Diplomarbeit

MODULATION OF THE PHENOTYPICAL POLARIZATION OF PLACENTAL HOFBAUER CELLS IN VITRO – a pilot study

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Graz, am 09.07.2018 *Sandra Ibesich eh*

Medicine is not only a science; it is also an art. It does not consist of compounding pills and plasters; it deals with the very processes of life, which must be understood before they may be guided.

Paracelsus

Danksagungen

Ich möchte mich hiermit herzlich bedanken bei Christian Wadsack, der mir das Arbeiten in seiner Forschungsgruppe erst ermöglicht hat, für die Supervision und organisatorische Unterstützung.

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Zusammenfassung

Einleitung. Hofbauer Zellen (HBCs) sind feto-plazentare Makrophagen, die im Stroma-Kern von Chorionzotten lokalisiert sind. Sie tragen zur fetalen Immunabwehr gegen Antigene, zur Förderung der maternalen Immuntoleranz gegen den semi-allogenen Fetus, der Plazentamorphogenese, Angiogenese und Vasoregulation bei und spielen ebenfalls eine Rolle bei Schwangerschaftskomplikationen wie Schwangerschaftsdiabetes, Prä-Eklampsie, Villitis unklarer Genese und Chorionamnionitis.

Makrophagen können sowohl inflammatorische (M1) als auch regulatorische (M2) Funktionen einnehmen. Im Verlauf normaler Schwangerschaften weisen HBCs im Allgemeinen regulatorische Eigenschaften und zugehörige Marker auf. Es bleibt jedoch unklar, inwieweit und durch welche Modulatoren der Phänotyp von HBCs veränderbar ist.

Methoden. Es wurden terminal differenzierte HBCs aus gesunden humanen Plazenten isoliert (N = 5). Diese wurden Stimuli (LPS + IFNγ, IL-4 + IL-13, IL-10 + TGFβ, IL-6) ausgesetzt, welche Makrophagen in definierte Polarisationen (M1/M2) versetzen sollen. Gleichzeitig wurde eine THP-1-Zelllinie (humane immortalisierte Monozyten) als Kontrolle verwendet. Diese Zellen wurden zu Makrophagen differenziert und den gleichen Substanzen ausgesetzt. FACS wurde verwendet, um Oberflächenmarker zu quantifizieren, die für die verschiedenen Polarisationsphänotypen auf HBCs und THP-1-Zellen spezifisch sind. ELISA wurde verwendet, um die jeweiligen Sekretionsprodukte der Zellen aus Medienüberständen zu quantifizieren.

Ergebnisse. Bei HBCs, die mit pro-inflammatorischen Stimuli (LPS + IFNγ) behandelt wurden, konnte eine Abnahme von M2-Markern, aber kein Anstieg von M1-Markern gezeigt werden. Im Gegensatz dazu, konnte bei THP-1-Zellen, die denselben Stimuli ausgesetzt wurden, ein signifikanter Anstieg der M1-Marker CD80 bzw. CD86 sowie des Antigen-präsentierenden Oberflächenproteins HLA-DR gezeigt werden.

Im ELISA zeigten HBCs im Gegensatz zu THP-1-Zellen durchwegs einen IL-10_{high}/IL-12_{low} -Phänotyp, was als M2-Marker gilt.

Schlussfolgerung. HBCs sind in der Aufrechterhaltung ihres regulatorischen Phänotyps stabiler gegenüber inflammtorischen Stimuli als die Kontrollzelllinie. Weitere Untersuchungen sind erforderlich, um festzustellen, inwieweit eine

Verbesserung des M2-Phänotyps zum Erhalt einer gesunden Schwangerschaft und zur Behandlung schwangerschaftsbedingter Erkrankungen beitragen könnte.

Abstract

Introduction. Hofbauer cells (HBCs) are feto-placental macrophages residing in the stromal core of chorionic villi. They contribute to the fetal first line of defense against antigens, promotion of maternal immune-tolerance against the semi-allogeneic fetus, placental angiogenesis, vasoregulation and morphogenesis. They are also suspected to play a role in obstetric complications such as gestational diabetes mellitus, pre-eclampsia, villitis of unknown etiology and chorioamnionitis. Adapting to their environment, macrophages generally polarize to specific phenotypes, allowing them to fulfil a wide range of functions, e.g. as inflammatory (M1) or regulatory (M2) cells. In normal pregnancies, HBCs have generally been found to occupy a regulatory phenotype. However, it remains unclear in how far HBCs are able to undergo phenotypical switches.

Methods. Terminally differentiated HBCs from human term placentae (N=5) were isolated. Cells were exposed to stimuli (LPS + IFNγ, IL-4 + IL-13, IL-10 + TGFβ, IL-6) that have previously been defined to polarize macrophages into depending phenotypes. Simultaneously, THP-1 cells, a human immortalized monocyte/macrophage cell line, were used as control. These cells were differentiated into macrophages and exposed to the same stimuli. FACS was used to quantify surface markers specific for the various polarizations on HBCs and THP-1 cells. ELISA approach was used to quantify respective secreted proteins.

Results. In HBCs treated with pro-inflammatory stimuli (LPS + IFNγ), a decrease of M2 markers, but no increase of M1 markers was observed. In contrast, THP-1 cells treated with the same stimuli, a significant increase of M1 markers CD80 and CD86, respectively, as well as antigen-presenting surface marker HLA-DR was observed.

In ELISA, HBCs in contrast to THP-1 cells displayed consistently an IL-10_{high} /IL-12_{low} phenotype, which is regarded as M2 marker.

Conclusion. HBCs are more committed to a regulatory phenotype than the control cell lineage upon pro-inflammatory stimulation. Further research is needed to determine in how far an enhancement of M2 polarization could contribute to maintaining a healthy course of pregnancy and treatment of pregnancy-associated diseases.

Table of contents

Index of abbreviations

ABTS = 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
APC = allophycocyanin
CAM = chorioamnioitis
CCL5 = C-C motif chemokine ligand 5
DMSO = dimethyl sulfoxide
e.g. = example given
ELISA = enzyme-linked immunosorbent assay
FACS = fluorescence-activated cell sorting
FCS = fetal calf serum
FITC = fluorescein isothiocyanate
GDM = gestational diabetes mellitus
HBC = Hofbauer cells
HBSS = Hanks balanced salt soultion
HEPES = 4-(2-Hydroxyethyl) piperazine-1ethanesulfonicacid
HIV = human immunodeficiency virus
HRP = horse radish peroxidase
ICC = immuncytochemistry
IFNg = interferone gamma
IL = interleukin
LPS = lipopolysaccharide
M = molar
MaM = macrophage medium
MHC = major histocombatibility complex
ml = milliliter
MQ = Milli-Q, sterile aqua
NK = natural killer cells
PAMP = pathogen-associated molecular pattern
PBS = phosphate buffered saline
p.c. = post conceptionem
PE = phycoerythrin
PrE = pre-eclampsia
PMA = phorbol 12-myristate 13-acetate
RPMI = Rosewell Park Memorial Institute medium
TBE = tris buffered saline
TGF = transforming growth factor
TH1 = T helper 1 cells
TLR = toll-like receptor
TNFa = tumor necrosis factor alpha
VEGF = vascular endothelial growth factor
VUE = villitis of unknown etiology

Index of Figures

Index of Tables

1 Introduction

Hofbauer cells (HBCs) are macrophages which reside in the stromal core of chorionic villi in healthy human placentae and are believed to be of fetal origin(1–4). These cells play an important role in the fetal first line of defense against pathogens, the prevention of vertical transmission of pathogens during pregnancy(5) and as well contribute to placental angiogenesis and morphogenesis(2). These specific cells enable embryonal implantation without maternal rejection of the embryo(6).

It is widely accepted that macrophages in various tissues, following their broad spectrum of functions, are generally able to display a wide range of activations, reaching from classical pro-inflammatory activation (M1) to several subsets of alternative activation (M2)(6). Recent research reveals an ambiguous picture of the polarization spectrum of placental macrophages, HBCs. Whereas it is commonly assumed that HBCs hold an alternatively activated M2 polarization in the course of normal pregnancies(6,7), the stability of these cells in maintaining their phenotype is not clear.

Some studies suggest that the polarization of HBCs was influenced by the surrounding milieu(8), which differs from normal to complicated pregnancies such as presence of maternal Type 1 diabetes mellitus (T1DM) and therefore HBCs were able to switch their phenotype(9). On the other hand, major findings in another study performed at our laboratory proposed a stable phenotype of HBCs, comparing cells of healthy women and women with gestational diabetes mellitus (GDM)(10). A maintenance of phenotypical polarization of HBCs was also found in pregnancies complicated with chorioamnionitis (CAM)(11).

A clear consensus concerning the plasticity of HBCs polarization under different pathophysiological settings in pregnancy cannot be drawn according to the current knowledge. Therefore, the pursuit of this study was to determine whether the polarization of HBCs is terminally defined or if inflammatory stimuli can cause re-polarization.

1.1 Hofbauer cells

It has been more than 150 years that those specific cells, later referred to as Hofbauer cells firstly appeared on researchers' field of vision. Initially, they have been identified as part of the placental villi. At the beginning of the 20th century, Hofbauer described them, among others, as large, pleiomorphic cells containing a high number of vacuoles.(12)

Since then, HBCs have been under constant investigation and today we know some more details, nonetheless many characteristics remain still unexplored.

Hofbauer cells are phenotypically macrophages that reside within chorionic villi of the placenta and represent an important part of stromal cells, with a proportion of about 40%(13,14). The origin of these cells has been proven to be fetal, by demonstrating the presence of an Y-chromosome in the nucleus of macrophages in pregnancies with male fetus(15). Hofbauer cells are firstly described at day 18 *post conceptionem*(p.c.), decline in number but remain present within the whole course of pregnancy(16) and display a high phenotypic variability. With a diameter of 10-35μm they are round to elongated in shape and facilitate a large variety of cellular processes(1). *Castellucci et al.* have described four main phenotypic types, dependent on the presence of lamellipodia, blebs, microvilli and microplicae(1). Furthermore, HBCs have a highly vacuolated cytoplasm and express the Fc-receptor on their surface(17).

The human placenta is hemochorial, meaning that fetal trophoblastic structures directly interact with maternal blood, which loosely perfuses the intervillous space. The chorionic villous represents a functional unit of the placenta and resembles a tree, which is based on the chorionic plate and branches into the intervillous space. The trophoblast displays the outermost part of a chorionic villous and forms the feto-maternal transport barrier. It consists of a continuous layer of fused syncytiotrophoblast, under which lays a discontinuous layer of cytotrophoblast(18). The stroma in the center of the villus contains fetal vasculature, stromal cells and Hofbauer cells, that can be found in close proximity with trophoblasts and fetal capillaries(16).

1.1.1 Origin

Hofbauer cells seem to have diverse origins, depending on the gestational stage. They appear first in the early first trimester of pregnancy and are thought to arise

from hemangioblastic cells(16). These are mesenchymal stem cells and therefore act as progenitors of both endothelial- and blood cells(19). As the circulation of the fetus evolves, about 8 weeks p.c., the HBC population is established from fetal monocytes, which arise from hematopoietic stem cells residing within the fetal bone marrow(15,20). Depending on their descent, HBCs could have different functions in normal and complicated pregnancies(21).

1.1.2 Function

Equally diverse as the origin of HBCs is also their spectrum of functions during early and term pregnancy, and labor. As it lies in the nature of macrophages, one of the most essential functions of HBCs is to promote fetal immunological defense and prevent vertical transmission of pathogens(5). Following their close anatomical relation to the syncytiotrophoblast, the outermost border to maternal tissue, HBCs represent the active immune defense for the fetus(2). Therefore, cells cluster at potential pathogen entry portals, such as trophoblast leaks(22). Supporting this argument, HBCs have been found to express TLR4, which is a receptor crucial for pathogen recognition and the promotion of innate immune responses(23,24). Activated by direct binding of microbial particles, so called pathogen-associated molecular patterns (PAMPs), TLR4 induces intracellular signaling which triggers the transcription of many genes involved in immune response(25) and is therefore important for effective immunity(24). Moreover, HBCs have been shown to limit the replication and transmission of HIV-1, supposedly by expression of regulatory cytokines such as IL-10 and TGFβ(5).

Not only infection, but also placental inflammation may be controlled by HBCs. For instance, by mediating the uptake of apoptotic bodies HBCs could be able to reduce placental damage in non-infectious inflammation(26).

These cells also display immune-regulatory characteristics, inasmuch as they promote maternal immune tolerance towards fetal antigens by clearing up maternal IgG. These maternal antibodies against fetal patterns are gathered as immune complexes in the villous stroma and get engulfed by the macrophages, which identify them through their Fc – receptors(17). Not only pathogens, but also allergens have been shown to cross the placental barrier(27). Hofbauer cells trapping IgE in complexes on their surface and therefore retaining them in the placenta could protect the fetus from exposure to potentially harmful allergens(28).

In addition to these immunological functions, HBCs also play a role in placental morphogenesis. They express sprouty-proteins which are important for the development of vasculature within the villous tree(29). Moreover, their close proximity to trophoblasts is suggestive of their involvement in trophoblast differentiation and angiogenesis(2). Suitably, Hofbauer cells produce VEGF, an angiogenic protein which mediates not only angiogenesis, but also proliferation of trophoblasts. In addition, HBCs regulate the vascular tone of placental vessels by stimulating trophoblasts to release vaso-relaxing agents(30). Moreover, HBCs themselves play a direct role in vaso-regulation by producing both prostaglandin E2, which acts as vasodilator, and thromboxane, which acts as vasoconstrictor(31).

As stromal channels lack lymphatic drainage, HBCs supposedly intervene in the balance of stromal water content, exchange of ions and transport of serum proteins through the placental barrier(16,32).

1.1.3 Hofbauer cells in complicated pregnancies

Previous studies have suggested that alterations of HBCs can be found in pregnancies with obstetrical complications and infections, such as villitis of unknown etiology (VUE)(15), chorioamnionitis (CAM)(33), maternal Type 1 diabetes (T1DM)(9) and pre-eclampsia (PrE)(26). In VUE, CAM and PrE cell density of HBCs changes, whereas in diabetes the phenotypical polarization seems to change(9,15,26,33).

In the pathophysiology of VUE, maternal CD8+ (cytotoxic(34)) T cells infiltrate the chorionic villi and simultaneously the number of hyperplastic HBCs increases. This is analogous to graft-versus-host-disease (GVHD), with influx of immunocompetent maternal T cells as sign of maternal rejection of fetal antigens and a reactive increase of the HBC population as a rescue mechanism(15). It has been shown that in VUE HBCs express more CD14, a surface antigen which mediates innate immune response(35) and are positive for proliferation marker Ki67(15). Yet, these HBCs secrete more anti-angiogenic cytokines(20). Hence, it has been proposed that VUE results from a bilateral immunoreaction of both mother and fetus, where maternal allograft rejection and fetal GVHD lead to placental tissue destruction(20).

CAM has an infectious etiology and is also known as inflammation of the placental membranes. It usually arises from lower genital tract infections(36). Previous studies showed ambiguous results concerning HBC cell count in CAM. Whereas *Vinnars et al.* showed in 2010 that the overall cell density of HBCs in placentae of patients with CAM decreases significantly, compared with those of healthy control placentae(36), *Toti et al*. demonstrated in 2011 that HBC cell number increases at least focally as they cluster at some villi (33). This indeed needs further research in order to clarify this discrepancy. Independent from that, HBCs seem to maintain their regulatory M2 phenotype despite the presence of CAM(11).

Furthermore, if diabetes effects HBCs in the placenta is controversially discussed. On the one hand, one study performed at our laboratory showed that HBCs maintain their regulatory polarization under hyperglycemic GDM conditions, investigating upon women diagnosed with GDM compared to healthy women(10), on the other hand *Sisino et al.* showed an imbalance of the ratio pro-/anti-inflammatory macrophages in T1DM rats and humans and could provoke a phenotypic switch of rat HBC polarization under *high glucose* conditions *in vitro*(9). It has to be said that in these experiments, HBCs have been cultured in RPMI media, which represents an non-physiological environment for the cells, as it is too rich in L-arginine which might favor M1 over M2 polarization(6).

The pathophysiology of the early onset PrE, a pregnancy-specific hypertonic disease that goes along with proteinuria(26), is based in the first trimester of pregnancy. Subsequent to an inadequate development of the placenta by dysregulated angiogenetic factors, the placenta suffers from hypoxic-ischemic damage. *Tang et al.* observed a decrease of the HBC population in pre-eclamptic women and proposed that this loss might contribute to the severity of the disease. They postulated, that HBCs might switch their phenotype towards pro-inflammatory M1 polarization in the course of PrE(26). In accordance, *Medeiros et al.* observed that the pro-inflammatory surrounding environment in PrE may shift monocytes to a M1 polarization(37). *Yang et al.* observed recently that the pathogenesis of PrE goes along with a loss of HBC cell count as well as a downregulation of M2 phenotypical features such as CD163, DC-SIGN (CD209) and secretion of IL-10. Therefore, the immunosuppressive and regulating functions of HBCs are impaired(38).

Straight-forward, it seems obvious that HBCs play an important role not only in the course of normal pregnancy and labor, but also in the pathophysiology and development of some pregnancy-associated diseases. A profound research upon those special placental cells may reveal new strategies in treating those diseases.

1.2 Polarization of macrophages

Generally, macrophages fulfil a wide range of different tasks in the human body. In their function as “soldiers” of innate immunity and host defense they act along inflammatory and anti-microbial processes, which is what they are commonly known for. In addition to that, they fulfil anti-inflammatory, homeostatic and regulatory functions as well, especially in pregnancy. This seems to be a fundamentally different working field. To explain those apparently contrasting functions, a model of so called functional plasticity has been developed(39). According to this, macrophages sense their surrounding milieu and react to it by adopting a specific effector function(39). In this complex process of macrophage activation, the cells either respond with a classical pro-inflammatory M1 activation or an alternative M2 activation(40). Hence, the specific function of macrophages is an expression of their polarization and determined by the surrounding milieu(6).

“*The term macrophage activation (classical activation) was introduced […] to describe the antigen-dependent, but non-specific enhanced, microbicidal activity of macrophages toward BCG (bacillus Calmette-Guerin) and Listeria upon […] exposure to the pathogens*.”(40)

The term “classically activated macrophages” refers to their function as potent effector cells, which kill microbes and act in cell-mediated immune responses(41). Macrophages get recruited and polarized by natural killer cells (NK), which react to infections and stress by secreting pro-inflammatory stimuli, such as IFNγ, which will be described in depth later in this work. This further drives macrophages to adopt the M1 polarization to pursue their antigen-presenting and killing function by secreting additional pro-inflammatory cytokines like IL-12 and IL-23, superoxide

anions and oxygen, and nitrogen radicals(42–44). Subsequently, the innate immune system enhances the defense against infection *via* NK and macrophages. As NK only provide temporary stimuli for the macrophages, they are not very effective in sustaining an army of activated macrophages during infection. Hence, the adaptive immune system takes over this role and helps to maintain a stable immune response and thus an effective host defense. Protagonists in this are T helper 1 cells (Th1), which also produce IFNγ.(45)
In turn, macrophages lead T cell response to a Th1 or cell-mediated form, *via* chemokines such as CCL5(43,46) and therefore enhance their own stimulation.
As the primary purpose of M1 activated macrophages is host defense, the transcriptome following M1 polarization includes pro-inflammatory cytokines (TNF, IL-6, IL-12), cytokine receptors, chemokines (CCL5), microbicidal agents and co-stimulatory surface molecules such as CD80 and CD86(47).
M1 or classically activated macrophages display an IL-12_{high} and IL-10_{low} phenotype(46). *Verreck at al.* showed that macrophages require a stimulation with exogenous IFNγ combined with bacterial LPS to secrete IL-12 on a high level(46).

Several agents have been identified to induce and mediate M1 polarization, including external stimuli like intracellular pathogens(48), bacterial cell wall components, lipoproteins as well as cytokines such as IFNγ, tumor necrosis factor alpha (TNFα)(8) and granulocyte-macrophage colony-stimulating factor (GM-CSF)(40). Like mentioned before, IFNγ emerged as the main stimulus for polarization towards M1, produced by Th1, NK and macrophages itself. IFNγ promotes the expression of cytokine receptors, markers for cellular activation and adhesion molecules(8).
Bacterial antigens also cause a shift towards M1 polarization *via* binding to detection receptors. Toll-like receptor 4 (TLR4) is a transmembrane protein which recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs)(24,49). The Gram-negative bacterial cell membrane component LPS is a TLR4 ligand(50). By binding to this receptor, LPS induces expression of pro-inflammatory cytokines and chemokines, antigen presentation molecules (major histocompatibility complex (MHC) members), co-stimulatory agents and enzymes for antigen-processing(40).

Lately it has been found that GM-CSF, a cytokine produced by a variety of cells, also induces M1 phenotype in macrophages by enhancing cytokine production, antigen presentation, phagocytosis, killing capacity, chemotaxis of leukocytes and adhesion(40).

Polarization of macrophages to M2 is very diverse to M1 in both, in terms of the cell function and how they are stimulated. *Röszer at al*.(8) reviewed the complex M2 macrophage and described its variety.

M2 activation (alternative activation) is associated with host defense mechanisms such as phagocytosis, but also regulatory, tissue-remodeling processes such as angiogenesis, modulation of extracellular matrix proteins and chemokines. Also, M2 activated macrophages draw the T cell response in a Th2 and humoral antibody mediated direction(43,51). M2 induced macrophages play a role in clearance of apoptotic cells, reduction of inflammation and occupy trophic functions such as tissue repair, wound healing and organ morphogenesis(8). However, not only favorable functions have been linked to M2 polarization. Cells polarized in this direction can also mediate allergy, tumor growth and can serve as reservoir for intracellular pathogens(52).

Various M2 markers have been identified based on their protein expression including transmembrane glycoproteins and scavenger receptors, enzymes, hormones, growth factors and cytokines and cytokine receptors(8). In the following, some M2 markers, which will be further interesting for the study, are described.

CD206, also known as *C-type 1 mannose receptor*, recognizes carbohydrate structures on glycoproteins and mediates their endocytosis. This surface molecule enables cells to detect potentially endangering viruses, bacteria, and fungi and to consequently neutralize the germs by phagocytosis(53). It was identified as M2 marker(54) with its expression enhanced by helminth infections, IL-4, GM-CSF and TGFβ(55).

CD163 is a scavenger receptor which was found to be upregulated following stimulation with IL-4(51), as well as M-CSF, IL-6, IL-10 and glucocorticoids(54), whereas a reduction of CD163 was found after TNFα, IFNγ and LPS stimulation(8).

CD209 or *Dendritic Cell Specific ICAM-3 Grabbing Non-integrin* (DC-SIGN) is a transmembrane receptor important for the innate immune system by recognizing several pathogens(56). It was found to be upregulated following IL-4 stimulation and downregulated following exposure to IFNγ and TGFβ(57). Also, M-CSF and IL-10 enhance the expression of CD209(58). Although CD209 is known as dendritic cell marker, it has been found on placental macrophages as well(58).
HLA-DR (Human Leukocyte Antigen – antigen D Related) is a MHC class II cell surface receptor which is important for antigen presentation and therefore represents a characteristic of activated cells(59). As some M2 macrophages feature antigen presenting tasks, they also express HLA-DR, if they are stimulated appropriately(51,60). Intriguingly, another study showed that M2 macrophages downregulate the expression of HLA-DR (and also CD86, but not CD80)(46).
Hormones and growth factors, such as placental growth factor and *VEGF-A* (vascular endothelial growth factor-A), have also been found to be produced by M2 macrophages, especially by the subsets M2a and M2c (see below)(61).
In opposition to M1 macrophages, M2 macrophages display an IL-10_{high} and IL-12_{low} phenotype(46), which makes sense as IL-10 is linked to a decreased production and activation of various pro-inflammatory signals(45). IL-10 down-regulates the expression of Th1 cytokines and co-stimulatory molecules and enables B-cell proliferation and survival as well as antibody production(62).

The functional aspects of M2 polarization seem to be very wide spreading and inconclusive, and have therefore been further divided into several subgroups, which differ in stimuli and effector function.
The M2a stadium is also referred to as “alternative activated” and induced by stimulation with IL-4/IL-13. IL-4 is a product of Th2 cells, granulocytes and macrophages, and it lowers phagocytic activity and mediates the expression of CD206. As IL-13 also binds to IL-4 receptor(63), IL-13 stimulation resembles these phenotypical features, but less pronounced(40). M2a polarized macrophages have low expression of inflammatory molecules such as IFNγ and produce signal molecules associated with Th2 polarization(43).
M2b, or “Type II activation”(45), results from stimulation with immune complexes, TLR and IL-1 receptor agonists. These macrophages occupy a special position, as they produce high levels of inflammatory cytokines such as IL-1, IL-6 and TNFα(8)

whilst remaining the IL-10_{high}/IL-12_{low} phenotype(45). M2b activation represents the collaboration of macrophages with B-cells, as it triggers the activation of antibody receptors and mediates antigen presentation (through expression of surface markers such as MHC II and co-stimulatory CD86(8)) and Th2 response(40). M2b macrophages amplify Th2 response and play an important role in immunoregulation(43).

M2c is defined as “deactivated”, whereby “deactivated” refers to the deactivation of M1 genes and adoption of M2 phenotype(54). Triggering stimuli are glucocorticoids, IL-10 and TGFβ. Glucocorticoids are generally known as anti-inflammatory agents(64). They have been found to mediate, amongst others, IL-10, CD163 and TGFβ expression. IL-10, as anti-inflammatory cytokine, follows also the stimulation of TLR and CD209 and promotes e.g. the expression of some Fc receptors, chemo-attractants and TLR1(40). Moreover, it reduces the expression of pro-inflammatory molecules, mediates remodeling of extracellular matrix and is associated with B-cell function(43). Regulatory TGFβ controls cell proliferation, differentiation and growth(65). The secretion follows phagocytosis of apoptotic cells by macrophages and contributes to the immune-regulatory phenotype(66).

M2d polarization, induced by IL-6, was first described in tumor associated macrophages (TAMs). IL-6 mediates cell proliferation and differentiation, tumor resistance to apoptosis and therefore promotes cancer development and progression(67). M2d polarization of macrophages is a product of the tumor associated environment, with IL-6, IL-10 and VEGF as key stimuli(67,68) and is characterized by low production of inflammatory molecules like IL-12, co-stimulatory molecules such as CD80, CD86 and MHC-II and therefore poor antigen presenting skills. Considering that, M2d macrophages represent an immunosuppressive macrophage type(67).

Conclusively, whereas M1 macrophages are characterized by the production of pro-inflammatory cytokines and interact with Th1 cells to promote effective killing of microorganisms and tumor cells(43), the subgroups of M2 macrophages phenotypically regulate inflammatory responses, adaptive immune reactions and phagocytosis and mediate trophic features such as angiogenesis and tissue turnover(43).

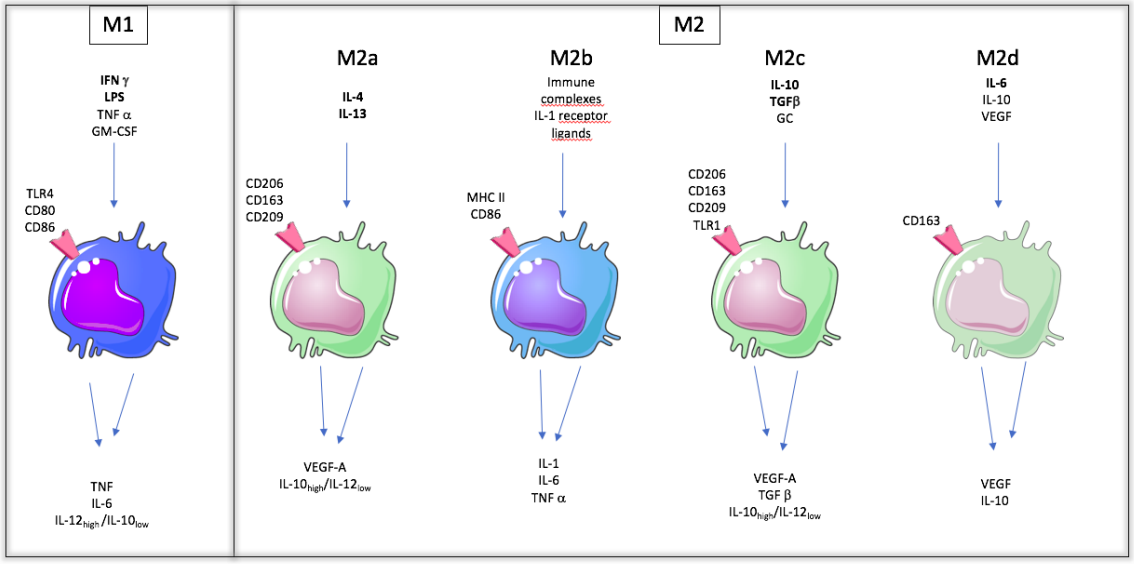


Figure 1| Classification of macrophage polarization

Figure adapted from *Martinez et al.,* (40). Patterns and drawings obtained from Smart Servier Medical Art (http://www.servier.com/Powerpoint-image-bank), licensed under a Creative Commons License (https://creativecommons.org/licenses/by/3.0/).

1.2.1 New nomenclature

It has to be said that the classification of macrophages into M1, M2 and subgroups is a simplified and nearly outdated classification. Newer reviews propose to classify the polarization status of macrophages terming the triggering stimulus. *Sudan et al*. recommend to refer to former "M1" as "M(LPS), M(IFNγ), M(GM-CSF)" etc., applied likewise to M2 and subtypes(69).

For simplification, within this study the former classification is used.

1.3 Macrophage polarization in the course of pregnancy

Pregnancy is an immunologic process, which is precisely regulated and shifts between inflammatory and regulatory milieu. Hence, one might assume that also macrophages are affected by this switch, as they are dependent on environmental stimuli(8). *Brown et al.*(6) described the polarization of maternal decidual macrophages in the changing milieu during course of pregnancy. Thus, embryonal implantation into the uterus requires a transitory inflammation, mediated through cytokines and prostaglandins in the semen. Accordingly, decidual macrophages are drawn to M1 polarization during peri-implantation period. As soon as

trophoblasts enter maternal tissue, decidual macrophages display a mixed profile, either a combined M1/M2 phenotype or a combination of M2 subtypes, with pro-inflammatory M2b and anti-inflammatory M2a/M2c phenotype.

For uterine vascular development and remodeling, this blend of polarization is maintained until the early second trimester, in order to guarantee the establishment of placental blood supply for the fetus. After completion of placental development, the milieu shifts towards pro-M2, to mediate maternal tolerance towards the fetus. This regulatory condition remains stable within the course of pregnancy, to avoid fetal rejection.

The initiation of parturition is also characterized by induction of inflammation and is triggered by infiltration of decidual macrophages into cervix, decidua and myometrium as well as fetal membranes. This inflammation drives uterine contraction, expulsion of child and placenta and subsequent involution of the uterus.(6)

Concerning macrophages derived from the fetus, there are not many results available. At term, Hofbauer cells, which already enter the chorionic villi by 4 weeks of gestation, have been shown to display M2 phenotype by analysis of methylation patterns of pro-M1 and pro-M2 genes. DNA methylation profile of HBCs in term placentae indicates silenced pro-M1 genes with concomitant hypo-methylated and therefore transcriptionally active pro-M2 genes(70). HBCs have further been shown to produce anti-inflammatory IL-10 and TGFβ, instigated by mesenchymal stem cells and trophoblasts, indicating a phenotypical commitment to M2 polarization.(6)

2 Hypothesis and Aim

The maintenance and favorable course of pregnancy requires precise regulation of the present milieu. An impairment of this regulation has been related to the development of pregnancy associated diseases(37). Macrophages sense and adapt to their surrounding milieu(39). Concluding, these cells as well act as protagonists in the process of milieu regulation(45).

Hofbauer cells seem to be committed to the regulatory M2 phenotype of polarization for successful pregnancy course(70). However, in contrast to the well examined phases of decidual macrophage polarization during the course of pregnancy(6), it has been made clear that current literature lacks equivalent characterization of fetally derived macrophages, HBCs.

This study aims to show for the first time how far inflammatory stimuli are able to influence HBCs phenotypical polarization and will therefore provide pioneering ideas of the importance of HBCs and their phenotype for pregnancy and associated diseases.

3 Material and Methods

3.1 Subject Characteristics

Placentae of healthy women with pre-gravid BMI of 21±1.6 kg/m^2 of three vaginal deliveries and two cesarean sections were gathered. The maternal mean BMI at delivery increased to 27±1.9 kg/m^2, the average pregnancy duration was 39+2 weeks. The mean placental weight was 578±69.1 g and the mean fetal ponderal index was 2±0.3 kg/m^3.

Table 1| Subject characteristics of women, placentae and children included in the study for macrophage isolation.

All data are shown as mean ± standard deviation.

	Mean (n=5)
Maternal pre-gravid BMI (kg/m^2)	21(±1.6)
Maternal BMI at delivery (kg/m^2)	27(±1.9)
Gestational age (GA) (weeks + days)	39+2
Placental weight (g)	578(±69)
Mode of delivery (vaginal/cesarean section)	3/2
Fetal ponderal index (kg/m^3)	2(±0.3)
Fetal sex (♂/♀)	2/3

3.2 Isolation of Hofbauer Cells from Term Placenta

3.2.1 Dissection of Villous Tissue

Isolation of Hofbauer cells was performed following a previously employed protocol at the gynecological laboratory of the Medical University of Graz, modified from *Tang et al.*(3). The study was approved by the institutional ethics committee of the Medical University of Graz (27-265 ex 14/15) and all mothers gave written informed consent.

On the first day, term placenta was brought just after either vaginal delivery or cesarean section by the research nurse, with masked patient data. Immediately upon receipt, placenta was transferred to cell culture laboratory and maternal

membranes were removed from placental tissue to avoid contamination with decidual macrophages. In the following, pieces of placental tissue (approx. 5cm^2) were cut out without interrupting the chorion plate. These pieces were washed in physiologic sodium chloride solution, and villous tissue was scraped off and finely minced. Tissue was transferred to a stericup with 150ml PBS (phosphate buffered saline, buffer solution(71)), weighed and stored at 4°C overnight.

3.2.2 Separation of Cytotrophoblasts

For digestion of villous tissue and separation of Hofbauer cells from cytotrophoblasts, the villous tissue was added to 150ml Trypsin/DNAse I digest buffer (50ml 10xHBSS, 12.5ml HEPES(1M), 1ml $CaCl_2$(1M), 1ml $MgSO_4$(0.4M), 2.5ml NaOH(2M), 450ml MQ, 2ml DNAse I (Roche, Mannheim, Germany), 50 ml 2.5% Trypsin (10×, Gibco, Lofer, Austria) in a sterile beaker and stirringly digested for 20 min at 37°C. Next, tissue was washed extensively with PBS, poured back into a beaker with 150ml Trypsin/DNAse I digest buffer and stirringly digested for 30min at 37°C. After PBS wash, previous digestion step with 150ml Trypsin/DNAse I was repeated for 30min at 37°C.

3.2.3 Isolation of Hofbauer Cells

3.2.3.1 Digestion of Villous Tissue and Collection of Single Cells

Subsequently, tissue was sieved through a 70μm sieve and sterile gauze. For further digestion of villous stromal tissue and collection of single Hofbauer cells, tissue was returned to beaker with 100ml Collagenase/DNAse I digest buffer (1 vial Collagenase A (Roche, Mannheim, Germany) + 50ml RPMI complete (500ml RPMI 1640 (Gibco, Lofer, Austria) + 5ml Penicillin/Streptomycin Aliquot + 12.5ml HEPES(1M) + 25ml FCS; this buffer needs to be kept cold to prevent the settlement of Hofbauer cells to plastic surfaces); 2ml of dissolved DNAse I (Roche, Mannheim, Germany) + 50ml RPMI complete; both filtrated using Steriflip) and stirringly digested for 1 hour at 37°C. Subsequently, digested tissue was filtered through sieve and gauze into sterile beaker, flow-through apportioned into 4 Falcon tubes, filled up to 45ml with RPMI and centrifuged at 300g for 10min in centrifuge (Eppendorf 5810 R). Supernatant was taken off, cells were washed in

cold RPMI complete twice, centrifuged and pellets were resuspended in a total volume of 24ml.

3.2.3.2 Separation of Hofbauer Cells by Percoll Gradient

For separation of Hofbauer cells by Percoll gradient (colloidal silica density separation), 6 tubes of gradients layering 40%, 35%, 30% and 20% Percoll were prepared. 4ml of cell suspension was layered onto each gradient and centrifuged at 2030g for 30min at 4°C with deceleration set to zero. After centrifugation, Hofbauer cells gather due to their specific density at the interface between 35% and 30% Percoll. Cells were taken off with a needle and syringe, transferred into Falcon tubes filling 25ml cold RPMI each, spun down at 300g for 10min, washed twice the same way and counted using CASY TT cell counter (Roche Innovatis; Bielefeld, Germany). Cells sized within the range of 7.5μm to 30μm were defined as viable.

3.2.3.3 Immune-Purification of Hofbauer Cells

After counting, cells were centrifuged and resuspended in 10ml cold RPMI media. For negative depletion of cytotrophoblasts,100μl anti-EGFR(72) (mouse monoclonal antibody, clone 528 + 199.12, 200μg/mL; NeoMarkers, Fremont, CA) magnetic beads (Dynabeads Goat anti-Mouse IgG, Invitrogen) per 100 million cells was added and incubated the suspension on ice on a horizontal shaker for 15min. Subsequently, cells were distributed to four vials and placed in a magnet. Cell suspension was taken off, spun down, and resuspended in 10ml cold RPMI complete. For negative depletion of remaining fibroblasts, 100μl anti-CD10(73) (mouse monoclonal antibody, clone MEM-78, 100μg/mL; SigmaAldrich) magnetic beads per 100 million cells was added to the suspension and proceeded the same way as before. Supernatant cell suspension was centrifuged, resuspended in cold RPMI complete, counted using CASY (again cells appearing with a diameter of 7.5μm to 30 μm were defined as viable) and resuspended in an appropriate media volume amount yielding $1x10^6$ cells per ml.

3.2.3.4 Culturing Hofbauer Cells

HBCs were seeded at a cell density of $1x10^6$ cells/ml in T-75 tissue culture flasks (12ml total vol. per flask) for treatment and FACS analysis and onto 4-well chamber slides (600μl total vol. per chamber) for treatment and analysis by ICC

(Immuncytochemistry) in macrophage medium (MaM, ScienCell, Carlsbad, USA; supplemented with 5% FCS, 1% Penicillin/Streptomycin Aliquot and macrophage growth supplements (MaGS; ScienCell, Carlsbad, CA). The cells were incubated at 37°C and 21% oxygen.

3.3 Induction of Cell Polarization

In order to induce polarization of macrophages, HBCs were treated by different stimuli which have been previously defined to polarize macrophages in M1, M2a, M2c and M2d phenotypes (see 1.3.1.2., 1.3.2.2.). The treatment was performed twice, each on day one and day four after isolation (see Figure 2 below).

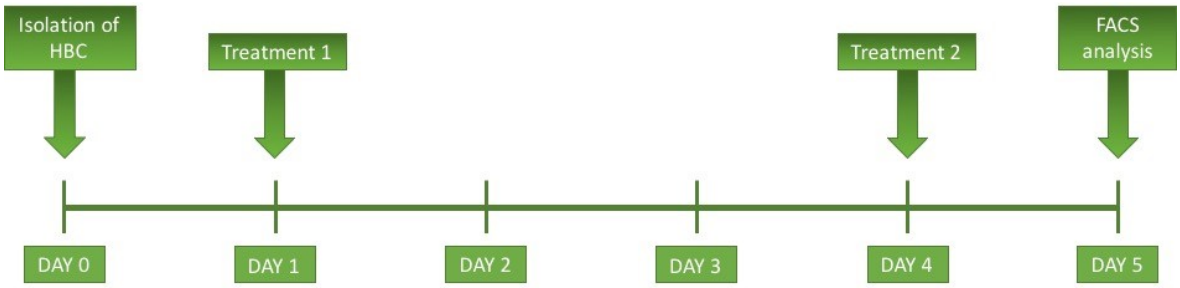


Figure 2| Time-line of HBC treatment regimen

One day after isolation of primary cells the first treatment was implemented. On day four cells were again boosted. FACS analysis was performed on day 5.

Cells were incubated at 37°C and 21% oxygen for the whole course of experiments.

For M1 activation, cells were exposed to a final concentration of 50ng/ml LPS + 20ng/ml INFγ. For the induction of M2a, M2c, and M2d phenotypes 20ng/ml IL-4 + 20ng/ml IL-13, 20ng/ml IL-10 + 20ng/ml TGFβ, and 20ng/ml IL-6 was used, respectively. The untreated controls were incubated under the same conditions but without any stimuli.

Table 2| Cytokines used for induction of cell polarization

Stimulus	*Concentration (ng/ml)*	*Solution*	*Stock*	*Company*
Lipopolysaccharide	50	1mg/ml	E.coli	Sigma Aldrich
Interferone γ	20	1mg/ml	Recombinant E.coli	Sigma Aldrich
Interleukine 4	20	10μg/ml	Recombinant E.coli	Sigma Aldrich
Interleukine 6	20	20μg/ml	recombinant	Sigma Aldrich
Interleukine 10	20	10μg/ml	Recombinant HEK 293	Sigma Aldrich
Interleukine 13	20	10μg/ml	Recombinant	Sigma Aldrich
Transforming growth factor β1	20	10μg/ml	Recombinant HEK 293	PeproTech

Used stimuli were LPS + IFNγ (M1), IL-4 + IL-13 (M2a), IL-10 + TGFβ (M2c), IL-6 (M2d).

3.4 FACS Analysis

On day 5 after isolation of HBCs (Figure 2), following two treatments with determined stimuli, the aim was to show whether changes in polarization of HBCs occurred. To do so, surface markers specific for the various polarization were quantified, as shown below, using fluorescence activated cell sorting technology (performed on LSR II instrument (BD Biosciences)).

3.4.1 Principle and Procedure

Flow cytometry is a method to detect characteristics of single particles, in our case, macrophages. Therefore, distinguishing properties of cells need to be marked using fluorochromes conjugated to antibodies (=fluorophores), which are directed to specific cellular epitopes. These fluorophores get excited by laser and can hence be detected in FACS device.

For detection of single cell parameters, cells get aligned in single row before passing through laser detection. The laser sends light to the passing particles,

which gets scattered and generates signals in surrounding detectors. The individual classification follows a three-step scheme. Firstly, the forward scatter channel scans light which is scattered at 20° and represents cell size, as larger particles typically reflect more light. Secondly, the side scatter channel detects scattered light at 90° and provides information about the granularity. Thirdly, the device performs a fluorescence measurement, by stimulating the earlier conjugated fluorophores and hence detecting molecules of interest on the cell surface or intracellularly by measuring the emitted light.
Like this, passing cells get classified by three parameters and quantitative analysis can be performed.(74)

For evaluation of secreted molecules *via* ELISA at a later time the supernatant media was frozen at -20°C.
HBCs show a strong adherence to plastic(3), therefore cells need to be detached from flasks by scraping gently in 3ml accutase. Cell suspensions were distributed according to prior treatment into Falcon tubes, adjusted to equal volume with RPMI complete + 10% FCS to stop accutase, counted using CASY (size range of viability 7.5μm to 30 μm) and incubated for 10 min in 3ml HBSS/3% FCS to block Fc-receptors, as HBCs presenting disproportionately high amounts of Fc receptors on their surface(17).
Cell suspensions were distributed to an adequate number of vials, yielding a minimum of $5x10^5$ cells/vial and centrifuged at 300g for 5 min.
After resuspending cells in 100μl PBS, suspension was incubated in darkness for 30 min at 4°C with the fluorochrome-conjugated antibody in appropriate dilution (Table 3). Cells were then washed with 500μl PBS and resuspended in 150μl PBS for FACS.
Expression of VEGF is detected intracellularly. Prior to intracellular staining, cells have to be permeabilized. Therefore, 150μl Permeabilization/Fixation solution (BD Cytoperm/Cytofix) was added and incubated for 15 min at 4°C, washed with 500μl BD Perm/Wash Buffer (BD Cytoperm/Cytofix) and resuspended in another 100μl BD Perm/Wash Buffer. Then, the directly conjugated anti-VEGF antibody in an appropriate dilution (Table 3) was added, incubated for 30 min at 4°C. Cells were washed with 300μl PBS and resuspended in 150μl PBS for FACS.

Table 3| Antibodies used for FACS analysis

Antibody target (Company)	*Description*	*Clone*	*Label*	*Dilution*
CD163 (BioLegend)	Mouse monoclonal	GHI/61	APC	1:7
CD206 (BDPharmingen)	Mouse monoclonal	19.2	FITC	1:10
CD209 (BDPharmingen)	Mouse monoclonal	DCN46	PerCP5.5	1:10
CD80 (BDHorizon)	Mouse monoclonal	L307.4	V450	1:10
CD86 (BDHorizon)	Mouse monoclonal	2331	V450	1:10
TLR1 (Abcam)	Mouse monoclonal	GD2.F4	FITC	1:20
TLR4 (RnDSystems)	Mouse monoclonal	610015	PE	1:20
HLA-DR (BioLegend)	Mouse monoclonal	L243	V450	1:20
VEGF (RnDSystems)	Mouse monoclonal	23410	PE	1:20

APC = allophycocyanin; FITC = fluorescein isothiocyanate; PE = phycoerythrin; PerCP= peridinin chlorophyll protein; V450 = PacificBlue, absorbed at 450nm

3.5 Immuncytochemistry

In parallel to FACS analysis, $6x10^5$ cells per well were seeded on 4-well glass chamber slides (Lab-Tek) in order to qualitatively corrobate changes is marker expression upon treatment of cells *via* ICC.

After isolation, seeding onto wells and culturing HBCs overnight with macrophage medium the same stimuli as for FACS analysis was used, namely LPS + IFNγ, IL-4 + IL-13, IL-10 + TGFβ and IL-6, yielding the same final concentrations. Three 4-well chamber slides per treatment and 3 chamber slides of untreated cells as negative control were prepared. HBCs have been treated on day 1 and 4 after isolation (Figure 2).

3.5.1 Principle and Procedure

Preparing for the ICC, chamber slides have been washed, air dried and cells were fixed with ice cold acetone. EnVision system (DAKO; Hamburg, Germany) was used, which consists of two steps of antibody labelling.

First, primary antibodies were added. For each treatment as well as the untreated control, primary antibodies in appropriate dilution (Antibody Diluent, DAKO) as given in Table 4 were added. After 30 min, slides were drained, incubated for 10 min with Antibody Enhancer (DAKO), and then rinsed extensively with TBE (Tris-buffered saline).

As second step, a large HRP-Polymer (Horse-radish peroxidase; ThermoScientific), which is an antibody-labeled enzyme binding to the Fc-region of the primary antibody was added. The peroxidase catalyzes the later added substrate(75). Slides were incubated for 30 min with HRP, and then washed as before. To visualize, samples were incubated for 10 min with Chromogene Solution (3-amino-9-ethylcarbazol (AEC-substrate solution, ready to use; DAKO)), washed with distilled water, counterstained for 5 min with haemalaun solution (Mayers Haematoxylin, Sigma Aldrich) and washed with tab water. Slides were then mounted with AquaTex (Merck, Darmstadt, Germany), covered with coverslips (Roth, Karlsruhe, Germany) and examined using BX53 microscope (Olympus; Hamburg, Germany) and AxioCam MRc5 (Zeiss, Oberkochen, Germany); AxioVision Rel. 4.8 software was used to acquire pictures.

Table 4| Antibodies used for ICC

Antibody (Company)	*Description*	*Clone*	*Dilution*
αCD163 (ThermoScientific)	Mouse monoclonal	10D6	1:50
αCD 90 (Dianova)	Mouse monoclonal	AS02	1:1000
αCD 206 (Novus)	Rabbit polyclonal	-	1:200
αCD 209 (Novus)	Mouse monoclonal	UW60.1	1:300
αCD 68 (Dako)	Mouse monoclonal	KP1	1:100
αCD 80 (Abcam)	Mouse monoclonal	2A2	1:300
αCD 86 (Novus)	Mouse monoclonal	Bu63	1:100
αTLR4 (Abcam)	Mouse monoclonal	76B357.1	1:300
αTLR1 (Abcam)	Rabbit polyclonal	-	1:100
αHLA-DR (Abcam)	Mouse monoclonal	L243	1:100
αVEGF-A (Abcam)	Rabbit monoclonal	EP1176Y	1:100
Isotype control (Dako)	Goat anti-mouse IgG		1:200

Classical macrophage markers (CD68(36)), M2 markers (CD 163, CD206, CD209, TLR1, HLA-DR, VEGF-A), M1 markers (CD 80, CD 86, TLR4) and fibroblast marker CD 90(76) for evaluation of cell population purity were selected.

3.6 Enzyme Linked Immunosorbent Assay

For detection of secretion products of both HBC and THP-1 (Control cell lineage, see 2.7.) cells, media supernatants were collected right before proceeding to FACS analysis. The media samples were frozen and stored until all samples of different experiments were collected. Samples were concentrated to 4:1, using Vivaspin Turbo centrifugation units (Sartorius; Stonehouse, UK) to a total volume of 2ml, aliquoted á 250ul and froze it for subsequent ELISA analysis.

3.6.1 Principle and Procedure

ELISA approach was selected where the antigen is quantified between two layers of antibodies, for quantifying specific protein. After the first antibody, or capture antibody, is bound to a surface (e.g. 96 well plate) and nonspecific binding sides

are blocked, the antigen-containing samples are added to the plate. If antigens are present, they will bind to the capture antibody. To remove unbound antigens, the plate is washed. Next, a specific antibody, or detection antibody, is bound to the antigen, followed by enzyme-linked secondary antibodies that bind to Fc-regions. After again washing the plate to remove unbound antibodies, a substrate is applied, which gets converted by the previously added enzyme and hence changes its color. This signal is measured as absorbency alteration to detect and quantify the presence of antigens.(77)

ELISA against IL-10, IL-12, TNFα and VEGF was performed using PeproTech (Rocky Hill, USA) Mini Development Kits on a 96 well microtiter plate. After coating the plate with the capture antibody and incubating at room temperature overnight, plates were washed 4 times using wash buffer (0,05% Tween-20 in PBS), blotted on paper towels and blocked by incubating with 300µl block buffer (1% BSA in PBS) for one hour at room temperature. Meanwhile standards were prepared, following a descending dilution of 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml and 15.63pg/ml. After washing again 4 times, 100µl of blanks, standards and samples in duplicates were loaded onto the plate and incubated for 2 hours at room temperature. Subsequent to another washing step, the detection antibody at a concentration of 0.5µg/ml was added and incubated for another 2 hours at room temperature. After that, Avidin-HRP Conjugate was diluted 1:2000, of which 100µl was added to the wells and incubated for 30min at room temperature. Following the last washing step, 100µl ABTS Liquid Substrate (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) for color development was added and immediately plate was measured by a plate reader (SPECTROstar Nano; BMG Labtech, Ortenberg, Germany).

For ELISA of TGFβ1 Human/Mouse TGFβ Ready-Set-Go ELISA Kit purchased from Invitrogen (ThermoFisher) following a slightly different protocol was used. Therefore, the plate was coated with the capture antibody and stored at 4°C. The next day, the plate was washed (0.05% Tween-20 in PBS) and blocked using ELISA/ELISPOT Diluent. As TGFβ1 resides in its latent form, it needs to be activated by acidifying samples for 10min at room temperature in 1N HCl. After neutralizing with 1N NaOH, samples and standards (at the same concentration as used in PeproTech protocol) were applied to the 96 well plate and incubated for 2

hours at room temperature. Subsequently, the plate was incubated with detection antibody for 1 hour, followed by 30min incubation with Avidin-HRP. After washing extensively, the substrate (TMB Solution (3,3′,5,5′-Tetramethylbenzidin)) was added for 15min at room temperature, then stopped using Stop Solution and placed into the plate reader (SpectroStar Nano; BMG Labtech, Ortenberg, Germany).

Data acquired from SpectroStar Nano was analysed using MARS data analysis software (BMG Labtech, Ortenberg, Germany).

Table 5| Kits used for ELISA

Target	*Company*	*Capture antibody (Concentration)*	*Standard*	*Detection Antibody*
IL-10	PeproTech	Rabbit Anti-Human IL-10(1μg/ml)	Recombinant Human IL-10	Biotinylated Rabbit Anti-Human IL-10
IL-12	PeproTech	Goat Anti-Human IL-12(1μg/ml)	Recombinant Human IL-12	Biotinylated Goat Anti-Human IL-12
TNFα	PeproTech	Rabbit Anti-Human TNFα(1μg/ml)	Recombinant Human TNFα	Biotinylated Rabbit Anti-Human TNFα
VEGF	PeproTech	Rabbit Anti-Human VEGF(0.5μg/ml)	Recombinant Human VEGF	Biotinylated Rabbit Anti-Human VEGF
TGFβ1	ThermoFisher	Anti-Human/Mouse TGFβ1	Recombinant Human TGFβ	Biotinylated Anti-Human/Mouse TGFβ

3.7 Control Cell Lineage

In parallel, the same protocols to a control cell lineage of different cellular origin were applied. These lymphatic cancer tissue cells (THP-1) represent a human monocyte/macrophage cell line (78).

The cells were stored in liquid nitrogen at -180°C and thawed at use. At thawing, cells have been washed to remove the cryopreservation agent (Dimethyl sulfoxide (DMSO), which reduces the formation of ice crystals and prevents cell death at freezing process) and then incubated with RPMI complete media.

For using the cell lineage as control in the experiments, cells were differentiated to macrophages by treating them with PMA (Phorbol 12-Myristate 13-Acetate) at a concentration of 100ng/ml for 4 hours. This protocol is referring to *Chanput et al.* (78) who successfully induced M1/M2 genes in THP-1 cells treated with PMA.

For induction of cell polarization of THP-1 the same stimuli in the same final concentrations as for the HBCs were used (see 3.3). The timeline of the treatment regimen differed to the protocol of primary cells (Figure 3). A treatment regimen of one polarization treatment 4 hours after PMA stimulation, then incubation at 37°C for 3 days was applied.

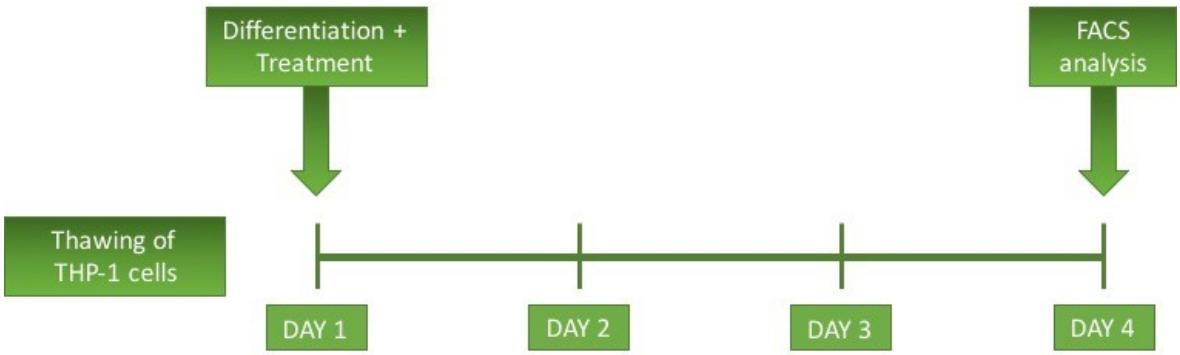


Figure 3| Time-line of THP-1 treatment

After thawing, removal of DMSO by changing the cell culture media and differentiation into macrophages with PMA, THP-1 cells were stimulated once and performed FACS analysis on day 4. Cells were incubated at 37°C and 21% oxygen for the whole course of experiments.

For analysis of cell polarization of THP-1 the same approaches and protocols as for HBCs were used.

3.8 Statistical Analysis

GraphPad Prism v7.0 has been used to perform statistical analysis and creation of graphs of all acquired data. T-test was used to compare two groups, for repeated-measures (RM) 2-way analysis of variance (ANOVA) was used to compare the four matched treatment-groups to the untreated control; Dunnets post-hoc test was applied to RM-ANOVA to correct for multiple comparisons. P-values below 0.05 were regarded as significant.

4 Results

4.1 Preliminary tests

At the beginning of the study two HBC-isolations following a slightly different protocol of stimulation were performed. HBCs were stimulated only once, on day 2 after isolation, and stained for FACS analysis on day 3. By observing the cells via light microscope, it was tested if treatment altered the morphology of the cells. The expectations of morphological alterations were not fulfilled within performed timeline of treatments. Retrospectively, also the quantitative FACS analysis of polarization markers of these experiments showed consistently absolute values lower than the means of experiments following our later established protocol.

Factors as the short time of exposition to stimuli and probably too low concentration of stimuli as well were outlined as an explorative approach of the resistivity of the cells to polarize. As the cells process the added cytokines, they reduce their concentration in supernatant over time. At this point, an adjustment of the protocol was conducted. The duration of stimulation was prolonged to four days, to counteract the time factor, and a second bolus of stimuli was added on day 4 after isolation (Figure 2), to maintain a higher concentration of stimulating factors over time in the supernatant, thereby exposing the cells more constantly to respective stimulus.

These two trials were considered as preliminary tests and were excluded from further analysis.

4.2 THP-1 cells can be considered as suitable control cell lineage

The expression of specific markers of both HBCs and PMA-stimulated THP-1 in untreated control cells were measured, to check whether THP-1 cell line were suitable as comparative control cells for this study. No significant difference between the two cell types was detected in the expression of CD163, CD209, CD80, CD86, TLR1, TLR4, HLA-DR or VEGF. Solely CD206 expression was significantly higher in untreated HBCs than THP-1 ($p<0.0001$), indicating a predominant M2 polarization of HBCs under normal cell culture conditions. Although differences in baseline phenotype compared to HBCs may exist, all other

M2 and M1 markers were expressed similarly in both cell types (Figure 4). Therefore, THP-1 cells were considered as suitable control cell lineage for this study.

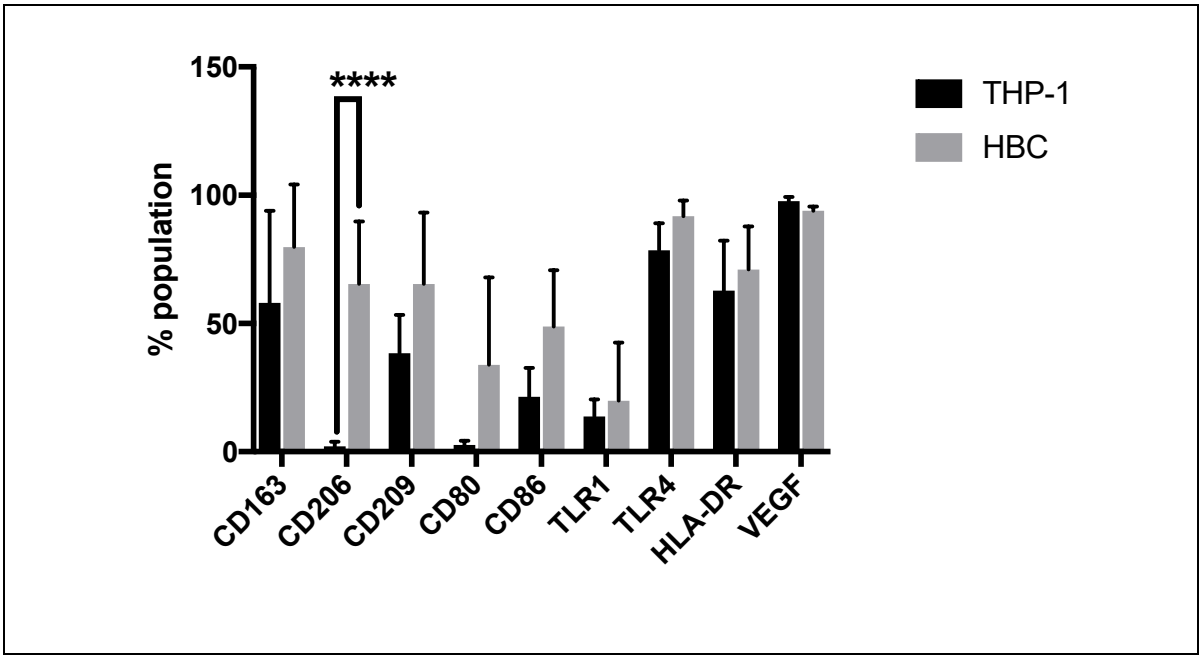


Figure 4| Comparison of marker expression in untreated HBC and THP-1.
By comparing each individual experiments (N=5), HBCs express baseline significantly more CD206 than THP-1. No significant differences in the expression of other outlined markers were observed.

4.3 HBCs remain more resilient in their regulatory phenotype upon stimulation compared to THP-1

For Hofbauer cells, quantitative changes of M1/M2 defined marker proteins on stimulated cells compared to untreated controls of the same isolation were examined. Figure 5 shows the quantitative results of FACS analysis, Figure 6 and 7 depict representative scatter plots of FACS data.

Figure 5 A shows consecutive changes of surface marker expression on stimulated HBCs. The absolute numbers of HBCs expressing respective markers are presented as percent population of total detected HBCs.

Regarding M2 polarization, a significant decrease of CD206 ($p < 0.0001$; -73.3% of total population, Figure 6A, B) and CD209 ($p < 0.0001$; - 66.5%, Figure 6C, D) in cells treated with LPS + IFNγ compared to untreated control was

observed, which indicates a decrease in number of M2 polarized cells following this treatment. Complementing, an increase of CD209 ($p = 0.0147$; + 35.6%, Figure 6C, E) in cells treated with IL-4 + IL-13 was detectable. Additionally, a significant decrease of intracellular VEGF protein in cells treated with LPS + IFNγ ($p = 0.0015$; - 30.82%, Figure 6F, G) was measurable.
Expression of the M1 marker CD86 was reduced in IL-4 + IL-13 and IL-10 + TGFβ treated cells, but only by trend ($p = 0.067$ and $p = 0.097$, respectively).
Finally, no significant differences in expression of CD163, CD80, CD86, TLR1, TLR4 and HLA-DR (Figure 5, A) was identifiable.

In comparison to HBCs, THP-1 cells were found to be less resilient and induction of different polarization phases was more effective, as FACS data reveals (Figure 5, B).
In particular, a highly significant increase of CD209 expressing THP-1 cells after IL-4 + IL-13 treatment was observed compared to untreated cells ($p < 0.0001$, + 86.1%, Figure 7A, B), suggesting a phenotypical shift towards M2 polarization. Moreover, a decrease of CD209 in IL-10 + TGFβ treated cells could be seen ($p = 0.0356$; - 46.7%, Figure 7A, C). Regarding M1 markers, CD80 was upregulated in both LPS + IFNγ (7D, E) and IL-6 (7D, F) treated cells by 15-fold and 8-fold ($p < 0.0001$; and $p = 0.0108$; respectively) as well as CD86 in LPS + IFNγ treated cells ($p < 0.0001$; + 162.7%, Figure 7G, H). TLR1 expression was increased in cells treated with IL-6 ($p = 0.0239$; + 235.9%, Figure 7I, J). The MHC class II molecule HLA-DR, which is important for antigen presentation(59), was found to be increased in LPS + IFNγ (7K,L) and IL-6 (7K,N) treated cells ($p = 0.0004$; + 44%, and $p = 0.0006$; + 39.8%, respectively) and decreased in IL-4 + IL-13 treated cells ($p = 0.002$; - 39%, Figure 7K, M).
No changes in CD163, DC206, TLR4 and VEGF expression of THP-1 treated cells were detectable (Figure 5, B).

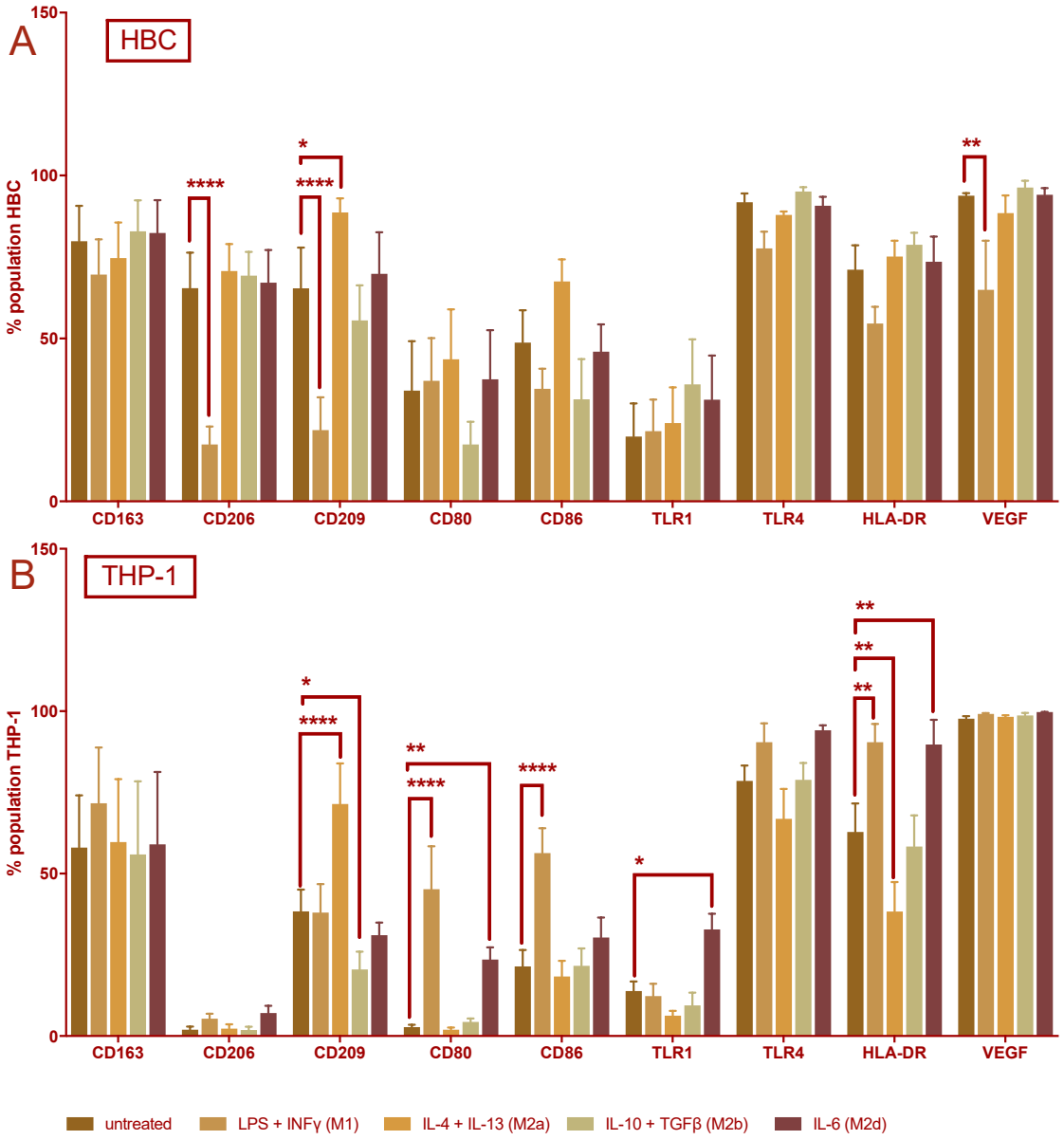


Figure 5| Expression of polarization markers on Hofbauer cells (HBC) and THP-1 cells quantified by FACS.

The leftmost bar of each specific marker represents the percentage of positive cells within the untreated population of cells. Significant changes have been marked; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

(A) In treated HBCs compared to non-treated cells significant changes in the expression of CD206, CD209, and VEGF were observed.

(B) In treated THP-1 compared to non-treated significant changes in the expression of CD209, CD80, CD86, TLR1, and HLA-DR were detectable.

Data are shown as mean ± standard deviation, using 2-way ANOVA for analysis, calculated and graphed by GraphPad Prism.

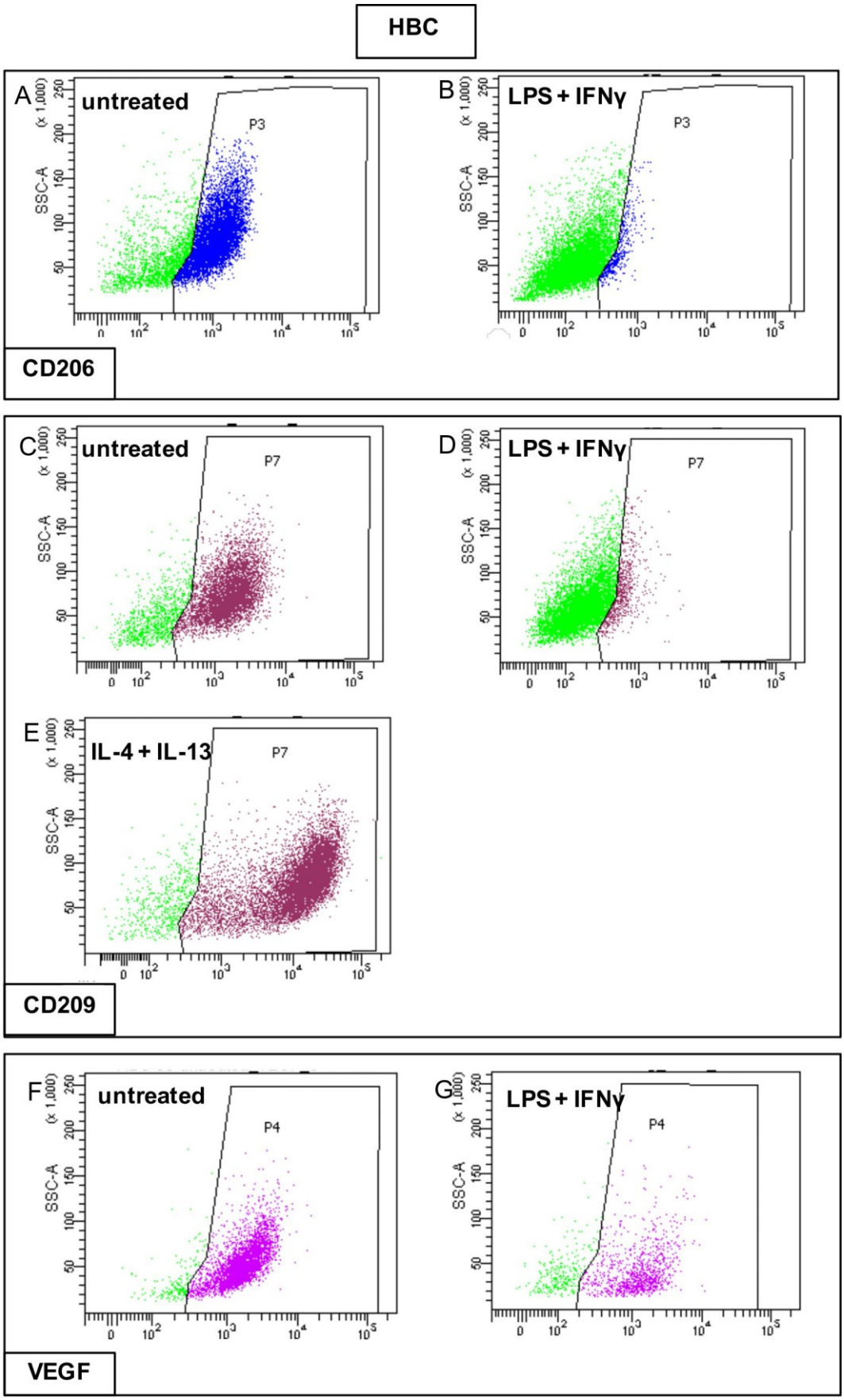

HBC
A
untreated
P3
B
LPS + IFNγ
P3
SSC-A
(x 1,000)
CD206
C
untreated
P7
D
LPS + IFNγ
P7
E
IL-4 + IL-13
P7
CD209
F
untreated
P4
G
LPS + IFNγ
P4
VEGF

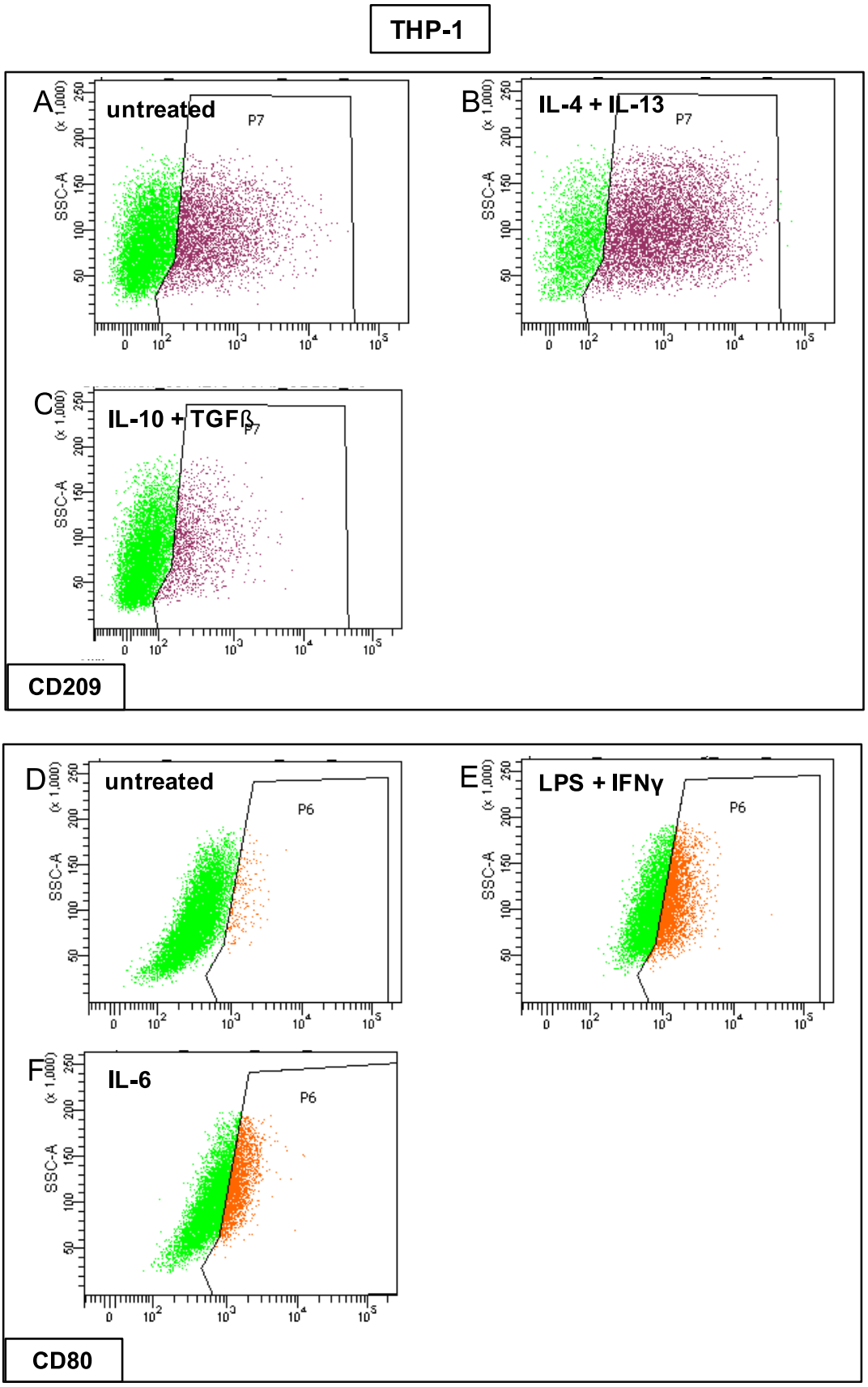
Figure 6| Scatter plots of flow cytometrical assessment of HBC.

This figure depicts original FACS data of HBCs marker expression. Differences considered as significant are presented at this point for illustration. Shown results are representative for FACS analysis of N=5 experiments.

Different colors of panels refer to different detection channels of the flow cytometer.

FITC, PerCP and PE were used to stain for CD206, CD209 and for VEGF, respectively.

Significant changes in marker expression were detectable in expression of CD206 (A, B), CD209 (C, D, E) and VEGF (F, G).


THP-1
A
untreated
P7
B
IL-4 + IL-13
P7
C
IL-10 + TGFβ
P7
SSC-A
(x 1,000)
CD209
D
untreated
P6
E
LPS + IFNγ
P6
F
IL-6
P6
CD80

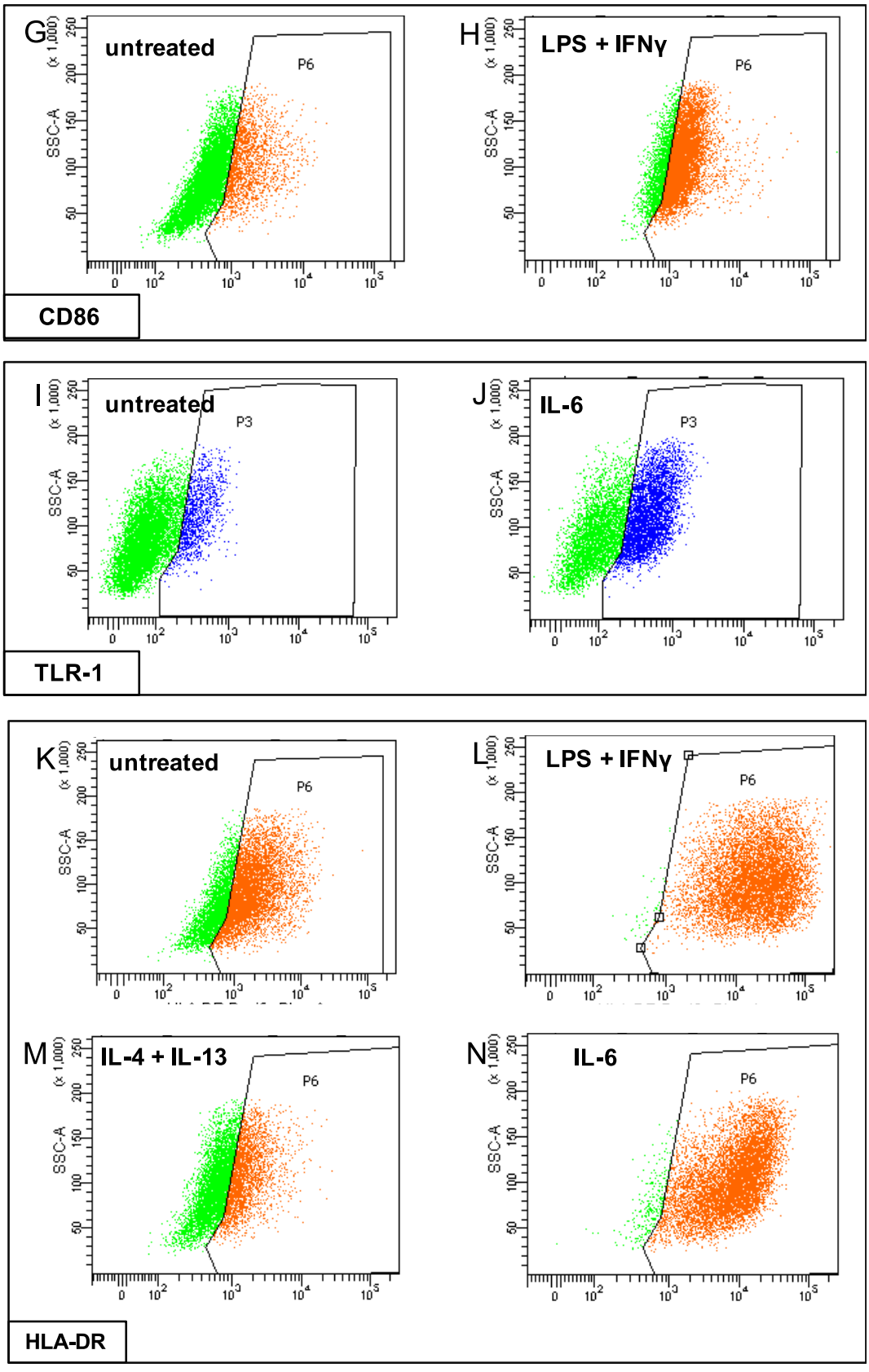

G
untreated
P6
SSC-A
(x 1,000)
H
LPS + IFNγ
P6
CD86
I
untreated
P3
J
IL-6
P3
TLR-1
K
untreated
P6
L
LPS + IFNγ
P6
M
IL-4 + IL-13
P6
N
IL-6
P6
HLA-DR

Figure 7| Scatter plots of flow cytometrical assessment of THP-1.

This figure depicts original FACS data of THP-1 cell marker expression. Differences considered as significant are presented at this point for illustration. Shown results are representative for FACS analysis of N=5 experiments.

Different colors of panels refer to different detection channels of flow cytometer.

PerCP was used to stain for CD209, Pacific blue was used to stain for CD80, CD86 and HLA-DR, FITC was used to stain for TLR1.

Significant changes in marker expression were observed in expression of CD209 (A, B, C), CD80 (E, F), CD86 (G, H), TLR1 (I, J) and HLA-DR (K, L, M, N).

4.4 THP-1 cells display a IL-12^{high}/IL-10^{low} baseline phenotype and HBCs remain regulatory IL-10^{high}/IL-12^{low} phenotype upon stimulation

The secretion of TGFβ, TNFα, IL-10, IL-12 and VEGF of HBCs and THP-1 upon different treatment conditions was investigated.

Both cell types, THP-1 and HBCs treated with LPS + IFNγ significantly increased their secretion of TNFα by 6-fold and 4-fold ($p = 0.0045$ and $p = 0.0005$, respectively). As TNFα is a pro-inflammatory cytokine, its increase indicates a shift towards M1 polarization (Figure 8A).

LPS + IFNγ treated THP-1 cells significantly lower their production of TGFβ compared to untreated control by 21-fold ($p = 0.0255$, Figure 8B). This finding is in line with literature data of other types of macrophages in M1 polarization, and therefore we postulate that THP-1 cells can be induced to M1 polarization by pro-inflammatory stimuli like LPS and IFNγ. In HBCs treated with IL-10 + TGFβ a significant increase of TGFβ by about 5-fold ($p = 0.003$, Figure 8B) was observed.

A significantly higher secretion of IL-12, as M1 marker, could be seen in LPS + IFNγ treated HBC compared to untreated ($p = 0.0027$, 4.5-fold increase, Figure 8D), which on the first sight suggests a polarization shift towards M1. But interestingly, the increase of IL-10, which has been described as anti-inflammatory cytokine assigned to M2 polarization, was significant in the supernatant of LPS + IFNγ treated HBCs ($p = 0.0003$, 3-fold increase, Figure 8C). As mentioned above, a widely used marker for macrophage polarization is the ratio of IL-12 to IL-10, where macrophages polarized to M1 display IL-12^{high}/IL-10^{low} phenotype, and regulatory M2-macrophages *vice versa*(79). Hence, the IL-12/IL-10 ratio was calculated (using the means of IL-10 and IL-12 for all experiments of THP-1 and HBCs). In HBCs, this ratio was found to always stays below 1 (Table 6 and Figure 8E). Conclusively, HBCs constantly secrete more IL-10 than IL-12 and this is considered as M2 characteristic. Untreated, LPS + IFNγ, IL-4 + IL-13 and IL-6 treated THP-1 cells produced more IL-12 than IL-10. Only for IL-10 + TGFβ treated THP-1 the ratio of IL-12 to IL-10 amounted to 0.33, which could be explained by the artificial addition of IL-10 to the supernatant for treatment.

As also untreated THP-1 display a ratio of 3.46, we postulate an IL-12^{high}/IL-10^{low} baseline phenotype of THP-1 cells. They constantly produce more IL-12 and less IL-10 than HBCs (Figure 8C,D).

Lastly, no significant changes of the secretion of VEGF in neither HBCs nor THP-1 were detectable (Figure 8F).

Table 6| IL-12/IL-10 ratio of THP-1 and HBCs untreated (= control) and following treatments.

Target	*THP-1*	*HBC*
Control	3,46	0,37
LPS + IFNγ	7,68	0,78
IL-4 + IL-13	2,9	0,45
IL-10 + TGFβ	0,33	0,17
IL-6	4,57	0,49

Hofbauer cells constantly express more IL-10 than IL-12. With application of IL-10 to supernatant for treatment, the ratio drops to below 1 for both HBCs and THP-1. This value needs to be regarded as critical, as the effect size of external IL-10 addition for absolute values was not previously determined.

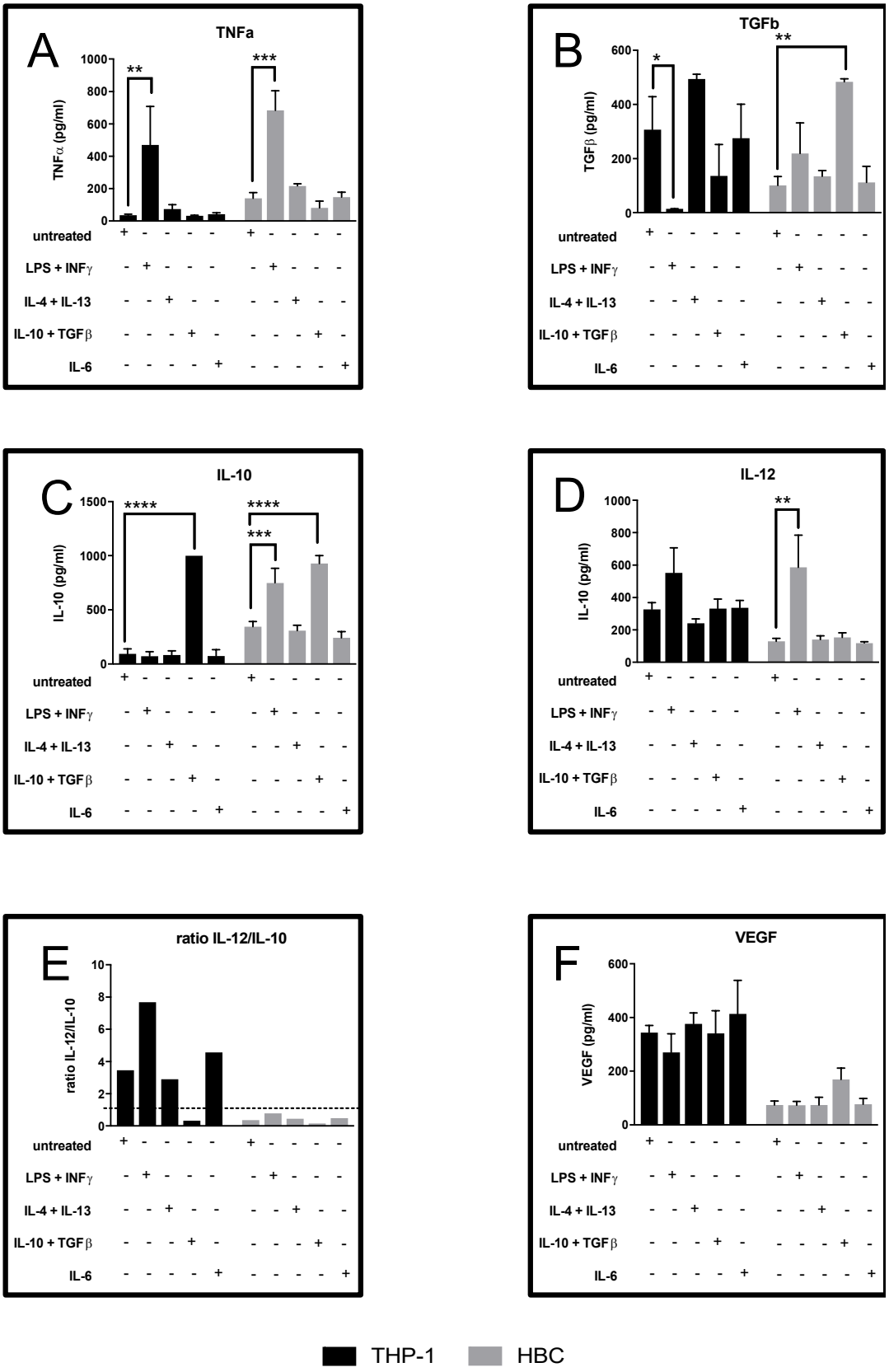

A
TNFa
TNFα (pg/ml)
1000
800
600
400
200
0
**
***
B
TGFb
TGFβ (pg/ml)
600
400
200
0
*
**
C
IL-10
IL-10 (pg/ml)
1500
1000
500
0
****
****
***
D
IL-12
IL-10 (pg/ml)
1000
800
600
400
200
0
**
E
ratio IL-12/IL-10
ratio IL-12/IL-10
10
8
6
4
2
0
F
VEGF
VEGF (pg/ml)
600
400
200
0
untreated + - - - - + - - - -
LPS + INFγ - + - - - - + - - -
IL-4 + IL-13 - - + - - - - + - -
IL-10 + TGFβ - - - + - - - - + -
IL-6 - - - - + - - - - +
THP-1
HBC

Figure 8| Secreted proteins of HBCs and THP-1 under different treatments.

Both THP-1 and HBCs increase their production of pro-inflammatory TNFα after treatment with LPS + INFγ (A). LPS + IFNγ treated THP-1 decreased their secretion of regulatory TGFβ (B). The supernatant of IL-10 + TGFβ treated cells showed a significantly higher amount of IL-10 (C) and TGFβ (B) in HBCs, as well as a significant increase of IL-10 in THP-1 (C). Intriguingly, LPS + IFNγ treated HBCs showed an increased production of IL-12 (D), which was determined as inflammatory cytokine. When below 1, the ratio of IL-12 to IL-10 is regarded as M2 characteristic (E). No significant changes in secretion of VEGF were detected(F).

Data are shown as mean ± standard deviation, compared using 2way ANOVA, calculated and graphed by GraphPad Prism.

Immunocytochemical assessment has been used to confirm the FACS data by a different approach.

Staining for macrophage marker CD163 and CD68 was used as positive control, fibroblast marker CD90 served as purity control and anti-mouse IgG was used as negative isotype control (Figure 9).

Anti-CD80 clone showed an inconsistent staining throughout all examinations and was therefore excluded from analysis.

Concerning HBCs, it could be shown that macrophage marker CD163 was constantly expressed through all treatments as well as untreated. Results of FACS analysis were supported with regard to CD206 (Figure 10A,B) and CD209 (10C,D,E). In contrast to FACS results, no differences of VEGF expression among the treatments were observed (10F,G).

Immunocytochemical assessment of THP-1 cells showed no difference in CD209 expression when comparing untreated cells (Figure 11A) to IL-4 + IL-13 treated cells (11B) and IL-10 + TGFβ (11C) treated cells. Also, no difference was detected in neither CD86 expression in untreated (11D) versus LPS + IFNγ (11E) treated cells nor TLR1 expression, respectively (11F,G). HLA-DR expression pattern resembled our FACS results. Compared to untreated (11H) the expression was upregulated in LPS + IFNγ treated cells (11I), downregulated in IL-4 + IL-13 treated cells (11J) and again upregulated in IL-6 treated cells (11K).

It has to be said that the evaluation of ICC results was conducted qualitatively and no quantitative cell counting was performed.

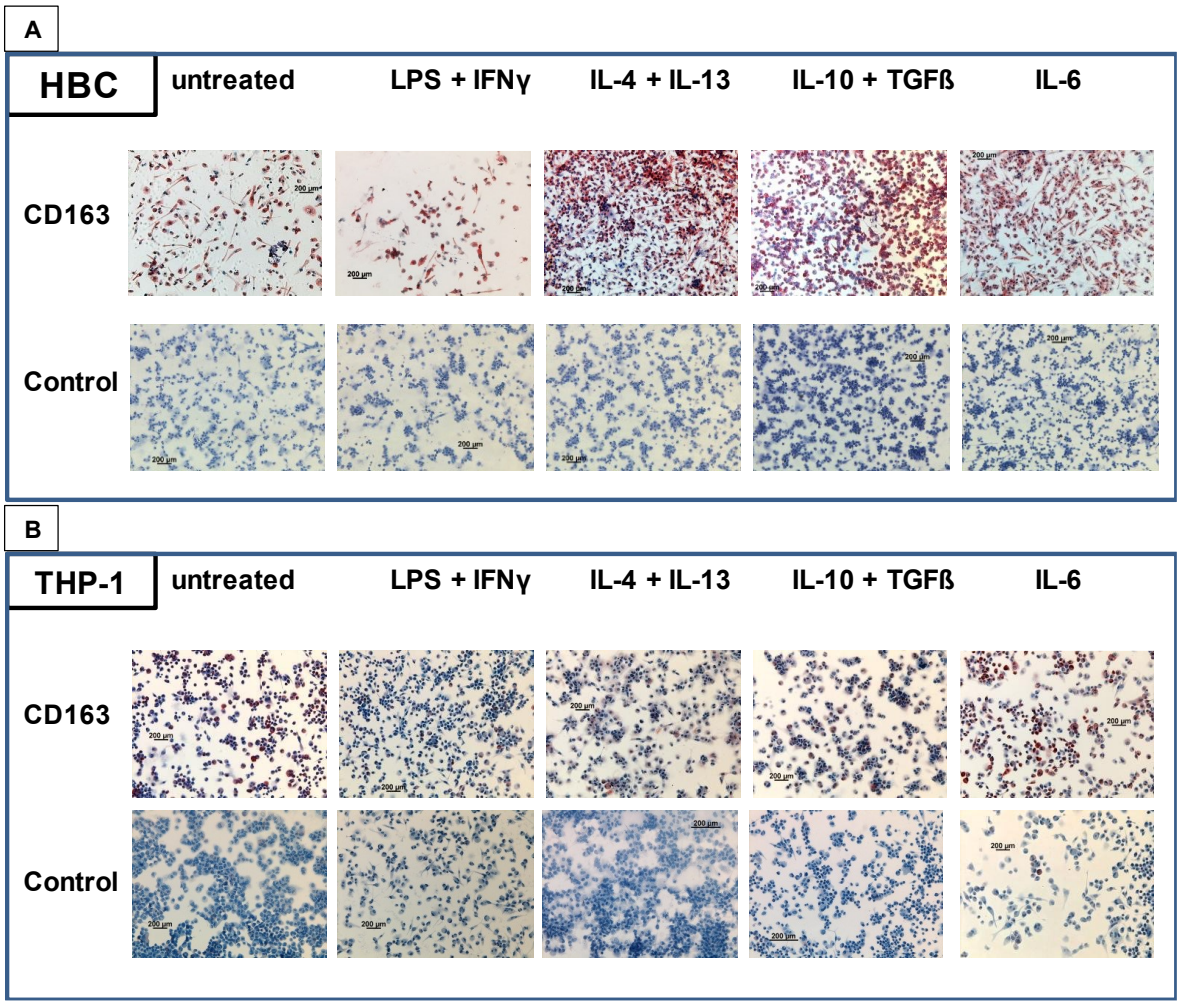


Figure 9| CD163 expression and negative isotype control.

For Hofbauer cells (A) as well as THP-1 cell (B) the evenly distributed expression of macrophage marker CD163 among all treatments (upper series of pictures) as well as the consistently negative isotype control (lower series of pictures) is depicted.

Supporting the quantitative data of cell sorting analysis, an even expression of macrophage marker CD163 was observed among all treatments as well as untreated control, in THP-1 cells constantly lower than in HBCs.

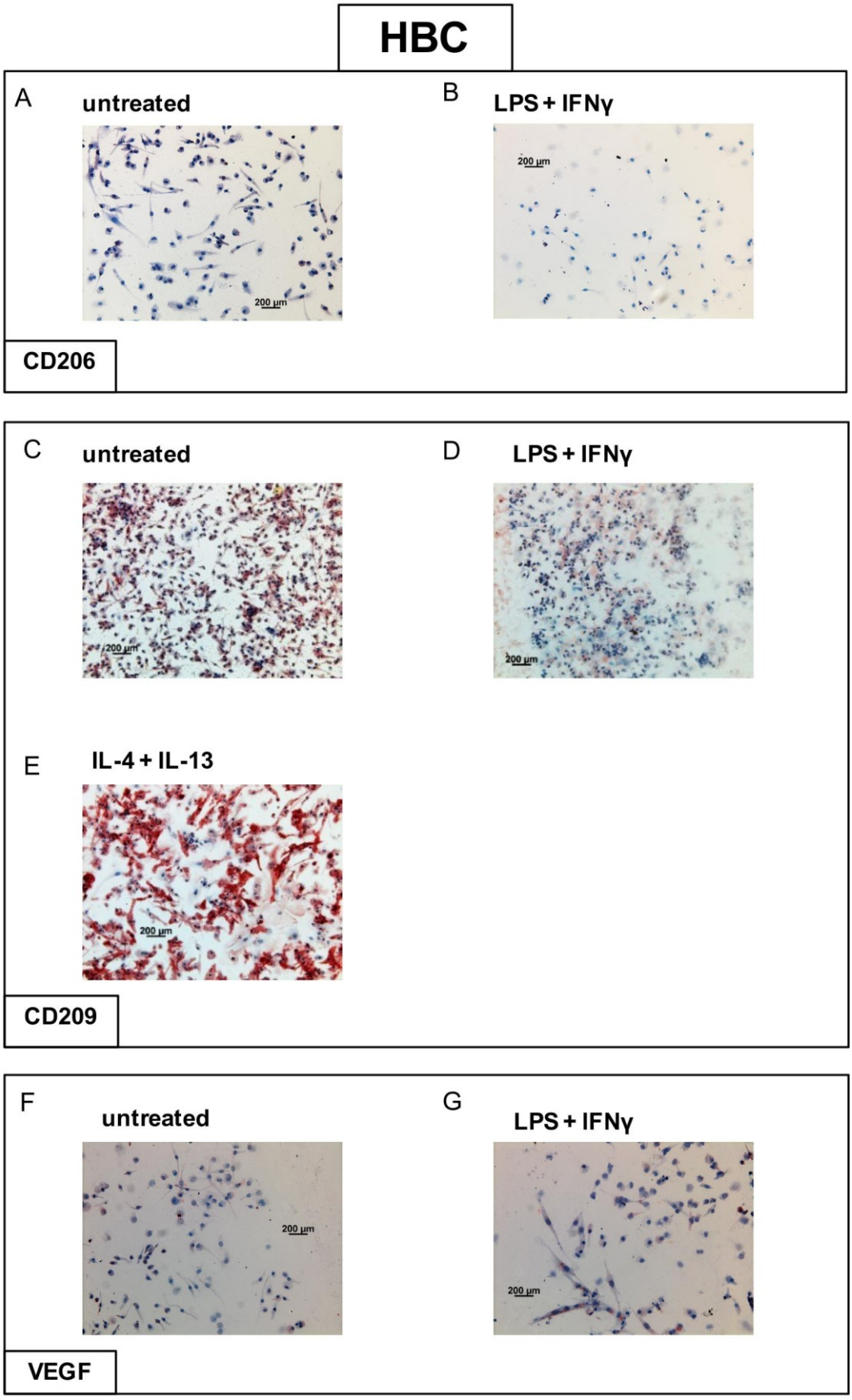

HBC
A
untreated
B
LPS + IFNγ
200 µm
CD206
C
untreated
D
LPS + IFNγ
200 µm
E
IL-4 + IL-13
200 µm
CD209
F
untreated
200 µm
G
LPS + IFNγ
200 µm
VEGF

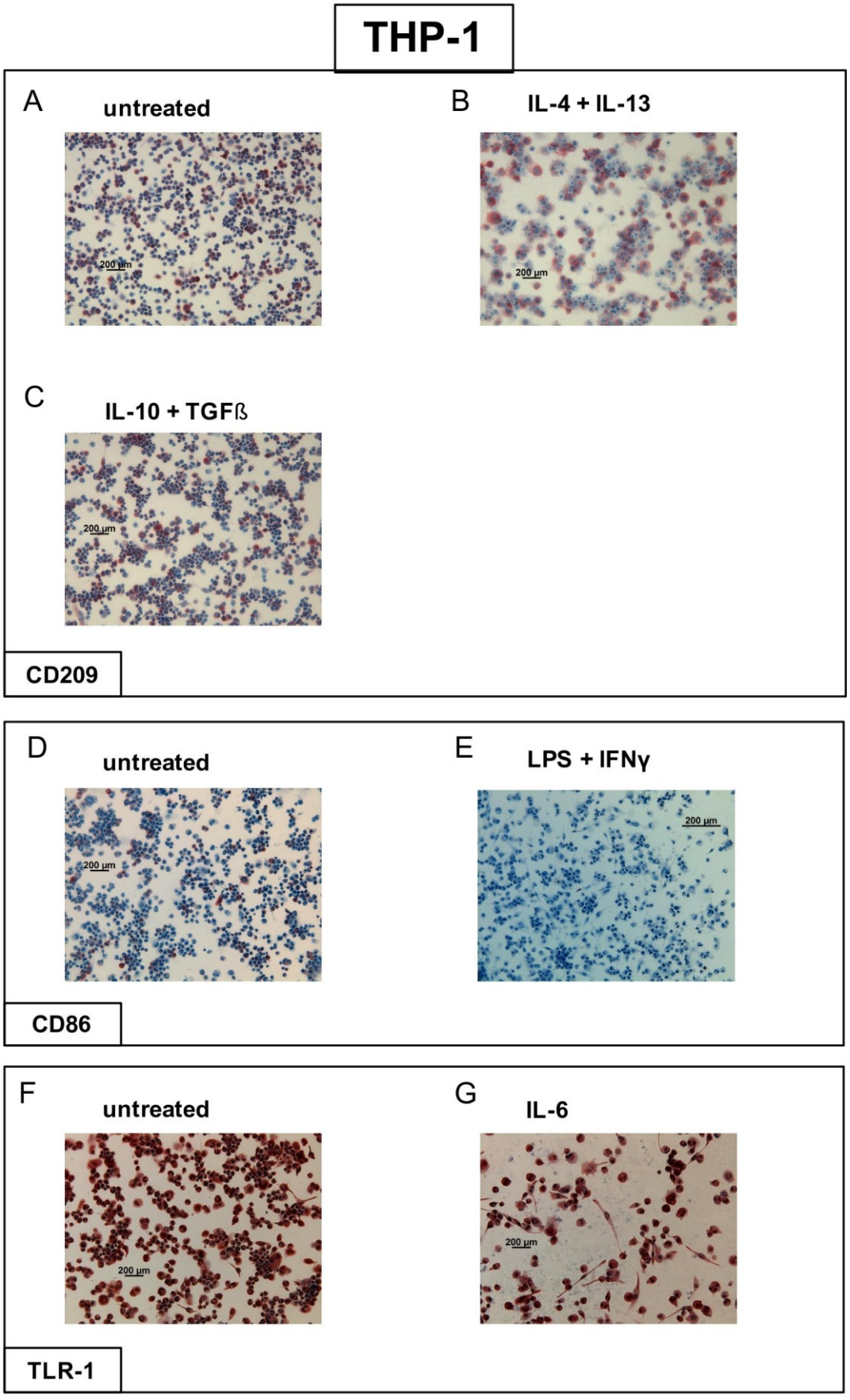
Figure 10| Immunocytochemistry of HBCs.

Images are representative for the cohort of n=3 individually performed experiments.

Compared to untreated (A) CD206 expression impresses decreased in LPS + IFNγ treated cells (B).

Compared to untreated (C) CD209 expression was decreased in LPS + IFNγ treated cells (D) and increased in IL-4 + IL-13 treated HBC (E). In contrast to FACS results, no difference in VEGF expression was observed in untreated (F) versus LPS + IFNγ treated cells (G).

Black scale bars equate 200μm.


THP-1
A
untreated
200 µm
B
IL-4 + IL-13
200 µm
C
IL-10 + TGFß
200 µm
CD209
D
untreated
200 µm
E
LPS + IFNγ
200 µm
CD86
F
untreated
200 µm
G
IL-6
200 µm
TLR-1

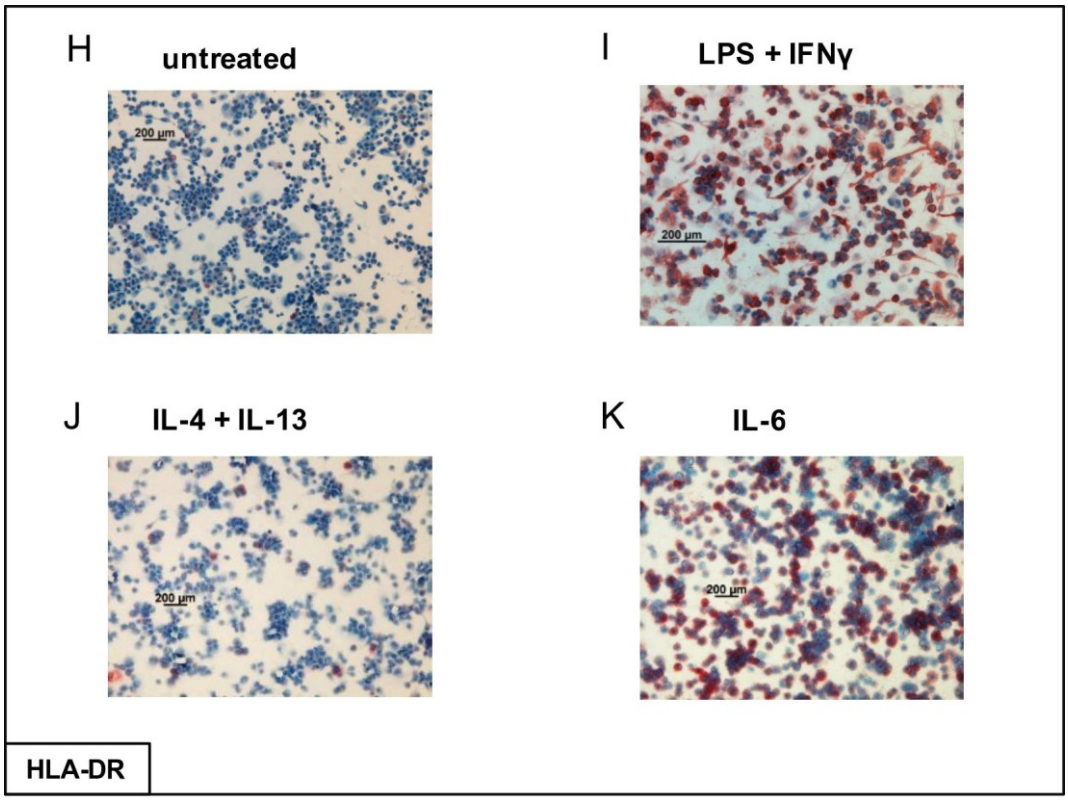


Figure 11| Immunocytochemistry of THP-1 cells.

Images are representative for the cohort of n=3 individually performed experiments.

CD209 (M2) expression was equally distributed among untreated cells (A) IL-4 + IL-13 treated cells (B) and IL-10 + TGFβ treated cells (C). The same applied for CD86 (D,E) and TLR1 expression (F,G). HLA-DR expression of untreated cells (H) increased following LPS + IFNγ (I) and IL-6 treatment (K) and slightly decreased following IL-4 + IL-13 (J) treatment.

Black scale bars equate 200µm.

4.5 HBCs alter cell morphology dependent on stimulus

Throughout the course of our experiments cells were constantly observed through light microscope to check if pro-inflammatory treatments may mediate morphologically changes of the cells. Directly after isolation, HBCs settled on the plastic surface of cell culture flasks. At that time, they impressed as roundish cells, dense in vacuoles, which were consistent in their size. One day later, when first stimulus was given into the supernatant media, the phenotype was not changed. On day 4 after isolation, when cells were boosted again and cells had already been exposed to respective stimuli for three days (Figure 2), drastic differences in their appearance were visible even under the microscope. Untreated cells had stretched and formed some protrusions (Figure 12A). HBCs treated with LPS + IFNγ remained their round shape. They even seemed to condense and did not form any protrusions (12B). The presence of IL-4 + IL-13 in the media forced HBCs to stretch much more and form longer processes (12C). Cells treated with IL-10 + TGF-β and IL-6 predominantly resembled the phenotype of untreated cells (12D, E).

Comparatively, THP-1 did not display such obvious differences in morphology (Figure 12F-J).

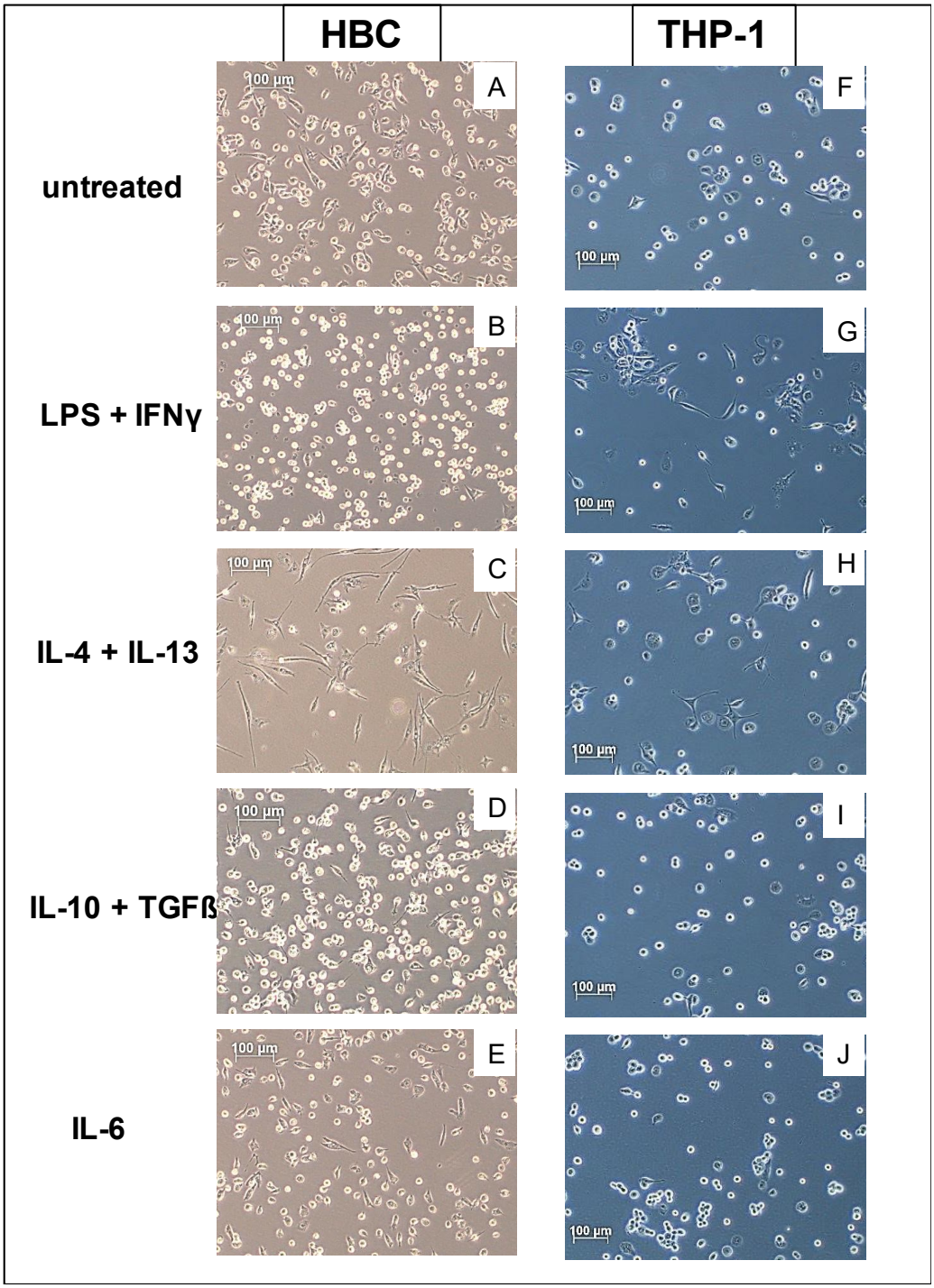


Figure 12| Morphology of HBC and THP-1 on day 6 after isolation.

Untreated HBCs lengthen and form some protrusions (A), LPS + IFNγ treated HBC remain round (B), IL-4 + IL-13 treated HBCs stretch widely (C), IL-10 +TGFβ and IL-6 treatment did not induce obvious changes of phenotype, compared to untreated (D,E). THP-1 cells remained stable in their appearance, independent from treatment (F-J). Images are representative for the cohort of N=5 individually performed experiments. White scale bars equate 100µm.

5 Discussion

Hofbauer cells are considered to maintain a regulatory M2 phenotype during pregnancy for a favorable course of embryonal implantation, pregnancy progress and labor, as well as mediation of maternal tolerance against the fetus(5,6).

This study showed for the first time that isolated HBCs from term placentae express significantly more CD206 than PMA differentiated THP-1 cells at baseline, which points towards a pre-determined M2 polarization of these primary cells.

Even upon LPS + IFNγ treatment of HBCs, solely a significant decrease of the expression of M2 markers CD206 and CD209 was observed. An induction of classical M1 markers like CD80, CD86 or TLR4 could not be detected. These results suggest maintenance of the HBC phenotype, rather than mediating M1 polarization upon LPS + IFNγ induction. These pro-inflammatory cytokines are just capable of depressing the production of M2 markers rather than shifting the macrophage phenotype towards M1. In contrast, following treatment with M2a-inducing agents as the combination of IL-4 and IL-13, HBCs increased their expression of CD209 by 35.6% over baseline. In total, HBCs seem to retain their functional capacity to adapt to the prevailing stimulus and to shift their polarization only modestly to a stimulus-directed phenotype.

In addition, LPS + IFNγ did not significantly change the secretion of TGFβ and revealed a constant IL-12^{low}/IL-10^{high} phenotype throughout all treatments, determined by ELISA. These are both indications again for a maintained M2-phenotype of HBCs. Moreover, a significant 5-fold increase of TGFβ secretion upon IL-10 + TGFβ treatment was observed. However, this effect needs to be viewed with suspicion. The effect size of external TGFβ addition for absolute values was not measured. Therefore, it remains masked whether this increase follows a drawing of HBCs towards M2 or is a mere effect of the external addition of the cytokine to supernatant for treatment.

Contrastingly, the secretion of pro-inflammatory TNFα was increased by LPS + IFNγ-treated HBCs. But, as this indirect finding is solely pointing towards a M1 polarization of HBCs, it is postulated that HBCs maintain their M2 phenotype and are more adaptable upon exogenous stimuli. Of note, induction of M1 polarization

in HBCs was not verifiable, but indeed an enhanced proportion of M2 polarized cells after respective treatment.

In THP-1 cells induction of polarization following proper stimulus was more effective, a higher number of cells were found to polarize upon treatment. Like HBCs, expression of CD209 increased after IL-4 + IL-13 treatment by 86.1%, an even more pronounced change than in HBCs. In contrast to HBCs, expression of M1 markers CD80 and CD86 was significantly increased upon LPS + IFNγ treatment, observing 15-fold and 8-fold increases, respectively. Also, antigen presenting HLA-DR expression was increased following LPS + IFNγ treatment.

Concerning protein marker secretion, LPS + IFNγ exposure increased pro-inflammatory TNFα and decreased anti-inflammatory TGFβ production significantly in THP-1 cells. Considering all these significant changes, it can be concluded that THP-1 cells are more flexible than and not as committed as HBCs in maintaining their phenotypical polarization.

For over 70 years now, cells are known to change morphology and form protrusions in cell culture. Already in 1951 *Lumsden at al.* have described elongation and process formation in cultured oligodendrocytes of rats(80).

When observing Hofbauer cells through light microscope drastic changes in cell morphology upon treatments were observed (Figure 12A-E). Whereas untreated HBCs in culture lengthened and formed some protrusions (12A), LPS + IFNγ treated cells remained spherical and condensed (12B) and IL-4 + IL-13 treatment caused the cells to stretch widely (12C). This phenomenon was observed throughout all experiments (N=5). It is already known that macrophages change their phenotype depending on their function and polarization. *McWhorter et al.* showed in bone-marrow derived macrophages that cell elongation is associated with M2 polarization (IL-4 and IL-13) and roundish, “pancake-like shape” is associated with M1 polarization (LPS and IFNγ)(81). This phenotypical categorization is confirmed by own similar results in primary placental Hofbauer cells (Figure 12A-E).

Comparatively, THP-1 did not display such obvious differences in morphology by treatment (Figure 12F-J).

The exposure to different stimuli alters phenotype of HBCs, but not of THP-1 cells. Conversely, quantitative assessment of surface markers showed that HBCs are more committed to their phenotypical polarization and variations in polarizations were observed in higher cell proportions of THP-1 cells. This effect lacks a suitable explanatory model and requires further research.

McWhorther et al. also proved that not only stimulation leads to adjustment of cell shape, but also *vice versa*. By forcing macrophages into elongated cell shape, they could induce M2 polarization. Hence, they postulated that cell morphology contributes to regulation of polarization. In cells forced to elongate they found a reduced secretion of pro-inflammatory cytokines and an upregulation of M2 markers. When combined with chemokine signals, elongation of cells enhanced effects of IL-4 + IL-13 stimulation and prevented them from M1 polarization following inflammatory stimuli. This indicates the importance of the environment for macrophage polarization, were extracellular matrix interacts *via* cell surface adhesion proteins with the cytoskeleton, mediating intracellular contractility(81). For this study, primary HBCs from healthy term placentae were isolated. Speculatively, this healthy environment leads to cell elongation and maintains M2 polarization of HBCs. For expressing and maintaining phenotypical polarization, both soluble factors, like cytokines, and cell shape are of crucial importance. Some pregnancy-associated diseases are associated not only with pro-inflammatory milieu but also with destruction or undesirable development of placental architecture, for instance early-onset PE(82). This may also affect HBCs potential of adapting regulatory polarization. Subsequently, HBCs are impaired in their complex functions, which could contribute to the course of such diseases.

In how far these morphological changes correlate with pregnancy associated diseases remains unclear and further research is indicated.

5.1 Limitations & Strengths

This work was carried out as pilot study and performed experiments were conducted *in vitro. As in vivo* research in pregnant women is merely impossible, *in vitro* use of human placental tissue and of isolated primary cells is considered a major strength of the study in order to tackle outlined objectives. Nonetheless, results acquired *in vitro* only provide first pioneering, which needs to be further considered with precise concern.

To compare results of placental HBCs with a human control monocyte/macrophage cell line of different cellular origin (lymphatic cancer tissue) represents a further strength of the study.

Experiments were performed using primary biological tissue, which always implements variability in source material per experiment. Concomitant, the cell yield from isolation of primary tissue differed per isolation. As the priority of this study was set upon quantitative analysis *via* FACS, an appropriate cell count for these experiments was primarily endeavored. Therefore, only three immuncytochemical stainings of HBCs, meaning three independent biological replicates, were conducted, whereas five individual FACS experiments were performed.

Finally, used ELISA approach failed to determine the absolute effect size of externally added stimuli TGFβ and IL-10. No plain control, containing the exogenous stimuli but no cell supernatant, was included to the ELISA. Therefore, it could not be calculated absolutely how much IL-10 and TGFβ the cells produce and thusly the results of TGFβ and IL-10 in respective supernatant media contain little informative value.

5.2 Conclusion and clinical implication

It has been made clear that HBCs fulfil many important functions for initiation, preservation and completion of normal pregnancy.

In the development of pregnancy associated diseases such as pre-eclampsia, a loss of M2 phenotypical features, corroborated by changes in the functionality of HBCs, has been observed(38). It has been shown that HBCs isolated from normal, healthy placentae are quite resistant to pro-inflammatory stimuli *in vitro*. However, the regulatory M2 phenotype of HBCs was mediated by stimulation with regulatory IL-4 + IL-13 (Figure 5, Figure 6 C,E, Figure 10, Table 6). It is conceivable that *in vivo* stimulation of M2 polarization of HBCs could be beneficial for several functions of these cells beyond immunity, including maintenance of physiologic homeostasis and tissue repair, thereby preventing development and/or regulation of such diseases. However, it has yet to be investigated i) in how far HBCs are involved in the precise pathophysiology of those diseases ii) whether *in vivo* stimulation of HBCs reveals the same effects as *in vitro,* and iii) how triggering stimuli could be applied in a targeted manner to their placental effector site.

6 Bibliography

1. Castellucci M, Zaccheo D, Pescetto G. A three-dimensional study of the normal human placental villous core. I. The Hofbauer cells. *Cell Tissue Res*. 1980;210(2):235-247.
2. Benirschke K, Burton GJ, Baergen RN. Basic Structure of the Villous Trees. In: *Pathology of the Human Placenta*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:55-100. doi:10.1007/978-3-642-23941-0_6.
3. Tang Z, Tadesse S, Norwitz E, Mor G, Abrahams VM, Guller S. Isolation of Hofbauer Cells from Human Term Placentas with High Yield and Purity. *Am J Reprod Immunol*. 2011;66(4):336-348. doi:10.1111/j.1600-0897.2011.01006.x.
4. Young OM, Tang Z, Niven-Fairchild T, et al. Toll-like Receptor-Mediated Responses by Placental Hofbauer Cells (HBCs): A Potential Pro-Inflammatory Role for Fetal M2 Macrophages. *Am J Reprod Immunol*. 2015;73(1). doi:10.1111/aji.12336.
5. Johnson EL, Chakraborty R. Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by induction of immunoregulatory cytokines. *Retrovirology*. 2012;9(1):101. doi:10.1186/1742-4690-9-101.
6. Brown MB, von Chamier M, Allam AB, Reyes L. M1/M2 macrophage polarity in normal and complicated pregnancy. *Front Immunol*. 2014;5(NOV):1-10. doi:10.3389/fimmu.2014.00606.
7. Reyes L, Wolfe B, Golos T. Hofbauer Cells: Placental Macrophages of Fetal Origin. *Results Probl Cell.* 2017:45-60. doi:10.1007/978-3-319-54090-0_3.
8. Roszer T. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm*. 2015;2015:16-18. doi:10.1155/2015/816460.
9. Sisino G, Bouckenooghe T, Aurientis S, Fontaine P, Storme L, Vambergue A. Diabetes during pregnancy influences Hofbauer cells, a subtype of placental macrophages, to acquire a pro-inflammatory phenotype. *BBA - Mol Basis Dis*. 2013;1832:1959-1968. doi:10.1016/j.bbadis.2013.07.009.
10. Schliefsteiner C, Peinhaupt M, Kopp S, et al. Human Placental Hofbauer Cells Maintain an Anti-inflammatory M2 Phenotype despite the Presence of Gestational Diabetes Mellitus. *Front Immunol*. 2017;8:888.

doi:10.3389/fimmu.2017.00888.

11. Joerink M, Rindsjö E, van Riel B, Alm J, Papadogiannakis N. Placental macrophage (Hofbauer cell) polarization is independent of maternal allergen-sensitization and presence of chorioamnionitis. *Placenta*. 2011;32(5):380-385. doi:10.1016/j.placenta.2011.02.003.
12. Tang Z, Abrahams VM, Mor G, Guller S. Placental Hofbauer cells and complications of pregnancy. *Ann N Y Acad Sci*. 2011;1221(1):103-108. doi:10.1111/j.1749-6632.2010.05932.x.
13. Takahashi K, Naito M, Katabuchi H, Higashi K. Development, differentiation, and maturation of macrophages in the chorionic villi of mouse placenta with special reference to the origin of Hofbauer cells. *J Leukoc Biol*. 1991;50(1):57-68. doi: 10.1002/jlb.50.1.57
14. Goldstein J, Braverman M, Salafia C, Buckley P. The Phenotype of Human Placental Macrophages and Its Variation with Gestational Age. *Am J Pathol*. 1988;133(3).
15. Kim J-S, Romero R, Kim MR, et al. Involvement of Hofbauer cells and maternal T cells in villitis of unknown etiology. *Histopathology*. 2008. doi:10.1111/j.1365-2559.2008.02964.x.
16. Demir R, Erbengi T. Some new findings about Hofbauer cells in the chorionic villi of the human placenta. *Acta Anat (Basel)*. 1984;119(1):18-26.
17. Simister NE, Story CM. Human placental Fc receptors and the transmission of antibodies from mother to fetus. *J Reprod Immunol*. 1997;37(1):1-23.
18. Benirschke K, Burton GJ, Baergen RN. Microscopic Survey. In: *Pathology of the Human Placenta*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:17-25. doi:10.1007/978-3-642-23941-0_3.
19. Vogeli KM, Jin S-W, Martin GR, Stainier DYR. A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature*. 2006;443(7109):337-339. doi:10.1038/nature05045.
20. Kim MJ, Romero R, Kim CJ, et al. Villitis of Unknown Etiology is Associated with a Distinct Pattern of Chemokine Up-regulation in the Feto-maternal and Placental Compartments: Implications for Conjoint Maternal Allograft Rejection and Maternal Anti-fetal Graft-versus-Host Disease 1. *J Immunol J Immunol March*. 2009;15(1826). doi:10.4049/jimmunol.0803834.
21. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage

plasticity and polarization in tissue repair and remodelling. *J Pathol*. 2013;229(2):176-185. doi:10.1002/path.4133.

22. Bulmer JN, Johnson PM. Macrophage populations in the human placenta and amniochorion. *Clin exp Immunol*. 1984;57:393-403.
23. Kumazaki K, Nakayama M, Yanagihara I, Suehara N, Wada Y. Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis. *Hum Pathol*. 2004;35(1):47-54.
24. TLR4. http://www.ncbi.nlm.nih.gov/pubmed/7099. Accessed December 29, 2017.
25. Kornat, GW, (2008). Signaling by Toll-Like Receptors. Boca Raton, FL 33487-2742: Taylor & Francis Group, pp.1-15.
26. Tang Z, Buhimschi IA, Buhimschi CS, et al. Decreased levels of folate receptor-β and reduced numbers of fetal macrophages (Hofbauer cells) in placentas from pregnancies with severe pre-eclampsia. *Am J Reprod Immunol*. 2013;70(2):104-115. doi:10.1111/aji.12112.
27. Szépfalusi Z, Loibichler C, Pichler J, Reisenberger K, Ebner C, Urbanek R. Direct evidence for transplacental allergen transfer. *Pediatr Res*. 2000;48(3):404-407. doi:10.1203/00006450-200009000-00024.
28. Rindsjö E, Joerink M, Papadogiannakis N, Scheynius A. IgE in the human placenta: why there? *Allergy*. 2010;65(5):554-560. doi:10.1111/j.1398-9995.2010.02345.x.
29. Anteby EY, Natanson-Yaron S, Greenfield C, et al. Human placental Hofbauer cells express sprouty proteins: a possible modulating mechanism of villous branching. *Placenta*. 2005;26(6):476-483. doi:10.1016/j.placenta.2004.08.008.
30. Ahmed A, Li XF, Dunk C, Whittle MJ, Rushton DI, Rollason T. Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta. *Growth Factors*. 1995;12(3):235-243.
31. Wetzka B, Clark DE, Charnock-Jones DS, Zahradnik HP, Smith SK. Isolation of macrophages (Hofbauer cells) from human term placenta and their prostaglandin E2 and thromboxane production. *Hum Reprod*. 1997;12(4):847-852.
32. Ingman K, Cookson VJKW, Jones CJP, Aplin JD. Characterisation of

Hofbauer cells in first and second trimester placenta: incidence, phenotype, survival in vitro and motility. *Placenta*. 2010;31(6):535-544. doi:10.1016/j.placenta.2010.03.003.

33. Toti P, Arcuri F, Tang Z, et al. Focal Increases of Fetal Macrophages in Placentas from Pregnancies with Histological Chorioamnionitis: Potential Role of Fibroblast Monocyte Chemotactic Protein-1. *Am J Reprod Immunol*. 2011 May;65(5):470-9. doi: 10.1111/j.1600-0897.2010.00927.x.
34. CD8. http://www.ncbi.nlm.nih.gov/pubmed/925. Accessed December 30, 2017.
35. CD14. https://www.ncbi.nlm.nih.gov/gene/929. Accessed December 29, 2017.
36. Vinnars M-TN, Rindsjö E, Ghazi S, Sundberg A, Papadogiannakis N. The Number of CD68+ (Hofbauer) Cells is Decreased in Placentas with Chorioamnionitis and with Advancing Gestational Age. *Pediatr Dev Pathol*. 2010;13(4):300-304. doi:10.2350/09-03-0632-OA.1.
37. Medeiros LTL, Peraçoli JC, Bannwart-Castro CF, et al. Monocytes from Pregnant Women with Pre-Eclampsia are Polarized to a M1 Phenotype. *Am J Reprod Immunol*. 2014;72(1):5-13. doi:10.1111/aji.12222.
38. Yang SW, Cho EH, Choi SY, et al. DC-SIGN expression in Hofbauer cells may play an important role in immune tolerance in fetal chorionic villi during the development of preeclampsia. *J Reprod Immunol*. 2017;124:30-37. doi:10.1016/j.jri.2017.09.012.
39. Ginhoux F, Guilliams M, Naik SH. Editorial: Dendritic Cell and Macrophage Nomenclature and Classification. *Front Immunol*. 2016;7:168. doi:10.3389/fimmu.2016.00168.
40. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;6. doi:10.12703/P6-13.
41. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *J Immunol*. 2017;199(7):2194-2201. doi:10.4049/jimmunol.1701141.
42. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. *Blood*. 2008 Aug 15;112(4):935-45. doi: 10.1182/blood-2007-12-077917.
43. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The

chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004;25(12):677-686. doi:10.1016/j.it.2004.09.015.

44. Mills CD. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. *Crit Rev Immunol*. 2001;21(5):399-425.
45. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969. doi:10.1038/nri2448.
46. Verreck FAW, De Boer T, Langenberg DML, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A.* 2004 Mar 30;101(13):4560-5. doi: 10.1073/pnas.0400983101.
47. Benoit M, Desnues B, Mege J-L. Macrophage polarization in bacterial infections. *J Immunol*. 2008;181(6):3733-3739. doi:10.4049/JIMMUNOL.181.6.3733.
48. Pfeffer K, Matsuyama T, Kündig TM, et al. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell*. 1993;73(3):457-467. doi:10.1016/0092-8674(93)90134-C.
49. Li J, Yang F, Wei F, Ren X. The role of toll-like receptor 4 in tumor microenvironment. *Oncotarget*. 2017;8(39):66656-66667. doi:10.18632/oncotarget.19105.
50. Nau GJ, Richmond JFL, Schlesinger A, et al. Human macrophage activation programs induced by bacterial pathogens. *Proc Natl Acad Sci U S A*. 2002;99(3):1503-1508. doi:10.1073/pnas.022649799.
51. Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. 2014;41(1):14-20. doi:10.1016/j.immuni.2014.06.008.
52. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. 2012;122(3):787-795. doi:10.1172/JCI59643.
53. CD206. https://www.ncbi.nlm.nih.gov/gene/4360. Accessed December 29, 2017.
54. Porcheray F, Viaud S, Rimaniol A-C, et al. Macrophage activation switching: an asset for the resolution of inflammation. *Immunol Clin Exp Immunol*. 142:481-489. doi:10.1111/j.1365-2249.2005.02934.x.

55. Dupasquier M, Stoitzner P, Wan H, et al. The dermal microenvironment induces the expression of the alternative activation marker CD301/mMGL in mononuclear phagocytes, independent of IL-4/IL-13 signaling. *J Leukoc Biol*. 2006;80(4):838-849. doi:10.1189/jlb.1005564.
56. CD209. https://www.ncbi.nlm.nih.gov/gene/30835. Accessed December 29, 2017.
57. Relloso M, Puig-Kröger A, Pello OM, et al. DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. *J Immunol*. 2002;168(6):2634-2643. doi: 10.4049/jimmunol.168.6.2634
58. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the Fetal–Maternal Interface Express Markers of Alternative Activation and Are Induced by M-CSF and IL-10. *J Immunol*. 2011;187(7):3671-82. doi: 10.4049/jimmunol.1100130.
59. Tang MX, Hu XH, Liu ZZ, Kwak-Kim J, Liao AH. What are the roles of macrophages and monocytes in human pregnancy? *J Reprod Immunol*. 2015;112. doi:10.1016/j.jri.2015.08.001.
60. Nagamatsu T, Schust DJ. The Immunomodulatory Roles of Macrophages at the Maternal—Fetal Interface. *Reprod Sci*. 2010;17(3):209-218. doi:10.1177/1933719109349962.
61. Jetten N, Verbruggen S, Gijbels MJ, Post MJ, De Winther MPJ, Donners MMPC. Anti-inflammatory M2, but not pro-inflammatory M1 macrophages promote angiogenesis in vivo. *Angiogenesis*. 2014;17(1):109-118. doi:10.1007/s10456-013-9381-6.
62. IL-10. https://www.ncbi.nlm.nih.gov/gene/3586. Accessed December 29, 2017.
63. IL-4. https://www.ncbi.nlm.nih.gov/gene/3565. Accessed December 29, 2017.
64. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol Cell Endocrinol*. 2011;335(1):2-13. doi:10.1016/j.mce.2010.04.005.
65. TGFbeta1. https://www.ncbi.nlm.nih.gov/gene/7040. Accessed December 29, 2017.
66. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM.

Macrophages That Have Ingested Apoptotic Cells In Vitro Inhibit Proinflammatory Cytokine Production Through Autocrine/Paracrine Mechanisms Involving TGF-$_{NL}$, PGE2, and PAF. *J Clin Invest*. 1998;101(4):890-898. doi: 10.1172/JCI1112

67. Wang Q, Ni H, Lan L, Wei X, Xiang R, Wang Y. Fra-1 protooncogene regulates IL-6 expression in macrophages and promotes the generation of M2d macrophages. *Cell Res*. 2010;20(6):701-712. doi:10.1038/cr.2010.52.
68. Duluc D, Delneste Y, Tan F, et al. Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood*. 2007;110(13):4319-4330. doi:10.1182/blood-2007-02-072587.
69. Sudan B, Wacker MA, Wilson ME, Graff JW. A systematic approach to identify markers of distinctly activated human macrophages. *Front Immunol*. 2015;6(MAY):1-18. doi:10.3389/fimmu.2015.00253.
70. Kim SY, Romero R, Tarca AL, et al. Methylome of Fetal and Maternal Monocytes and Macrophages at the Feto-Maternal Interface. *Am J Reprod Immunol*. 2012;68(1):8-27. doi:10.1111/j.1600-0897.2012.01108.x.
71. Phosphate buffered saline, used in diagnostic assay manufacturing | Sigma-Aldrich. http://www.sigmaaldrich.com/catalog/product/sigma/p4417?lang=de&region=AT. Accessed September 6, 2017.
72. Mühlhauser J, Crescimanno C, Kaufmann P, Höfler H, Zaccheo D, Castellucci M. Differentiation and proliferation patterns in human trophoblast revealed by c-erbB-2 oncogene product and EGF-R. *J Histochem Cytochem*. 1993;41(2):165-173. doi:10.1177/41.2.8093455.
73. Mariotti E, Mirabelli P, Abate G, et al. Comparative Characteristics of Mesenchymal Stem Cells from Human Bone Marrow and Placenta: CD10, CD49d, and CD56 Make a Difference. *Stem Cells Dev*. 2008;17(6):1039-1042. doi:10.1089/scd.2008.0212.
74. Flow Cytometry Basics Guide. https://facs.stanford.edu/sites/default/files/documents/scheduler/flowcytometry-intro.pdf. Accessed September 13, 2017.
75. ICC. http://www.proteinatlas.org/learn/method/immunocytochemistry. Accessed

September 13, 2017.

76. Abumaree MH, Al Jumah MA, Kalionis B, et al. Human Placental Mesenchymal Stem Cells (pMSCs) Play a Role as Immune Suppressive Cells by Shifting Macrophage Differentiation from Inflammatory M1 to Anti-inflammatory M2 Macrophages. *Stem Cell Rev Reports*. 2013;9(5):620-641. doi:10.1007/s12015-013-9455-2.
77. Sandwich ELISA, Highly Sensitive. http://www.elisa-antibody.com/ELISA-Introduction/ELISA-types/sandwich-elisa. Accessed October 10, 2017.
78. Chanput W, Mes JJ, Savelkoul HFJ, Wichers HJ. Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds. *Food Funct*. 2013;4(2):266-276. doi:10.1039/c2fo30156c.
79. Gerber JS, Mosser DM. Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol*. 2001;166(11):6861-6868. doi: 10.4049/jimmunol.166.11.6861
80. Lumsden C, Pomerat C. Normal oligodendrocytes in tissue culture: A preliminary report on the pulsatile glial cells in tissue cultures from the corpus callosum of the normal adult rat brain. *Exp Cell Res*. 1951. doi:10.1016/0014-4827(51)90010-9.
81. McWhorter FY, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci*. 2013;110(43). doi:10.1073/pnas.1308887110.
82. Murphy DJ, Stirrat GM. Mortality and morbidity associated with early-onset preeclampsia. *Hypertens pregnancy*. 2000;19(2):221-231.