resistance[207, 221], on the other hand, IL-1RA was found to diminish markers of inflammation in blood samples of diabetic patients and enhance beta cell function[209].

In general, we found differences between Hp1-1 and Hp2-2 effects in hyperglycemic conditions in terms of dynamics and spectrum of inflammatory response. Hb-Hp1-1 complex uptake was the strongest stimulus for M(IFNγ) for acute (6h) cytokine release, however, cytokine secretion was diminished after 24h. An elevated acute cytokine secretion indicates the release of pre-synthesized cytokines in storage vesicles in macrophages as a part of the physiological scavenging process[177].

Contrarily, Hb-Hp2-2 complex uptake resulted in the increased secretion of all read out cytokines after 24h, indicating a transcriptional upregulation of cytokine production. Our *in vitro* finding that Hb-Hp2-2 complex uptake is able to create a long-lasting elevated release of pro-inflammatory cytokines goes in line with the numerous clinical observations of Hp2-2 possessing a lesser antioxidative capacity than Hp1-1[126, 127], inducing a lesser anti-inflammatory response than Hp1-1[128] and even being an independent risk factor for the development of cardiovascular disease of diabetic patients[118, 222].

As an element of the pathophysiology of diabetes, these mechanisms promote the low-grade inflammation present in the vascular system of diabetic patients and support the development of both micro- and macrovascular complications. This shows that – in addition to a more pro-inflammatory setting – diabetes mellitus is a disease characterized by a less effective anti-inflammatory damage control[81, 191].

5 SUMMARY

Hyperglycemia is a hallmark of diabetes and can induce inflammatory programming of monocytes and macrophages. Scavenging function of monocytes and macrophages is essential for the clearance of both exogenous components and unwanted self-products and the control of inflammatory reactions. Scavenger receptor CD163 internalizes hemoglobin-haptoglobin (Hb-Hp) complexes to initiate their degradation. The genetic variants haptoglobin1-1 (Hp1-1) and haptoglobin2-2 (Hp2-2), considerably differing in their molecular structure, form complexes with free hemoglobin in situations of intravascular hemolysis which especially occurs in inflammatory conditions. Inflammation is an essential pathological process leading to the development of micro- and macrovascular diabetic complications. Clinical studies have found a correlation between an impaired scavenging process of Hb-Hp complexes via CD163, particularly in combination with the presence of the genetic variant Hp2-2, and vascular complications of diabetes mellitus. Our aim was to identify the effect of hyperglycemia on CD163 expression and function in human primary macrophages. We used the model system based on human primary monocyte-derived M(IFNγ) and M(IL-4) macrophages that was established in our laboratory. In this system, M(IFNγ) and M(IL-4) macrophages as well as non-stimulated M0 macrophages were differentiated out of human monocytes in normal (5mM) and high (25mM) glucose conditions in the presence of IFNγ and IL-4 or without further cytokine stimulation. The effects of hyperglycemia on CD163 gene and surface expression were quantified by RT-qPCR and flow cytometry. The function of CD163 was analysed by flow cytometry using fluorescently labeled Hb-Hp1-1 and Hb-Hp2-2 complexes, the uptake being visualized by confocal microscopy. The effect of hyperglycemia on the release of soluble CD163 and of read-out inflammatory cytokines TNFα, IL-1β, IL-1RA, IL-6 and IL-8 was detected by ELISA. CD163 gene expression was decreased 5.53 times in M(IFNγ) and 4.76 times in M(IL-4) compared to non-stimulated M0 macrophages. Hyperglycemia elicited an additional suppression of CD163 gene expression only in M(IFNγ) (1.99 times). CD163 surface expression was downregulated in a statistically significant way by hyperglycemia in M(IFNγ) (1.43 times); but not in M(IL-4). In normoglycemic conditions, the shedding of surface CD163 proved to be most effective in pro-inflammatory conditions, whereas in hyperglycemia, the cleavage of CD163 was most pronounced in M(IL-4). Flow cytometry and confocal microscopy demonstrated that hyperglycemia did not impair Hb-Hp complex uptake and delivery to endosomal pathway, neither in inflammatory nor in anti-inflammatory macrophages. Hb-Hp1-1 complex uptake was the strongest stimulus for M(IFNγ) for the acute (6h) release of TNFα, IL-1β, IL-6, IL-8 and IL-1RA, with IL-6 secretion being enhanced the most (3.03 times). However, 24h after Hb-Hp1-1 complex uptake, cytokine release decreased in normoglycemia, the strongest effect being detected for IL-6 (5.78 times). Hyperglycemia interfered with Hb-Hp1-1 mediated suppression (only 3.05 times suppression of IL-6 release). Contrarily, Hb-Hp2-2 complex uptake did not affect cytokine release in a significant way after 6h, but increased the secretion of all read-out cytokines after 24h, IL-6 release being stimulated the most (3.06 times). Overall, the strongest enhancing effect of hyperglycemia was detected for IL-1β release 24h after M(IFNγ) were stimulated with Hb-Hp1-1 complexes (6.75 times). Our data suggest that the inflammatory response of M(IFNγ) after Hb-Hp1-1 and Hb-Hp2-2 complex uptake in hyperglycemic conditions can enhance low-grade inflammation in patients with diabetes mellitus and promote their susceptibility to vascular complications by a dysfunctional control of tissue damage.

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