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CLEVER-1 AS AN IMMUNE SUPPRESSIVE MOLECULE

Senthil Palani

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ABSTRACT

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CLEVER-1 as an immune suppressive molecule

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The immune response and immune suppression are equally essential for the immune system to protect the host against an infection and to protect self-molecules in different pathophysiological conditions. Pregnancy is one of the conditions where the maternal immune system remains resistant against microbes and yet attains tolerance to protect the fetus, whose genetic material differs partially from the mother's. However, if the balance of immune suppression is not precise in the host it can favor conditions which lead to diseases, such as cancer and autoimmune disorders.

This study was initiated to investigate the expression and functions of CLEVER-1/Stabilin-1, a multifunctional protein expressed on subsets of endothelial cells and type II macrophages, as an immune suppressive molecule. Firstly, the expression of CLEVER-1/stabilin-1 and its function in human placental macrophages were examined. Secondly, the expression profile and functional significance of stabilin-1 on healthy human monocytes was investigated.

The results clarified the expression of CLEVER-1/stabilin-1 on placental macrophages, and verified that CLEVER-1/stabilin-1 functions as an adhesion and scavenging molecule on these cells. The data from normal monocytes revealed that the monocytes with low stabilin-1 expression carried a pro-inflammatory gene signature, and that stabilin-1 can directly or indirectly regulate pro-inflammatory genes in monocytes. Finally, it was shown that monocyte CLEVER-1/stabilin-1 dampens IFNγ production by T cells.

To conclude, CLEVER-1/stabilin-1 is defined as an immune suppressive molecule on monocytes and macrophages. Strikingly, anti-stabilin-1 antibodies may have the potential to promote the Th1 dependent inflammatory response and counteract the tumor induced immune suppression.

Keywords: Macrophages, Monocytes, T cells (Th1 cells), CLEVER-1, Immune suppression

TIIVISTELMÄ

Senthil Palani

CLEVER-1 tulehdusreaktiota vaimentavana molekyylinä

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Elimistön puolustusjärjestelmä torjuu mikrobi-infektioita ja muita vieraita molekyylejä immuunivasteeksi kutsutun mekanismin avulla. Samalla immuunivasteen pitää kuitenkin suojella elimistön normaalisoluja ja kyetä palaamaan taas normaalitilaan uhkan torjumisen jälkeen. Raskaus on yksi esimerkki tilanteesta, jossa äidin immuunisysteemi säilyttää reagointikykynsä mikrobeja kohtaan samalla kun se sikiön suojelemiseksi sietää sikiön sisältämää vierasta geneettistä materiaalia. Immuuunireaktion tasapainon säätelyhäiriöt ovat osallisina syövän ja autoimmuunitautien synnyssä.

CLEVER-1/stabilin-1 on tietyissä endoteelisoluissa ja makrofaageissa ilmentyvä molekyyli, joka osallistuu valkosolujen liikkumiseen ja vieraiden molekyylien tunnistamiseen. Tässä työssä selvitin CLEVER-1/stabilin-1:n ilmentymistä ja toimintaa ihmisen veren monosyyteissä ja istukan makrofageissa.

Väitöskirjatyön tulokset osoittivat, että CLEVER-1/stabilin-1 ilmenee istukan kaikissa makrofageissa ja että se toimii näissä soluissa sekä tarttumismolekyylinä että ns. scavenging-molekyylinä. Normaaleilla monosyyteillä tehdyt tutkimukset paljastivat, että niukasti CLEVER-1/stabilin-1 molekyyliä ilmentävät solut olivat tulehduksellisesti aktiivisempia kuin runsaasti CLEVER-1/stabilin-1:tä ilmentävät solut. Lisäksi kokeissa pystyttiin osoittamaan, että monosyyttien CLEVER-1/Stabilin-1 säätelee tulehdusta vahvistavien geenien ilmentymistä monosyyteissä ja tulehduksen välittäjäaineiden (gamma-interferoni) tuottoa T-soluissa.

Väitöskirjatyöni osoittaa, että CLEVER-1/stabilin-1 on immunosuppressiivinen molekyyli monosyyteissä ja makrofageissa. CLEVER-1/stabilin-1- vasta-aineilla voisikin olla mahdollista tehostaa interferoni-gamma riippuvaista tulehdusvastetta ja näin kumota syövän aiheuttamaa immunosuppressiota.

Avainsanat: makrofagit, monosyytit, T-solut (Th1-solut), CLEVER-1/Stabilin-1, immuunivaste

TABLE OF CONTENTS

ABSTRACT **3**

TIIVISTELMÄ **4**

ABBREVIATIONS **8**

LIST OF ORIGINAL PUBLICATIONS **10**

1 INTRODUCTION **11**

2 REVIEW OF THE LITERATURE **12**

2.1 Immune system 12
2.1.1 Innate immune system 12
2.1.2 Adaptive immune system 14
2.1.2.1 T Cells 16
2.2 Development and polarization of monocytes and macrophages 19
2.2.1 Polarization 20
2.2.1.1 M1 Polarization 21
2.2.1.2 M2 Polarization 23
2.2.2 Markers 24
2.2.3 Functions 27
2.3 CLEVER-1 28
2.3.1 Structure 29
2.3.2 Expression 30
2.3.3 Function 31
2.4 Monocytes and macrophages in health and disease 32
2.4.1 Pregnancy 33
2.4.2 Hypersensitivity and allergy 35

3 AIMS OF THE STUDY **38**

4 MATERIALS AND METHODS **39**

4.1 Antibodies 39
4.2 Cells, cell lines and transfectants 40
4.3 Methods 40
4.3.1 Immunofluorescence staining 41
4.3.2 In vitro polarizations 41
4.3.3 ELISPOT 41

5 RESULTS 43
5.1 Characterization of CLEVER-1^{+} placental macrophages (I) 43
5.2 Functional analysis of CLEVER-1 on placental macrophages (I) 44
5.3 Identification of CLEVER-1^{high} and CLEVER-1^{low} population on monocytes and their gene expression profiles (II) 45
5.4 Functions of CLEVER-1 on monocytes (II) 46

6 DISCUSSION 49
6.1 CLEVER-1 expression on monocytes/macrophages 49
6.2 New functions of CLEVER-1 51
6.3 Potential therapeutic manipulation of CLEVER-1 52

7 SUMMARY 54

ACKNOWLEDGEMENTS 56

REFERENCES 58

ORIGINAL PUBLICATIONS 71

List of figures

Figure 1 Simplified schematic overview of the immune system 14

Figure 2 Model of the structure and domain organization of CLEVER-1 29

List of tables

Table 1 Simplified version of immune cells and their key functions 16

Table 2 Markers and key functions of heterogeneous monocyte population 25

Table 3 Markers for macrophages of selected stimulus 26

Table 4 Information of the primary antibodies used in the studies 39

Table 5 Secondary antibodies used in the studies 40

Table 6 Primary cells and cell lines used in the studies 40

Table 7 Methods used in the studies 40

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ABBREVIATIONS

AAM	alternative activated macrophages
acLDL	acetylated low density lipoprotein
APC	antigen presenting cells
β-ME	β -mercaptoethanol
BSA	bovine serum albumin
CCR	chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
CLEVER-1	common lymphatic endothelial and vascular receptor-1
CLR	C-type lectin receptors
DC	dendritic cells
Dex	dexamethasone
DTH	delayed type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
F(ab)2	fragment antigen binding fragments
FCS	fetal calf serum
FEEL-1	fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor
FITC	fluorescein isothiocyanate
GATA3	GATA binding protein 3
GM-CSF	granulocyte macrophage-colony stimulating factor
HEK	human embryonic kidney cells
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cells
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
MRC-1	mannose receptor C type 1

PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	phycoerythrin
PRR	pattern recognition receptor
PV-1	plasmalemma vesicle-associated protein-1
RNA	ribonucleic acid
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
Th	T helper cells
TLR	toll like receptor
TCR	T cell receptor
TNF	tumor necrosis factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-II)

I. Palani S, Maksimow M, Miiluniemi M, Auvinen K, Jalkanen S, Salmi M. Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. European Journal of Immunology, 2011 Jun (41): 2052-2063

II. Palani S, Elima K, Ekholm E, Jalkanen S, Salmi M. Monocyte stabilin.1 suppress the activation of Th1 lymphocytes. Journal of Immunology, 2016 Jan 1;196(1): 115-23

1 INTRODUCTION

The immune system is a complex network composed of cells, tissues and molecules that defends the host against pathogens like bacteria, viruses or any other foreign substances. This process of defense by the immune system is called the immune response. After the removal of pathogens the immune system enters to resolve the inflammation and helps the host return to its normal condition by using a process called immune suppression to prevent the self-destruction of the host tissues. Immune suppression is regulated by the functional characteristic change of immune cells with differential protein expression patterns. Any imbalance in immune suppression can lead to pathophysiological conditions like cancer and autoimmune disorders.

Monocytes and macrophages are particularly important among the immune cells, since they play a role in both immune responsive (pro-inflammatory) and immunosuppressive (anti-inflammatory) conditions by changing their phenotype. Therefore, the identification of the particular monocyte/macrophage populations is a crucial factor in many studies in the field of immunology and cancer biology. Cell surface proteins expressed specifically on these cells are commonly used as markers to distinguish the different monocyte and macrophage subpopulations. The information regarding these cells and shifts in their characteristics with the differential expression of marker proteins is an important factor used in several studies to diagnose and target disease conditions caused by an imbalance in immune suppression.

However, the role of monocyte and macrophage subpopulations, and their marker protein functions in normal immune suppressive conditions, like pregnancy, still need to be studied further. Pregnancy is a complex biological process, where the maternal immune system remains resistant against pathogens and yet attains tolerance to protect the fetus, whose genetic material is partially different from that of mother's. Furthermore not all immune suppressive marker protein functions have been studied in normal healthy individuals. More knowledge is needed for investigating the functional characteristic of immune suppressive protein molecules, which may be useful for diagnoses and specific therapies.

2 REVIEW OF THE LITERATURE

2.1 Immune system

The immune system is composed of cells, tissues and molecules that mediate defense mechanisms in the host against pathogens like bacteria, viruses, fungi and parasites or against any abnormal activity like cancer growth or foreign substances. This process of defending the host against a pathogen is called the immune response and the resistance to disease is known as immunity. The function of immune system is based on two principles, 1. Immune response – recognition and elimination of pathogens, 2. Immune suppression – prevents the self-destruction of host tissues. To function efficiently the immune system first has to recognize and discriminate between self-molecules (self-antigens) and non-self (foreign) antigens. Generally, the immune system is tolerant or non-reactive against cells expressing self-antigens, but when it encounters a non-self (foreign) antigen, a pathogen or a cell expressing non-self-antigens it responds by the action of neutralization and elimination. After the clearance of pathogens by the inflammatory immune response, the immune system begins to resolve the inflammation by returning to its normal condition by the process called immune suppression.

The immune system consists of two subsystems; 1. The innate immune system refers to the native or naturally existing system, which combats pathogens in general. 2. The adaptive immune system refers to the acquired or specific response, which develops after encountering a pathogen.

2.1.1 Innate immune system

The innate immune system is a defense mechanism which is always present in a host to defend against an infection. It forms the first line of defense known as the native or natural immune system. The innate immune system consists of epithelial barriers, cells in tissues and circulation and proteins in the plasma. Epithelial barriers protect the host by providing both physical and chemical barriers to infection. Microbes usually enter through tissues that are exposed to the environment, such as the skin, respiratory and gastrointestinal tract, and these sites are protected by an epithelial cell lining. Physical barriers include the skin and the mucus lining the respiratory tract. Chemical barriers like the acidic environment in the stomach, and the secretion of alpha-defensins with antimicrobial properties in the intestine prevent microbial growth. Pathogens that invade the epithelium are phagocytosed or attacked in the circulation and in tissues by

specific cells, like macrophages, neutrophils, basophils, eosinophils, mast cells, natural killer (NK) cells and monocytes. These cells express proteins called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Mannose receptors (MR), Nod-like receptors (NLRs) and RIG-like helicase receptors (RLHs). PRRs recognize structures in pathogens called pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS) or endotoxin in bacteria, double-stranded RNA in viruses, and unmethylated CPG oligonucleotides. Host cells on the other hand do not express PAMPs. The innate immune system can also recognize the damage associated with molecular patterns expressed on necrotic cells or cells under stress in order to clear these cells from the host. The host system expresses regulatory molecules which prevent the innate immune reaction against self-molecules or antigens (molecules expressed by host cells).

Cells of the innate immune system not only attack pathogens but also recruit other leukocytes to the site of infection for the enhanced elimination of pathogens. For instance, macrophages involved in phagocytosis simultaneously release proteins, such as TNF, IL-1 and chemokines that alter vascular permeability to improve leukocyte trafficking (adhesion, rolling and transmigration) to the site of infection. The recruited leukocytes, including different granulocytes, release molecules, such as defensins, cathepsin, myeloperoxidase and histamine at the site of infection, which leads to inflammation and improves the clearance of pathogens. Another aspect of the innate immune system is the complement proteins in circulation. These proteins bind to pathogens to eliminate them directly by lysing them or by neutralizing them to prevent further activation in the host. Complement proteins can also enhance phagocytosis by opsonization of the pathogen.

For decades, the innate immunity was viewed as non-specific which is no longer considered correct. The specificity of the innate immunity differs from that of the adaptive immunity; the innate immune system recognizes proteins or other structures on pathogens, which are essential for the pathogen to live in the host. If pathogens try to evade the innate immune response, the antigen presenting cells (APCs), including dendritic cells (DCs) and macrophages of the innate immune system, present the antigen to the adaptive immune system to elicit the next level of immune protection. In this way, the components of the innate and adaptive immune system work together to achieve and efficient immune response.

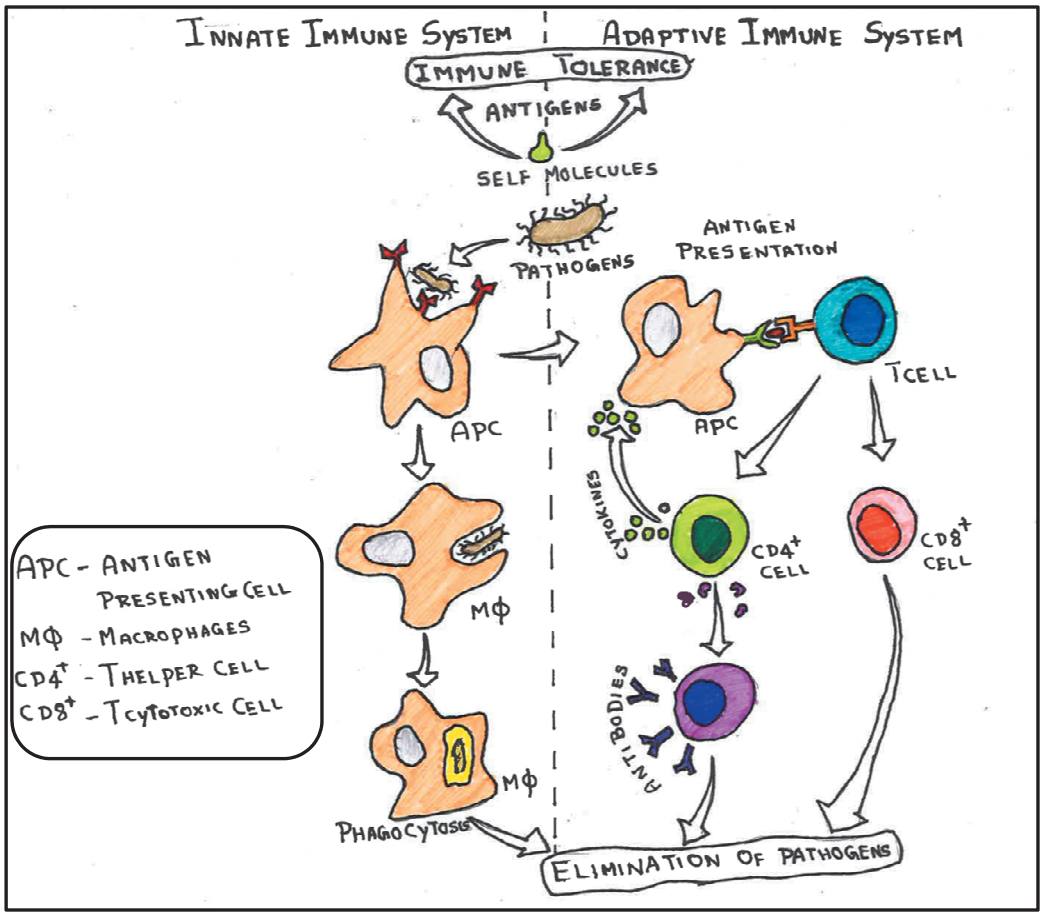

Figure 1 Simplified schematic overview of the immune system

2.1.2 Adaptive immune system

The adaptive immune system, also known as the acquired or specific immune system, as its name suggests recognizes pathogens specifically (based on their antigens). In contrast to the fast innate immune system, it takes several days to launch an adaptive immune response. The most important features of adaptive immune system are specificity and diversity, clonal expansion, memory, contraction and homeostasis and non-reactivity to self-antigens. The adaptive immune system ensures that it recognizes specific antigens to mount an immune response, and that it also covers a wide range of antigens. After recognizing the antigen, the adaptive immune system expands the specific cell population by clonal expansion, and saves some of these cells as memory cells for subsequent encounters with the same antigen in the near future. Further, it also clears the effector cells by homeostasis once the pathogens are eliminated from the host. Finally, the adaptive immune system has the ability to recognize the self/non-self-

antigens and launch an immune response specific for non self-antigens (molecules expressed by pathogens).

There are two types of adaptive immune systems, namely humoral immunity and cell-mediated immunity, which are mediated by B and T lymphocytes, respectively. These lymphocytes are activated upon an antigen exposure and undergo differentiation and clonal expansion to mediate the immune response. B cells do so by differentiating into antibody producing plasma cells and T cells by differentiating into T helper or T cytotoxic cells producing cytokines or by being cytotoxic. The antibody molecules (a class of proteins called immunoglobulins) are expressed on the surface of B cells as a B cell receptor, and are later secreted in soluble forms into the body fluids to mediate humoral immunity. B cells are produced and matured in the bone marrow. When these cells encounter a pathogen or a foreign antigen they undergo clonal expansion to become antigen specific effector B cells or plasma cells, which then secrete antibodies into the circulation to eliminate the pathogen. After the clearance of the pathogens, some of these cells live long in the host as memory cells, waiting for future encounters with the same pathogen. The important functions of the antibodies are to neutralize the pathogen by binding to them and preventing the adhesion of the pathogen to host tissues, initiating the phagocytosis by macrophages through opsonization of the pathogens, and finally by binding and activating the complement system and lysis of the pathogens. Antibodies can only act on pathogens in extracellular fluids in the circulation and in tissues, and cannot affect pathogens living inside cells. To defend against such intracellular pathogens the host requires cell mediated immunity.

The cell mediated immunity is mediated by T cells, which are produced as T precursor cells in the bone marrow and which migrate into the thymus in order to mature into T cells. T cells are activated in lymphoid organs by antigen presenting cells (APCs), such as dendritic cells, macrophages. APCs process the antigens of a pathogen and present them as a peptide to T cells via the major histocompatibility complex (MHC) I and II molecules, which are a set of cell surface proteins encoded by a large gene family. Stimulated T cells proliferate and differentiate into distinct subsets of effector T cells and memory T cells with different functional properties. CD4 (CD, cluster of differentiation) is a protein displayed on the surface of T cells which recognize MHC II and thereby these naïve $CD4^+$ T cells become helper T cells (Th cells). Naïve $CD8^+$ T cells recognize MHC I and becomes cytotoxic T cells (CTLs). These effector T cells migrate into the blood stream and to the site of infection and eliminate the pathogens. Helper T cells express the cell surface molecule CD4 and secrete various soluble signaling molecules known as cytokines. Cytokines are a group of proteins produced by a broad range of cells including immune cells, and they play a crucial role in the regulation of the immune system. Cytokines bind to receptors on B cells, neutrophils and macrophages, promoting antibody production or phagocytosis to destroy pathogens. Cytotoxic T cells recognize and directly kill the cells that carry pathogens,

which have escaped into their cytoplasm from phagocytosis or other mechanisms, to avoid any further infection (1–4).

Hence, the immune response to microbes invading the host is composed of complex interactions between different cell types in the immune system. This thesis is focused on monocytes, macrophages and T cells, which will be explained in more detail in the following chapters. However, to simplify the description of the overall immune system Table-1 highlights some of the immune cells and their key functional properties.

Table 1 Simplified version of immune cells and their key functions (Adopted - Basic immunology by Abdul K Abbas et al (1))

Immune cell type	*Basic functions*
T lymphocytes	Mediators of cell-mediated immunity CD4 Helper T lymphocytes: assist other white blood cells in immunological processes, activate maturation of B cells into plasma cells and cytotoxic T cells and macrophages CD8 Cytotoxic T cells: destroy the viral infected cells and tumor cells
B lymphocytes	Mediators of humoral immunity Plasma cells: produce antibodies which help phagocytosis also functions as antigen presenting cells
Natural killer cells	Act in innate immunity and do not need activation by major histocompatibility complex (MHC)
Dendritic cells	Antigen presenting cells: capture and display antigen and initiate T cell and B cell response
Macrophages	Play a role in both innate and adaptive immunity. Phagocytose cellular debris and pathogens, also stimulate lymphocytes as antigen presenting cells
Monocytes	Under inflammatory condition can quickly move into site of infection and differentiate into macrophages and dendritic cells
Neutrophils	Phagocytose or ingest pathogen and degrade them inside their vesicles
Eosinophils and Basophils	Involved in host defense against parasite attacks and allergic reactions
Mast cells	Release histamine upon encountering antigens

2.1.2.1 T Cells

T cells can differentiate into different effector subsets after being activated by antigen presenting cells (APCs). Naïve $CD4^+$ T cells differentiate into functionally distinct effector T helper cells (Th cells) and regulatory T cells (Tregs) (5–7). They are called helper and regulatory, because T helper cells help the phagocytes to destroy pathogens and help B lymphocytes to get activated which produce antibodies, whereas regulatory T cells regulate the immune system by preventing or limiting immune responses. Ex-

tensive studies on $CD4^+$ T cells have identified and characterized different subsets like Th1, Th2, Th9, Th17, Th22, Tregs and T follicular helper cells (Tfh), which have been shown to play various roles in defending against pathogens (6–13).These subsets are identified by the expression of their key cell surface chemokine receptors, lineage specific transcriptional regulators and the secretion of vital cytokines. Th1 cells express transcription factors such as STAT4 (Signal Transducer and Activator of Transcription 4) and TBX21, which promotes the secretion of key cytokine such as IFNγ and IL12 (14–18). Th2 cells express STAT6 and GATA3 as their transcription factors which stimulate the transcription of *Il4, Il5 and Il13* (19–22). Th17 cells have been reported to express interleukin receptors, including IL1R1, IL23R1, IL12RB2, and transcription factors such as STAT3, RORA and RORC (RAR-related orphan receptors). In addition, Th17 cells secrete IL9, IL17A, IL17F, IL21, and IL22 cytokines (23–25). Th9 and Th22 cells have not been characterized in detail yet, but it is known that Th9 cells express transcription factors PU.1 and STAT6, and secrete IL9 (26), whereas Th22 express aryl hydro carbon receptor (AhR) and secrete IL22 as a key cytokine (12). Tregs are identified mainly by the expression of transcription factor FOXP3 and cell surface proteins like CD25, CD39 and CD73, and they are known to secrete TGFβ (27) . Tfh cells express cell surface receptors like CD84, CXCR5, IL6R and IL21R, and transcription factors BCL6 and STAT3, and secrete IL6 (13). This thesis is focused on the activation of Th1 and Th2 cells by monocytes and macrophages. Hence, Th1 and Th2 are discussed in detail in the following paragraphs.

After Mosmann discovery of T cell polarization in mouse (16), the early 1990s studies in human patients with allergic or infectious diseases found that Th1 or Th2 profiles were from CD4 T cells (28). Around the same time this was confirmed by an another study, in which CD4 T cells of same individual were found to produce different cytokine patterns for different infectious agents. T cells produced Th1 cytokines including IFNγ for stimulation with purified protein derivative (PPD) of *Mycobacterium tuberculosis*, whereas during stimulation with excretory/secretory antigen of *Toxocara canis* T cells were found to produce Th2 cytokines IL4 and IL5 (29). Th1 cells are important for eradicating intracellular pathogens and for the pathogenesis of several autoimmune diseases. The differentiation of Th1 is stimulated by IL12, which signals through the receptor complex made up of IL12Rb1 and IL12Rb2. The expression of the complex is induced by the activation of the T cell receptor (TCR). Binding of IL12 to its receptor complex activates STAT4, which subsequently translocates to the nucleus and binds to the regulatory sequences of its target genes and promotes their transcription. For example, *Ifnγ,* a key Th1 cytokine gene is activated by this pathway (14, 17, 18). When secreted by T cells or other cells, IFNγ also promotes the further differentiation of Th1 cells, stimulates STAT1 phosphorylation and subsequently triggers the transcription of T-bet (T-box transcription factor, a Th1 specific gene). T-bet induces the expression of *Ifnγ* through a positive feedback mechanism and promotes Th1 differentiation (15, 30).Therefore, Th1 cells can be identified by the receptors they ex-

press, such as IL12R and IFNγR (Interferon gamma receptors), transcription factors, including T-bet, STAT1 and STAT4, secretion of key cytokine IFNγ. IFNγ is shown to enhance the microbicidal and phagocytic activity of mononuclear phagocytes, including monocytes and macrophages. It induces the NADPH dependent phagocyte oxidase (NADPH oxidases) system, the production of nitric oxide (NO) and the up-regulation of lysosomal enzymes that promote the destruction of microbes (31–33). Therefore, IFNγ shifts the immune response to the Th1 phenotype by stimulating the Th1 effector mechanisms and by activating monocytes/macrophages into classical macrophages (34).

Th2 cells promote humoral responses and are important for defense against extracellular parasites and critical for the stimulation and the progress of several allergic states. The differentiation of Th2 cells is initiated by IL4, which activates the phosphorylation of STAT6, whereby it translocates to the nucleus and stimulates the transcription of Th2 specific genes, such as Gata3, *Il4, Il5, Il13 and IL25*. Hence, Th2 cells are recognized by their receptors, such as IL4R, the transcription factors Gata3 and STAT6, and the secretion of the cytokines IL4, IL5, IL13 and IL25 (10, 19–22, 35–37). These cytokines play many roles in the immune response either individually or together. IL4 has pleiotropic effects on many cell types, and it alone promotes the class switch of immunoglobulins (Ig) in B cells to secrete IgG1 and IgE or along with IL13.IL4 is needed for protection against gastrointestinal parasites (38, 39). IL4 and IL13 share similar characteristic features, and these together coordinate the expression of IL4, IL13 and IL5 (40, 41). IL5, another key cytokine secreted by Th2 cells, binds to its receptor on basophils and eosinophils, and regulates their differentiation, growth and function. In addition, IL5 activates the production of inflammatory molecules such as histamine and leukotrienes in these cells and stimulates the proliferation and differentiation of B cells (19, 42–44). Studies using the *Litomosoides sigmodontis* (parasite) model system in IL4 or IL5 deficient mice demonstrated that the mutant mice were more susceptible to infection than wild-type controls, and needed IL5 or IL4 for protection (45, 46). The elimination of IL4 or IL5 also resulted in a poor response to the protective effects of vaccination against larval pathogen *Onchocerca Volvulus* (47). The Th2 cytokine response to an infection also activates macrophages into alternative activated macrophages (AAM) (48, 49). These AAMs are involved in wound healing and metabolic homeostasis (50, 51), but they also suppress T cell responses, which lead to several immunosuppressive conditions (52, 53). Hence, Th2 cell dominance and the activation of AAMs also has a negative impact on the immune system (immunosuppression) in certain disease conditions (53, 54). Moreover, products of Th1 and Th2 cells function as autocrine growth factors for their own expansion and serve as inhibitory mediators for the opposite cell type. For instance, IFNγ supports the clonal expansion of Th1 cells and decreases the proliferation of Th2 cells. Conversely, IL4 stimulates the growth of Th2 cells and reduces the growth of Th1 cells, which explains the need for a Th1 and Th2 balance in the immune system (6, 8, 15, 31, 55).

2.2 Development and polarization of monocytes and macrophages

Monocytes and macrophages are phagocytic mononuclear cells. These cells have a vital and distinct role in homeostasis and immunity (56). Monocytes are circulating blood leukocytes, which are heterogeneous in shape with sizes ranging from 10 to 30μm in diameter, and have a horseshoe or kidney shaped nucleus. Monocytes constitute from 2 to 10% (in humans) and 4% (in mice) of all blood leukocytes. They are produced in the bone marrow, circulate in the bloodstream for 1 to 3 days, and if they receive activation signal they migrate into tissues. If a monocyte is not activated it undergoes apoptosis and dies. Half of the monocytes generated are stored in the spleen, clustering in red pulp cords of billroth as a reserve (57, 58). In tissues monocytes are divided into dendritic cells and various types of macrophages. Macrophages are large phagocytosing cells, which reside in essentially all tissues of the body. They have different names in different tissues, such as Kupffer cells in the liver, microglia in the brain and osteoclasts in bone. Macrophages were discovered by Metchnikoff in the early 1880s as phagocytic cells, which ingest and degrade cell debris, apoptotic cells and pathogens.

The development of monocytes and macrophages from hematopoietic stem cells in the bone marrow takes many commitment steps and intermediate progenitor stages, such as common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), and macrophage/dendritic cell progenitor (MDP) (59, 60). PU.1, which is a transcription factor of the Ets family, plays a vital role in the differentiation of these cells. PU.1 can stimulate the commitment of myeloid cells into immature progenitor cells and is needed for the generation of CMP in the initial myelopoiesis (61–63). PU.1 is essential for stimulating monocytic differentiation, at the expense of granulocytic development, by antagonizing C/EBPα (a transcription factor needed for granulocytic development) (63, 64). PU.1 can activate Egr transcription factors and their cofactor Nab, which determines monocyte and macrophage differentiation. Egr1 can distinctly activate macrophage differentiation (65, 66). There are other transcription factors, which drive monocytic and macrophage differentiation instead of PU.1. For instance, ICSBP/IRF-8 drives monocytic differentiation (67), KLF4 induces macrophage fate and was also reported to rescue monocyte differentiation in $PU.1^{-/-}$ deficient mice (68), Maf-B and c-Maf can selectively drive monocytic fate in myeloid progenitors (69, 70). Both Maf-B and c-Maf inhibit the transactivation of Ets-1 and c-Myb, indicating that these factors can shift the homeostasis balance between progenitor cell proliferation and terminal differentiation (71–73). Further, mutations in the genes for PU.1 or CSF-1R resulted in perinatal mortality and growth retardation (74, 75). CSF-1R (Colony Stimulating Factor-1 Receptor) is a hematopoietic growth factor receptor expressed in monocytes, macrophages, dendritic cells and their precursor cells (76, 77), Some of its known ligands are CSF-1 (also known as Macrophage-colony stimulating factor (M-CSF)) and IL34 (78, 79). The number of blood monocytes is significantly reduced in

mice devoid of Csf-1R or its ligands (75, 80–82). Evidence for the need of CSF-1 for the development of a subset of tissue macrophages came from a natural mutation in the CSF-1 gene (83). Finally, these monocyte derived cells have numerous biological functions, which include activities related to tissue macrophages and dendritic cells, including migration to lymph nodes, antigen presentation and bactericidal activity (84, 85).

The development of tissue macrophages and their origin has been discussed extensively in the literature. Although the monocytic origin of tissue macrophages was the basis of the mononuclear phagocyte concept, it has now been established that most tissue macrophages are independent of the monocyte input and are of embryonic origin (56, 86). Tissue resident macrophage numbers were largely unaffected in studies with monocytopenic animals and human patients suffering from monocytopenia, revealing the non-monocytic origin of macrophages from a prenatal stage of the embryonic yolk sac (56, 80, 81). Primitive embryonic macrophages, which are generated without monocytic intermediates appear in the yolk sac blood island E8.5/9.0 (Embryonic days). These early fetal tissue macrophages retain their proliferative self-renewal capacity and are likely to be involved in the clearance of cell debris during tissue remodeling in development (87, 88). The macrophage precursors in fetal liver were found to considerably contribute to the macrophage population in adult tissues in the lungs, dermis and spleen (89). In addition, adult lung alveolar macrophages have been shown to develop from fetal liver monocytes in a GM-CSF directed pathway (90). Moreover, parabiosis studies in mice whose circulations are surgically joined also supported the notion that adult tissue macrophages are independent from the circulating monocyte input (91). Cytokines like Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Fms-like receptor tyrosine kinase 3 (Flt3) and lymphotoxin $\alpha 1\beta 2$, which control the development and homeostasis of macrophages and dendritic cells, were reported to be unimportant for monocyte development (92–94). Together, all these studies suggest the existence of non-monocytic origin of tissue macrophages and that tissue macrophages do not originate from monocytes. This finding is now widely accepted, but the function of these macrophages based on its origin needs to be clarified.

2.2.1 Polarization

Macrophages act in both the innate and adaptive immune systems, as modulators and effector cells of the immune response. Macrophages change their functional phenotype according to the cytokines and other signals in the tissue environment. Macrophages are classified into either classical or alternative activated macrophages, M1 or M2 macrophages, depending on the activation signals they receive. This classification, however, became complicated when differences in gene and protein expression in these cells were studied in more detail. In 1960 Mackaness introduced the term activation (classical activation) of macrophages in an infection setting, in order to explain the improved

activity of macrophages towards *Bacillus Calmette-Guerin (BCG)* and *Listeria* upon secondary exposure to the microbes (95). This was later linked with IFNγ production and a Th1 response (96), followed by cytotoxic and antitumoral effects (97, 98). The alternative activation phenotype of macrophages different from the IFNγ activation was proposed by the group of Stein and Doyle. They found that the Th2 cytokines, IL4, and IL13 selectively enhanced the expression of the macrophage mannose receptor (MRC-1) in murine macrophages, and prompted the high endocytic clearance of mannosylated ligands and the higher expression of the MHC class II antigen, and decreased the production of pro-inflammatory cytokines (99, 100). The term M1 and M2 macrophages was proposed by Mills and colleagues, when they found that the macrophages in mouse strains with a Th1 (C57BL/6, B10D2) and Th2 (BALB/c, DBA/2) background responded differently to the classical stimulation of IFNγ or lipopolysaccharide (LPS) or both. They defined the activated macrophages producing nitric oxide (NO) as M1 macrophages and macrophages producing tropic polyamines as M2 macrophages (101). There are also other cytokines and factors, which do not fall self-evidently into the class of Th1/Th2 responses shown to prompt similar macrophage phenotypes. For example, IL10 and glucocorticoids were reported to induce the expression of MRC-1 on the surface of macrophages and to reduce the production of pro-inflammatory cytokines and nitric oxide (102–104).In their review Mosser and Edwards proposed three major populations of macrophages based on homeostatic activities: 1) host defense/classical activation, 2) wound healing and 3) immune regulation/regulatory macrophages (105). Furthermore, this model suggests that different subsets can exist between these three main populations depending on various activation stimuli/states. To link the phenotypic similarities and differences created by various stimuli, Mantovani et al. grouped these stimuli in a continuum between two functionally polarized states of macrophages, based on their effect on selected macrophage markers (106). They designated macrophages as M1 (when the stimulus is IFNγ combined with LPS or tumor necrosis factor (TNF)) and M2 as a continuum (M2a when the stimulus is IL4, M2b when stimuli is Fc receptors and Immune complex and M2c when stimuli is IL10 and GCs).This classification created the distinctions between the M2 groups, such as Th2 products and immunoregulation (106). But this categorization did not include the finding of GM-CSF and M-CSF as stimuli for M1 and M2, respectively (107). Some of the major stimuli for M1 and M2 polarization and their effects on macrophages are discussed in the following section and reviewed in (34).

2.2.1.1 M1 Polarization

The M1 stimuli are grouped, according to their ability to stimulate a prototypic inflammatory response and the expression of markers on macrophages. However, M1 stimuli sources and signaling cascades differ strikingly. IFNγ is the key cytokine of Th1cells but is also produced by many other cells and is associated with M1 activation.

IFNγ binds to IFNγR-1 and IFNγR-2 and recruits the JAK1 and JAK2 adaptor proteins, which activates STAT1 and IRFs (Interferon regulatory factors) such as IRF1 and IRF8, subsequently controlling many cytokine receptors, adhesion molecules and others (108). INFγR or IFNγ deficient mice are viable and their macrophage numbers are normal under steady state (normal) conditions (109, 110). However, these mice are susceptible to *Mycobacterium bovis* and *Listeria monocytogenes*, and their macrophages show impaired production of antimicrobial agents (111). In humans, a mutation in the IFNγ gene leads to a severe immunodeficiency in patients with a familial disseminated atypical mycobacterial disease (112).

Lipopolysaccharide (LPS) is the other major stimulant for M1 cell differentiation. LPS is a component of the bacterial cell wall and it signals via Toll-like receptor 4 (TLR4). TLR4 is best known for its role in the M1 stimulation pathway, although recent studies have shown that LPS can also signal via a TLR-independent mechanism (113, 114). Conventionally, the binding of LPS to TLR4 stimulates MyD88 and Mal/Tirap (Toll-interleukin 1 receptor domain containing adaptor protein) dependent pathways, which induces strong pro-inflammatory cytokines (e.g, IFNβ, IL6, IL12 and TNF), and chemokine production (e.g CCL2, CXCL10 and CXCL11), and expression of antigen presenting molecules, including MHC members and co-stimulatory molecules. This profile is controlled by nuclear factor of kappa-light-enhancer of activated B cells or nuclear factor kappa B(NF-κB), activator protein 1 (AP-1), IRFs, STAT1 and many other transcription factors, which contribute to the IFN response (108). IFNγ is combined with LPS in the M1/M2 paradigm, but the gene expression profile of the combination is different from that induced by IFNγ or LPS alone (115, 116). Like IFNγ deficient mice TLR knockout (KO) mice also have a normal number of macrophages, but their activation status is defective and they are susceptible to infections (117–119). Also, a mutation in the human TLR4 gene causes susceptibility to infections (120).

Another major stimulant for M1 is TNFα. Interestingly, macrophages, which are the main producers for TNFα, are also highly reactive to TNFα in an autocrine or paracrine manner. TNFα binds to TNFR1 and TNFR2 transmembrane receptors, which activate adapter proteins, and which subsequently induce activation of NF-κB in the nucleus, resulting in an increase in TNFα production. TNFα stimulation has been shown to be effective when combined with primary M1 stimuli, such as IFNγ or LPS. In this combined stimulus, IFNγ or LPS activates STAT1 and other adapter proteins for enhanced TNFα induced stimulation of NF-κB (121). TNFα deficient mice have macrophages with normal phagocytic activity, but these mice were reported to be susceptible to infections, such as those caused by *Candida albicans* (122). TNFα and its receptors have been shown to play a major role in many inflammatory diseases, such as rheumatoid arthritis and Crohn's disease (121).

GM-CSF is the most recent addition to the M1 stimuli. It binds to the GM-CSF receptor and recruits JAK2, which prompts the activation of STAT5, and extracellular

signal regulated kinase (ERK), subsequently leading to the translocation of NF-κB and IRF5 into the nucleus (123, 124). GM-CSF enhances phagocytic activity, antigen presentation, and favors the production of pro-inflammatory cytokines, including TNFα, IL1β and IL8. However, the activation status of macrophages by GM-CSF is lower compared to the LPS stimuli. Cell surface molecules, such as CD14, CD163 were shown to be regulated by GM-CSF in a transcriptome analysis (125). GM-CSF KO mice have normal numbers of macrophages in some tissues, but have defects in the maturation of alveolar macrophages (126). Similarly, a mutation in the GM-CSF receptor beta chain in humans leads to defects in alveolar macrophages (127, 128). IL1β and IL6 are examples of other factors that share pro-inflammatory properties, which further increase the heterogeneity of the group.

2.2.1.2 M2 Polarization

M2 stimuli are grouped according to their ability to antagonize the pro-inflammatory responses and markers in macrophages. Nevertheless, like the M1 stimuli, their sources and signaling cascades differ prominently. IL4, a major cytokine of Th2 cells, drives M2 polarization. The binding of IL4 to its receptor activates JAK1 and JAK3, which in turn stimulates the activation of STAT6 and its translocation to the nucleus. A transcriptome analysis of the effect of IL4 in different species revealed transcriptomes like MRC-1 and transglutaminase 2 (TGM2), as well as transcription factors, such as IRF4 and KLF4, and signaling molecules, including CISH and SOCS1 (129–132). IL4 alone or combined with glucocorticoids has been shown to stimulate MS-1/stabilin-1 in human monocyte derived macrophages (133). IL13 and IL5 are other cytokines which drive M2 polarization. The transcriptional signature of IL13 resembles that of IL4, but it does not overlap with it (134). IL4 and IL13 upregulate the expression of MRC-1 and MHC class II molecules on the surface of macrophages, which induces endocytosis and antigen presentation (53). The effects of IL5 on macrophages are not as clear. IL5 combined with IL4 or IL13 (or both) contributes to M2 polarization. Interestingly, it has been shown that monocytes induced with IL5 reduce the production of TNFα (135). In IL4 KO animals, the number and maturation of macrophages are normal, but the cells shows defects in an immune challenge against nematodes and some viral infections (136, 137). A polymorphism in the human IL4R on other hand has been linked to the development of asthma and atopy (138, 139).

The macrophages stimulated by immunocomplexes are defined as type II activated macrophages by Mosser and Edwards (105) and classified as M2b by Mantovani (106). Immunoglobulins bind to the family of Fc gamma receptors (FcγR), and the binding of FcγRIIB (CD32) appears to be critical for type II activation of human monocytes and macrophages (140). The binding of IgG to FcγR activates spleen tyrosine kinase (Syk) and phosphoinositide 3-kinase (PI3K) (141). FcγR KO animals have normal macro-

phage numbers, but have defects in opsonic phagocytic capabilities. Genetic alterations in human FcγRs result in autoimmune diseases (142).

The binding of IL10 to the IL10 receptor (a dimer of IL10R1 and IL10R2) leads to the autophosphorylation of the receptor molecules, which leads to the activation of JAK1 and STAT3, and subsequently the inhibition of a pro-inflammatory cytokine profile (143, 144). IL10 deficient mice have normal macrophage numbers, but colonization of the gut with resident bacteria made them develop inflammatory bowel disease (145), and an elevated inflammatory response to parasite infections (144). Defects in the human IL10 receptors contribute to colitis and elevated inflammation (146).

Glucocorticoid hormones are secreted by the adrenal glands and metabolized by cellular enzymes in macrophages. Glucocorticoids, together with Th2 cytokines, drive M2 polarization. Synthetic glucocorticoid compounds, including dexamethasone, have been used in many studies to suppress M1 and support M2 polarization (133, 147, 148). Glucocorticoids and IL10 represent different stimuli in the activation of macrophages, but they have been classified as M2 stimuli. Glucocorticoids diffuse through the plasma membrane and bind to the glucocorticoid receptor (GCR) alpha, which leads to the translocation of this complex to the nucleus, subsequently binding to DNA in order to regulate gene transcription directly or indirectly by interacting with transcription factors, including NF-kB. Glucocorticoids induce the expression of CD163, IL10, thrombospondin 1(THBS1), TSC22 domain family, member 3 (DSIPI), complement component 1 subunit A (C1QA), MRC1 and stabilin-1 (133, 149). GCR deficient mice do not survive long after birth (150), and GCR polymorphisms in the human gene have been reported to be involved in various malignancies, as well as in inflammatory and autoimmune disorders (151).

M-CSF is a recent addition to the M2 group of stimuli. The binding of M-CSF to the M-CSF receptor leads to the dimerization, and autophosphorylation of the receptor, activation of ERK, phosphatidylinositol 3-kinase, and phospholipase C, and ultimately the translocation of the Sp1 transcription factor to the nucleus. Cell cycle genes (like cyclins A2, B1, D1 and E1) are over-represented by the M-CSF stimulus, it also downregulates human leukocyte antigens (HLA) molecules (115). Gene defects in M-CSF in mice resulted in decreased levels of monocytes and selected macrophages, as well as osteopetrosis (152). In humans, a mutation in M-CSF receptor is associated with hereditary diffuse leukoencephalopathy (153).

2.2.2 Markers

Monocytes are heterogeneous in size and shape. They were identified initially based on their morphology and with cytochemistry, and flow cytometry using cell surface markers. Ziegler-Heitbrock's group discovered the expression of CD16 (FcγR-III) on a

minor monocyte population, and the expression of CD14 on a major subset of monocytes, which lacks CD16 and has higher phagocytic activity and lower cytokine production levels than the minor subset with high CD16 expression (154, 155). Later work by Grage-Gribenow and his group has shown that CD16+ monocytes contain two populations with different functions (156). Monocytes that express CD16 and CD14 ($CD14^{+}CD16^{+}$) have phagocytic activity and are responsible for the production of TNF-α and IL-1 in response to LPS, whereas the monocytes with very low CD14 ($CD14^{dim}CD16^{+}$) are weak in phagocytic activity and the production of TNF-α in response to LPS (157, 158). As a result, human monocytes have been classified into three subsets, based on the expression of CD14 and CD16, which also have distinct transcriptomes (57, 159–161). The majority (80-90%) of blood monocytes belong to the $CD14^{+}CD16^{-}$ subset, which along with the $CD14^{+}CD16^{+}$ subset forms pro-inflammatory monocytes similar to mouse $Ly6C^{hi}CCR2^{hi}CX_3CR1^{low}$ cells. The $CD14^{dim}CD16^{+}$ minor monocyte population mimics mouse $Ly6C^{low}CCR2^{low}CX_3CR1^{hi}$ cells, which regularly patrol the vessels and are involved in tissue maintenance and healing (160–162). Studies have also reported an intermediate population in mice with $Ly6C^{mid}$ (163). The Table-2 below shows the widely accepted heterogeneous monocyte classification, their markers and key functions.

Table 2 Markers and key functions of heterogeneous monocyte populations (adopted from Chao Shi et al (160) and Yang et al (163))

Subsets	***Markers***	***Chemokine receptors***	***Key functions***
Human			
Classical	$CD14^{+}CD16^{-}$	CX_3CR1^{low} CCR2hi	Phagocytosis & Pro-inflammatory
Intermediate	$CD14^{+}CD16^{+}$	$CX_3CR1^{high}CCR2^{mid}$	Pro-inflammatory
Non classical	$CD14^{dim}CD16^{+}$	$CX_3CR1^{high}CCR2^{low}$	Patrolling, anti-viral roles
Mouse			
$Ly6C^{high}$ ($Ly6C^{+}$)	$CD11b^{+}CD115^{+}Ly6C^{high}$	$CX_3CR1^{low}CCR2^{high}$	Phagocytosis & Pro-inflammatory
$Ly6C^{mid}$ ($Ly6C^{+}$)	$CD11b^{+}CD115^{+}Ly6C^{mid}$	$CX_3CR1^{low}CCR2^{high}$	Pro-inflammatory
$Ly6C^{low}$ ($Ly6C^{-}$)	$CD11b^{+}CD115^{+}Ly6C^{low}$	$CX_3CR1^{high}CCR2^{low}$	Tissue repair and patrolling

Ly6C -lymphocyte antigen 6C, CCR2 -CC-chemokine receptor 2, CX3CR1, CX3C-chemokine receptor 1.

The vast heterogeneity in monocytes led researchers to identify new markers for monocytes. CD11b, HLA-DR/MHC-II, CD80, CD86 and CD163 are some other key cell surface markers of monocytes, which have been used extensively to define the status of monocytes in inflammatory and disease conditions. These markers are differentially expressed depending on the status of the inflammatory setting and they have been elaborately discussed in several reviews (163–165).

As mentioned in the previous section, macrophages respond to various stimuli and change their phenotype and function depending on the particular stimulus. For the same reason they can execute distinctive function in the tissues they reside in. Macrophages perform these functions by activating transcription factors, via the differential expression of specific proteins on their surface and by the secretion of cytokines. These transcription factors, cell surface molecules and cytokines are used as markers to identify the difference in the macrophage phenotype. Since this thesis is centered on monocyte derived macrophages, I will focus mostly on macrophage markers for different stimulus. The Table-3 below shows the list of markers, stimulus and denomination used for polarization, and identification.

Table 3 Markers for macrophages of selected stimulus (adopted from Murray et al (166)).

Macrophage types	***Markers***					
	Species	***Scavenger receptors***	***Cytokines***	***Chemokines***	***Transcription factors, Socs protein***	***Others***
M(IFNγ)	Mouse Human				pSTAT1+++,SOCS1 pSTAT1+++IRF5	
M(LPS+IFNγ)	Mouse Human	Marco	Tnf,IL6,IL27, IL23a,IL2a TNF,IL6,IL1 B,IL12A,IL1 2B,IL23A	 CCL5,CXCL 9,CXCL10, CXCL11	pSTAT1+++,SOCS1 ,NFKBIZ, IRF5 pSTAT1+++,IRF5, IRF1	 CCR7,CD40 IDO1,KYNU
M(LPS)	Mouse Human	Marco	Tnf,Il6,Il27 TNF,IL6, IL1B	 CXCL10, IL8	pSTAT1+++SOCS1, NFKBIZ IRF5	 PTX3,MM9
M(GC)	Mouse Human	 CD163 stabilin1 MARCO				 TGFBR2, ADORA3, F13A1
M(IL10)	Mouse Human		IL10		pSTAT3+,Nfil3, SBNO2,SOCS3	 IL4RA
M(Ic)	Mouse Human		IL10,IL6	CXCL13,CC L1 CCL20		
M(IL4)	Mouse Human	 stabilin1 MRC-1		CCL17,CCL 24, CCL22 CC4,CCL13 CCL17,CCL18	pSTAT6+++,Nfil3 SBNO2,SOCS3 IRF4,SOCS1,GATA3	 TGM2,ADORA 3,IL17RB,ALO X15,CD200R

ADORA- adenosine A3 receptor, ALOX15- arachidonate 15-lipoxygenase, CCR- chemokine (c-c motif) receptor, CCL- chemokine (c-c motif) ligand, CD-cluster of differentiation, CXCL- chemokine (c-x-c motif) ligand, F13A1- coagulation factor XIII A1 receptor GC- glucocorticoids, Ic- immunocomplexes, IL- interleukins, IFNγ – interferon gamma, IRF- interferon regulatory factor, LPS- lipopolysaccharide, MMP- matrix metallopeptidase, MRC-1 – macrophage mannose receptor 1, Nfkbiz- nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta. Nfil3-nuclear factor interleukin 3 regulated protein, SBNO-strawberry notch homolog, SOCS- suppressor of cytokine signaling, STAT- signal transducer of activator of transcription, TGFBR- transforming growth factor beta receptor, TGM- transglutaminase

2.2.3 Functions

Monocytes and macrophages are highly plastic in their response to environmental factors. They play a role in the both innate and adaptive immunity in forming the first line of defense by phagocytosis and acting as antigen presenting cells to activate lymphocytes. Monocytes/macrophages express a wide range of PRRs, including scavenging receptors, Toll like receptors, Nod-like receptors, and others, which coordinate the clearance of apoptotic cells, cell debris, and pathogens, and regulate homeostasis. Importantly, these PRRs stimulate T cell priming in adaptive immunity (56, 167–170). Phagocytosis and maintaining of tissue homeostasis are the central functions of monocytes and macrophages under steady state conditions, which is emphasized by their role in resolving the inter-digit regions during limb formation (56, 75). Scavenger receptors, such as Scavenger receptor-A, CD36 and stabilin-1 are actively involved in recognizing and clearing apoptotic cells, cell debris and in wound healing (171–173). The binding of CD36 to oxidized low density lipoprotein (Ox-LDL) displayed by apoptotic cells, leads to the activation of peroxisome proliferator-activated receptor gamma (PPARγ), which further increases the expression of CD36 on these cells for effective phagocytosis (171). Monocytes/macrophages accomplish pathogen clearance and wound healing in three major stages: 1) an inflammatory stage, where these phagocytes recognize the pathogen and phagocytosis them for destruction. They also secrete a repertoire of pro-inflammatory molecules that recruit T cells to initiate cell mediated immunity for the effective elimination of pathogens and other cells for the removal of necrotic tissue; 2) a proliferative stage in which they activate and induce proliferation of endothelial cells, and fibroblasts for the formation of extracellular matrix and new blood vessels to the site of infection; 3) a resolution stage where they suppress the inflammation caused by the infection or inflammatory cells, by producing anti-inflammatory molecules and triggering the apoptosis of recruited cells, followed by the formation of new tissue and wound remodeling (174).

In humans, the classical $CD14^{+}CD16^{-}$ and intermediate $CD14^{+}CD16^{+}$ monocytes have been reported to have higher phagocytic activity, antigen presentation activity and pro-inflammatory profile than $CD14^{dim}CD16^{+}$. However, the intermediate population has been shown to produce more TNFα and IL12 in response to LPS stimulated TLR activation compared to the classical cells, whereas the classical cells produce high levels of reactive oxygen species (ROS) (162, 175). The non-classical $CD14^{dim}CD16^{+}$ monocytes have been reported to be weak in phagocytosis, and have been defined as patrolling monocytes, which crawl on the luminal side of the endothelium, inspect tissue damage and play a role in wound healing (159, 162, 176). $CD14^{dim}CD16^{+}$ cells have also been reported to sense viral nucleic acids via TLR7 and TLR8 (161), indicating that they play a role in viral recognition. Variations in these monocyte and macrophage (M1/M2) populations have been used as potential markers for various disease conditions (162, 165, 176, 177).

In tissues, macrophages have different names and they perform various functions. In the brain, macrophages are called microglia, which play a role in brain development, synaptic remodeling and immune surveillance. In bone they are known as osteoclasts and function in bone modeling and remodeling. Macrophages are known as kupffer cells in the liver, they perform multiple roles in lipid metabolism, toxic substance removal, and clearance of erythrocytes, cell debris, and microbes. Lung macrophages are known as alveolar macrophages and they execute the clearance of surfactants, and process the inhaled pathogens (176, 178, 179). Since this thesis is focused on the activation status of monocytes and macrophages in line with Th1 and Th2 cells activation, and their cytokine environment and immune suppressive settings. The functions of monocytes and macrophages in the activation and suppression of T cells, and their role in immune suppressive conditions and diseases are discussed in more detail in the following paragraphs and section 2.4.

In pathogen clearance and immune suppression, the recognition of LPS by the TLR4 receptor on monocytes/macrophages activates MyD88 dependent/independent pathways. This leads to the stimulation of NF-κB and the subsequent production of pro-inflammatory cytokines, including TNFα, IL-12 and type I interferons (180). These cytokines activate naïve T cells to Th1 cells, which favor the elimination of the pathogen. Th1 cells produce IFNγ to activate monocytes and macrophages to become a more destructive phenotype (M1 like). After the pathogen is eliminated monocytes/macrophages enter the resolution stage, and activate Th2 and Tregs cells to produce anti-inflammatory molecules, including IL4, IL13, IL10, TGFβ and prostaglandins, which dampen the immune system to protect the host tissue (34, 49, 53, 102, 174). Therefore, the environmental Th1/Th2 status after the infection can determine the activation status of macrophages or the M1/M2 polarization. M1 macrophages in general produce effector molecules, such as reactive oxygen and nitrogen intermediates, and pro-inflammatory cytokines (IL1β, TNFα, and IL6), which contribute to Th1 responses, as well as mediate resistance against tumor and intracellular pathogens (181). Conversely, M2 macrophages produce less pro-inflammatory cytokines and express high levels of mannose receptors and scavenger receptors. M2 macrophages play a role in the clearance of parasites (182), function in diminishing inflammation and promote tissue remodeling (183). Notably, the M2 and Th2 immune suppressive state favors angiogenesis, tumor progression and immunoregulation (181, 184).

2.3 CLEVER-1

Irjala et al., produced antibodies against efferent lymphatic vessels in order to reveal novel molecules associated with cell migration in the lymphatic endothelium (185). Two of these antibodies (3-266 and 3-372) recognized small lymphatic vessels, lym-

phatic sinusoids and HEVs in lymphoid tissues, as well as afferent lymphatic vessels in non-lymphoid tissues. The expression of the target molecule or antigen recognized by these antibodies was upregulated on HEV-like vessels at sites of inflammation, but the expression was reported to be absent from peripheral blood leukocytes. This molecule was titled as common lymphatic endothelium and vascular endothelial receptor-1 (CLEVER-1) (185). The same molecule was named Stabilin-1 (the official name of this protein, and its official gene name, is *STAB1*), and FEEL-1 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1) by other groups (172, 186).

2.3.1 Structure

CLEVER-1 is a 270-300 kDa multidomain glycoprotein that has at least three different isoforms. CLEVER-1 consists of a type I transmembrane protein containing two RGD motifs, 7 Fasciclin domains, 17 Epidermal growth factor (EGF) domains, 4 EGF like domains, a EGF laminin domain and a proteoglycan link homology region (171, 173, 185). Stabilin-2, a protein homologous to stabilin-1 has been reported to be a definite hyaluronan receptor in hepatic sinusoidal endothelial cells (187, 188). Stabilin-1 and stabilin-2 have no significant DNA homology, but share 55% identity at the protein level (187). A major difference was found to be in their C-terminal region; Stabilin-1 contains a dileucine-based sorting motif and stabilin-2 has a classical tyrosine based endosomal sorting signal (189). The homology between the human and mouse stabilin-1 is 86% (187).

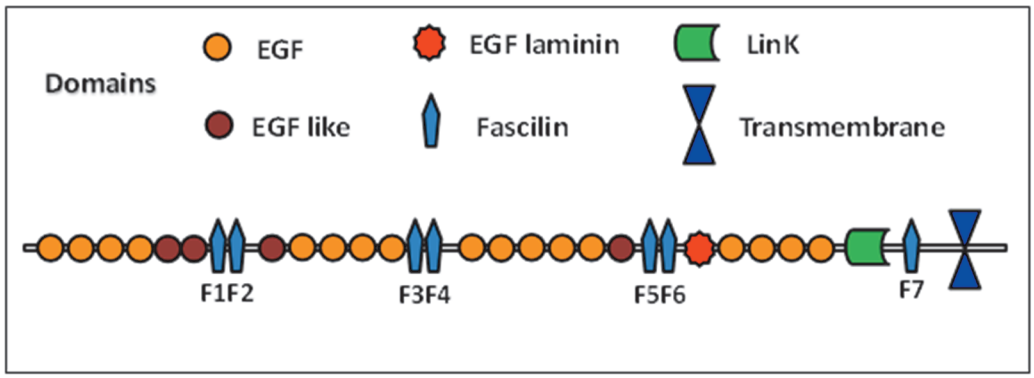

Figure 2 Model of the structure and domain organization of CLEVER-1 (modified from Irjala et al (185) and Canton et al (171))

2.3.2 Expression

Stabilin-1 was identified as the antigen for an MS-1 antibody on sinusoidal endothelial cells in human spleen by Goerdt et al., in 1991 (186). FEEL-1 in turn was cloned as a novel scavenger receptor by Adachi and Tsujimoto in 2002, while searching for a scavenger receptor on endothelial cells (172). Further studies on stabilin-1 revealed that it was expressed in alternatively activated macrophages or type II macrophages and in sinusoidal endothelial cells of the human spleen, liver and lymph nodes (187) and in the sinusoidal endothelial cells of the bone marrow (190). Human aortic endothelial cell-lines were found to express stabilin-1, but human umbilical vein endothelial cells were devoid of it based on a northern-blot analysis (187). Studies using human blood derived monocytes/macrophages cultured under inducing condition using IL-4 and dexamethasone or dexamethasone alone, showed an increase in stabilin-1 expression, whereas IFNγ was found to inhibit stabilin-1expression on these cells (133, 187, 189). Monocytes of familial hypercholesterolemia patients, but not monocytes from healthy individuals, were found to express stabilin-1 on their surface by flow cytometry (191). Stabilin-1 was used as a specific marker for alternatively activated macrophages *in vivo* (192, 193).

Our group has reported CLEVER-1 expression in normal lymphatic endothelial cells, in both lymphatic and blood endothelial cells of human psoriatic skin, and in cultured human vascular and lymphatic endothelial cells (194). A study on human liver showed increased expression of CLEVER-1 in endothelial cells at the sites of leukocyte recruitment to the inflamed human liver (195). Recent studies from our group have reported CLEVER-1 expression in M2 macrophages and tumor associated macrophages, and its role in tumor progression using cell specific knockouts of CLEVER-1 in mice (196–198). Studies performed by different research groups (including us) with different antibodies have revealed the expression of stabilin-1 in blood vessels under various conditions, including wound healing, tumor vascularization and chronic inflammatory conditions of the skin, such as psoriasis (133, 194). Stabilin-1 expression was observed in macrophages associated with tumorigenesis and angiogenesis (199), and in individual macrophages in the colon, stomach and skin (200).

Adachi and Tsujimoto cloned a scavenger receptor containing fascilin, EGF-like, laminin-type EGF and link domains (172). They named the molecule FEEL-1 because of its domain structures. FEEL-1 was found to be expressed in several tissues, prominently in the spleen, lymph nodes and in CD14 positive mononuclear cells in humans. Endothelial cells such as human coronary arterial endothelial cells and human microvascular endothelial cells clearly expressed FEEL-1. Adachi and Tsujimoto detected the mRNA of FEEL-1 by RT-PCR in human umbilical vein endothelial cells (172), which was reported to be negative for stabilin-1 (187). A northern-blot analysis of

mouse tissues revealed that FEEL-1 is expressed in the liver, lung, kidney, spleen, heart, aorta, white adipose tissue and peritoneal macrophages (201).

Stabilin-1 has been reported to shuttle between the extracellular and intracellular compartments of the cell, but it is predominantly localized in intracellular vesicles in both macrophages and sinusoidal endothelial cells (202), and a small percentage is found in enhanced alternative activated macrophages (189). Stabilin-1 is localized strongly in EEA-1 positive early endosomes and a minor percentage in late endosomes. Notably, a portion of stabilin-1 recycling has been confirmed using fluorescent labelled transferrin. In the same study stabilin-1 was also detected unexpectedly in the Trans Golgi network (TGN) using double immunofluorescence staining. Treatment of macrophages with brefeldin A induced the accumulation of stabilin-1 in the TGN and its depletion from early endosomes, demonstrating the shuttling of stabilin-1 between the endosomal compartment and the biosynthetic compartment (189).

2.3.3 Function

Our group reported that CLEVER-1 mediates leukocyte adhesion. In 2003, using *in vitro* studies, Irjala et al., found that an antibody against CLEVER-1 inhibited lymphocyte adhesion on the vascular and lymphatic endothelium in lymphoid organs (185). Monocyte, lymphocyte and granulocyte adhesion was inhibited by a CLEVER-1 antibody in HEV-like vessels in inflamed non-lymphoid organs. In the same year, in other studies the antibody against CLEVER-1 was reported to block the adhesion of malignant cell-lines on vascular and lymphatic endothelia (203). Studies using isolated vascular endothelial cells indicated that CLEVER-1 supported the rolling and transmigration of peripheral blood mononuclear cells, and mediated the transmigration of leukocytes through cultured lymphatic endothelium under static conditions (194). *In vivo* models revealed that the blocking of CLEVER-1 with an antibody significantly inhibited peritonitis in mice by reducing granulocyte entry to 50%. This blocking also completely inhibited the migration of monocytes and lymphocytes into the inflamed peritoneum (204). Studies on human liver have revealed that CLEVER-1 is involved in mediating lymphocyte transmigration, of mostly regulatory T cells, through cultured hepatic sinusoidal endothelial cells (195).

FEEL-1, which was cloned as a scavenger receptor is a receptor for acetylated low density lipoprotein (acLDL). Phagocytosis performed on FEEL-1 and FEEL-2 CHO cell transfectants suggested that both these proteins are involved in the internalization of Gram-negative and Gram-positive bacteria (172), but the authors did not show the co-localization of FEEL-1 and the internalized bacteria. In the same study the authors also suggested that FEEL-1 is involved in cell-cell interactions based on an *in vitro* matrigel tube formation assay. FEEL-1 was also shown to be the receptor for advanced glycation end products (AGEs) on transfected CHO cells, but the binding of AGEs was

stated to be effectively inhibited by acLDL and other inhibitors of scavenger receptors (201).

Stabilin-1 was reported to mediate the internalization of acLDL on CHO cells, which was demonstrated by confocal microscopy and a flow cytometric analysis. In the same study it was showed that stabilin-1 is involved in the delivery of acLDL from early to late endosomes, and that it is dependent on phosphatidylinositol 3 kinase (PI3K) for delivery, whereas internalization is not (205). Using the phage display technique and *in vitro* binding assays it was found that the EGF-like domain of Stabilin-1 on alternatively activated (M2) macrophages interacted with a secreted protein acidic and rich in cysteine (SPARC). This led to idea that stabilin-1 on M2 macrophages plays a role in the clearance of SPARC in the tumor microenvironment (206). Further, stabilin-1 on these macrophages has been shown to be involved in the phagocytosis of the apoptotic cells in phosphatidylserine dependent manner (207). Furthermore, stabilin-1 on M2 macrophages was also shown to be involved in the uptake of placental lactogen (208). A yeast two hybrid screen of a human placental cDNA library led to the identification of Stabilin Interacting-Chitinase Like Protein (SI-CLP) an intracellular ligand for stabilin-1 (209). A deficiency of both stabilin-1 and stabilin-2 in mice was shown to lead to a lack of proper hepatic clearance of noxious agents in the blood, and improper tissue homeostasis in the liver and in other distant organs (210). Mouse models with different CLEVER-1gene knockouts and CLEVER-1 antibody treatments have demonstrated that primary tumors and metastases were smaller in knockouts than in controls (197). Furthermore, treating mice bearing wild type tumors with a CLEVER-1 antibody reduced the size of the primary tumor, demonstrating that CLEVER-1 acts as an immune suppressive molecule in cancer (197).

2.4 Monocytes and macrophages in health and disease

Although the concept of monocyte recruited macrophages or dendritic cells was challenged by recent findings of tissue macrophages under steady state conditions or during certain states of inflammation (56), many studies have reported that the key function of monocytes is to migrate to the site of infection and develop into macrophages. The recruitment of monocytes to the site of inflammation regulates the inflammatory setting, depending on the infection. Monocytes recruit and activate T cells, and the recruited monocytes can also serve as markers to define disease conditions (56, 164, 179). During pregnancy, the recruitment of maternal blood monocytes to the placenta has been reported by several studies (211). Monocytes have also been shown to be recruited to the lungs as a part of Th1 and Th2 immune responses to pathogens and allergens (53). During bacterial and fungal infections recruited monocytes have been shown to differentiate into TNFα and iNOS producing dendritic cells and to control the clearance of microbe (212,

213). Numerous *in vivo* experiments have shown that mouse $Ly6C^+$ monocytes (which are equivalent to human $CD14^+CD16^-$) are recruited during inflammation, and can differentiate into the M1 like phenotype and initiate the inflammatory response. In the absence of inflammation the recruited $Ly6C^+$ monocytes can return to the bone marrow to differentiate in to $Ly6C^{low/-}$ (equivalent of human $CD14^{dim}CD16^+$) monocytes (164). $Ly6C^-$ monocytes have been shown to patrol better along the luminal surface of the endothelium, and to promote healing in the ischemic myocardium and tissue repair during an infection with *Listeria monocytogenes*. These properties of monocytes are related to the M2 direction (214). Recruited monocytes are highly plastic: an experimental liver fibrosis model has revealed that $Ly6C^+$ monocytes migrate to the injured area, and can differentiate into either iNOS (classically activated) or arginase (alternative activated) producing macrophages, depending on the Th1/Th2 environment (215). Another study on a muscle injury model has shown that monocytes recruited for the clearance of apoptotic cell bodies, can swift from a pro-inflammatory to an anti-inflammatory type after clearance by the secretion of TGFβ and IL10 for immune suppression and muscle regeneration (216). Monocytes/macrophages are important mediators of the inflammatory process, however they do have anti-inflammatory properties, since they are involved in the resolution of inflammation (176). This study focuses on the function of monocytes/macrophages in pregnancy (a strong immunosuppressive condition) and in a delayed hypersensitivity reaction. Hence, these aspects will be discussed in more detail in the following sections.

2.4.1 Pregnancy

Pregnancy is a complex biological process in which the maternal immune system remains resistant against microbes and yet attains tolerance towards the paternal antigens to protect the fetus. In normal pregnancy, the circulation of blood through the placenta brings maternal immune cells into contact with the placenta either directly or indirectly (217). The placenta is an organ that forms an interface between the maternal and fetal immune systems. In the placenta there is an increase of innate immune cells such as natural killer cells (NK), macrophages and fetal trophoblast cells right from the start of a healthy pregnancy (218). Further, circulating immune cells, particularly monocytes, have been shown to become activated when in contact with the placenta (219). Monocytes, macrophages and NK cells seem to have local immune functions and also play an important role in placental development by inducing the recruitment of trophoblasts, the remodeling of spiral arteries and angiogenesis (220). However, this function is impaired in disease conditions like pre-eclampsia.

Pre-eclampsia is the most common complication of pregnancy, identified by hypertension and proteinuria, which often results in early delivery of the infant (221). The two stage model proposed for pre-eclampsia is; 1) poor placentation and, 2) production

of pro-inflammatory factors by the abnormal placenta, which results in the stimulation of a systemic inflammatory response, prompting the signs of pre-eclampsia (222, 223).

In normal pregnancy, many changes occur in the peripheral circulation both in the adaptive and innate immune response. One of the key changes in the adaptive immune response is a decrease in the Th1/Th2 cell ratio, which is associated with changes in T regs and Th17 cells (224, 225). It has been suggested that to compensate for such changes, there has to be a change in the innate immune response to adapt to the pregnancy, which is shown by an experiment which demonstrated an increase in leukocytes, such as monocytes and granulocytes, during pregnancy (226, 227). A phenotypical activation of monocytes has been reported as the increased expression of CD11b, CD14 and CD64 on the monocytes of pregnant women compared to the monocytes of non-pregnant women (228). This study was later confirmed by others (227, 229). Further functional changes, including the increased production of oxygen free radicals by the monocytes of pregnant women (228), and cytokines by the non-stimulated or stimulated monocytes of pregnant women compared to the monocytes of non-pregnant woman has been reported (230, 231). However, this activation is stimuli specific. For instance, stimulation with LPS decreased the cytokine production of the monocytes of pregnant women, but this represents the activation and tolerance state of these monocytes in producing less inflammatory cytokines during normal pregnancy (231, 232). Regarding the monocyte subset, it is not clearly evident which population is predominant, because one study has shown an increase in the intermediate monocyte population and a decrease in the classical population (233). However contradictory results have also been published, reporting an increase in classical monocytes (211, 234).

In pre-eclampsia, the monocytes are more highly activated both phenotypically and functionally compared to those in normal pregnancy. This is shown by an increase in the expression of CD11b and CD14 on monocytes and an increase in the production of oxygen free radicals and an alteration in the production of cytokines (211, 219, 228, 235). Concerning the monocyte subsets, the increase in the intermediate monocyte population in pre-eclampsia compared to normal pregnancy has been shown (233). The mechanism involved in the activation of monocytes in pregnancy and pre-eclampsia has not been clearly elucidated, but some reports suggest that monocytes get activated when in contact with the placenta, where placental products like cytokines, microparticles and fetal DNA may activate the monocytes. Studies have also shown an increase in pro-inflammatory cytokines, including TNFα, IL1b, and IL18 in the placentas of pre-eclampsia pregnancies compared to normal ones, which explains the over-activation status of monocytes and their possible role in poor placentation in pre-eclampsia (211).

Macrophages comprise about 20 to 30 % of the decidual leukocytes during normal pregnancy. The decidua is a mucous membrane lining the uterus during pregnancy and forms the maternal part of the placenta (236). Macrophages in the decidua are associated with the formation of spiral arteries and angiogenesis, by producing factors related

to angiogenesis and tissue remodeling (237, 238). Apoptosis is the key step during the formation of spiral arteries and the invasion of trophoblasts. The involvement of decidual macrophages in engulfing the apoptotic cells and substances prevent the apoptotic cells from releasing pro-inflammatory factors into the decidua (239). In normal pregnancy, macrophages in the decidua are classified as M2 (immunomodulatory) and thus have been shown to express markers for M2 like CD206, CD163 and DC-SIGN (240, 241) They are not induced by Th2 cytokines like IL4, but by M-CSF and IL10, which are abundant in the decidua (241–243). Decidual macrophages express inhibitory receptors Ig-like transcripts (ILT)2 and ILT4, which can bind to HLA-G on invading trophoblasts, which leads to the delivery of negative signals to the macrophages and a subsequent tolerance and the stimulation of anti-inflammatory cytokines (244). It has also been suggested that the M2 phenotype is linked to the phagocytosis of apoptotic cells and trophoblast cell debris in the placenta (239).

Unlike in monocyte studies, it is more challenging to elucidate macrophage functions related to pre-eclampsia, given that most placental studies have been done after delivery. However, studies have reported differences in macrophage populations and functions between normal and pre-eclampsia pregnancies. Recently, a study has shown a decrease of M2 macrophages in the decidua of pre-eclampsia (245), which is consistent with the decrease in anti-inflammatory cytokines and the increase in pro-inflammatory cytokines (246, 247). However, other studies have reported an increase in the number of macrophages at the spiral artery region in cases of pre-eclampsia, without defining their phenotype clearly. The presence of these macrophages in the spiral artery region may be associated with the development of acute atherosis (248, 249). A study using early term decidua from women has revealed an increase in CD68 mRNA expression levels and a decrease of CD206/MRC-1 mRNA in pre-eclampsia, suggesting an increased number of macrophages and a decrease in total M2 macrophages (250). In a rat model, multiple doses of LPS have induced pre-eclampsia by poor spiral artery formation and trophoblast invasion, with an increase in macrophage numbers (251). Thus, the increased numbers of macrophages (possibly of the M1 phenotype) in the decidua in cases of pre-eclampsia, and the decrease in M2 like macrophages in pre-eclampsia, indicate that macrophages have an important role in normal pregnancy.

Originally, the discovery of macrophages in the decidua was considered an immune response to the semi-allogenic fetus, but it has now been established that M2 macrophages, monocytes and other immune cells are needed for proper implantation and protection of the fetus.

2.4.2 Hypersensitivity and allergy

The term allergy was initially defined by Clemens Von Pirquet as an altered capacity of the body to react to a foreign substance (reviewed in(252)). Later, allergy was rede-

fined as a class of immune system responses that are termed as hypersensitivity reactions to innocuous antigen/infectious agents. These reactions are harmful immune responses that cause tissue injury (like allergies) and may cause serious disease. In 1960's Coombs and Gell classified the hypersensitivity reaction into four types, type I, II, III and IV based on the time of the response and the mechanism involved (253). Although recent studies have shown that only limited reactions fit into this classification (254, 255), it is still a widely used categorization for hypersensitive reactions. Upon exposure to antigen hypersensitivity, reactions of type I, II, and III involve antibodies and respond in minutes to hours. Type IV differs from the other types; a type IV response is delayed and usually takes from 48 to 72 hours. Moreover, it is a cell mediated response elicited by T cells and monocytes/macrophages. Due to the delay in secondary cellular response after the antigen exposure it is also called delayed type hypersensitivity (DTH). Since this thesis focuses on monocytes/macrophages and T cells, type IV or delayed hypersensitivity reactions are discussed in more detail in the following paragraphs.

DTH is involved in the pathogenesis of many autoimmune and infectious diseases caused by bacteria, fungi and certain parasites (tuberculosis, leprosy, blastomycosis, histoplasmosis, toxoplasmosis, leishmaniasis, etc.). Another form of DTH is contact dermatitis caused by chemicals and heavy metals. The mechanism of DTH involves antigen presentation to T cells by monocytes/macrophages and the secretion of cytokines, including TNFα and IL12, which helps in the recruitment of monocytes, and in the stimulation and proliferation of T cells.

T cells and their activation status play a center role in DTH. The loss and impaired function of CD4 T cells has been reported to lead to a poor response to different antigens including the tetanus toxoid (256). Intracellular pathogens, including *Mycobacterium tuberculosis* are not killed effectively when there is loss of Th1 activity, whereas IL4 levels increase during a *Mycobacterium tuberculosis* infection (257). However an IL4, IL5, or Th2 response is needed for protection against parasitic infections and is involved in conditions like allergic asthma (39, 53).

Studies have shown that the activation of Th1/Th2 cells in DTH depends on some key factors, including antigens being recognized by monocytes/macrophages, the type of monocytes/macrophages involved, and the health condition of an individual (258–260). In a DTH recall assay monocytes/macrophages are co-cultured with T cells in the presence of antigens. In a DTH assay, most bacterial antigens, such as the purified protein derivative (PPD) of *Mycobacterium tuberculosis*, and the tetanus toxoid were shown to stimulate a strong IFNγ production/Th1 response, whereas allergens like pollen and timothy grass stimulated a Th2 response (261–263). However, the response and ratio of Th1/Th2 varies among patients. For example bacterial antigens, including streptokinase and PPD, stimulate Th1 cells in non-atopic individuals , whereas in atopic individuals the production of IL4 and IL5 is favored, because of the genetic modifi-

cation in the T cells of atopic persons (264). The phenotype of monocytes/macrophages and their antigen recognition determine the Th1/Th2 activation. For instance, an experiment on macrophages infected with mycobacteria stimulated naïve T cells into Th1 cells (265). Similarly, a study on allergic lung inflammation demonstrated that resident alveolar macrophages diminished the early events of allergic inflammation, whereas recruited monocytes promoted it (266). Moreover, it has been reported that inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype (267).

As described previously, different stimuli polarize macrophages into different types with a change in expression level of different molecules, and these molecules have an effect on the T cell activation into Th1/Th2. The interaction of co-stimulatory molecules like CD80/CD86 on APCs and CD28/CTLA of T cells seems to modulate the development of Th1 or Th2 together with the cytokines produced by monocytes/macrophages (268). MRC-1, a protein expressed on the cells of a M2 phenotype, has been shown to a bind a virulent form of *Mycobacterium tuberculosis*, and assist in the survival and growth of *Mycobacterium tuberculosis* by inhibiting the phagosome-lysosome fusion (269–271). Conversely, complement receptors (CRs) on macrophages bind to both opsonized virulent and attenuated strains of *Mycobacterium tuberculosis* and initiate phagocytosis (269). Moreover, when CRs and MRC-1 were blocked, *Mycobacterium tuberculosis* was indicated to enter via type A scavenger receptors (272). Furthermore, MRC-1 has been shown to support Th2 polarization, and the silencing of MRC-1 on monocyte derived cells in an antigen recall assay reversed the Th2 polarization (273).

Collectively, these studies suggest that targeting molecules expressed on monocytes/macrophages, which balance the Th1/Th2 ratio, can be used as immune therapy tools for allergic conditions.

3 AIMS OF THE STUDY

Immune response and immune suppression are the two critical phases used by the immune system to protect the host against an infection or any abnormal substance. These phases are crucial aspects for several studies in the field of immunology, since an imbalance between these two phases can lead to cancer and many inflammatory diseases, such as autoimmune diseases. To identify and target the diseases caused by this imbalance, immune cells like monocytes/macrophages and their marker proteins, which are differentially regulated and expressed respectively, are generally used in studies. However, in normal immune suppressive conditions like pregnancy not all the functions of proteins expressed on monocytes/macrophages are known. Likewise, the role of these proteins in normal individuals often has received little attention.

The aim of this study was to elucidate the expression and function of CLEVER-1/stabilin-1 on macrophages in a normal immune suppressive condition like pregnancy and to investigate the expression, and function of the same protein on the monocytes of normal individuals.

The specific aims of the study were:

1. To characterize the CLEVER-1/stabilin-1 positive macrophages in human placenta and study its immunological function.
2. To examine the expression of CLEVER-1/stabilin-1 on monocytes, and to analyze the gene expression profile of CLEVER-1/stabilin-1^{high} and CLEVER-1/stabilin-1^{low} monocytes and to investigate the potential function of stabilin-1 in immune regulation.

4 MATERIALS AND METHODS

A more detailed description of the materials and methods used in the studies is presented in the original publications (I and II).

4.1 Antibodies

Table 4 Primary antibodies used in the studies

Antibody	*Antigen*	*Isotype*	*Conjugate*	*Source*	*Study*
2-7	CLEVER-1/stabilin-1	rat IgG		Study I	I
3-372	CLEVER-1/stabilin-1	mouse IgG1		Irjala et al. (185)	I,II
3-372	CLEVER-1/stabilin-1	mouse IgG1	Alexa 488	Irjala et al. (185) Molecular Probes	I
9-11	CLEVER-1/stabilin-1	rat IgG2a		Study I	I
9-11 F(ab)2	CLEVER-1/stabilin-1	rat IgG2a		Study I	I,II
CD14	human CD14	mouse IgG2a	FITC	Southern Biotech	I,II
CD14	human CD14	mouse IgG2a	PE	BD Pharmingen	I,II
CD16	human CD16	mouse IgG1	PerCP cy5.5	BD Pharmingen	II
CD56	human CD56	mouse IgG1	Alexa 647	BD Pharmingen	I
CD68	human	mouse IgG1	Alexa 647	Sant Cruz Biotechnology	I
CD68	human CD68	mouse IgG1	FITC	Dako	I
CD206	macrophage mannose receptor-1	mouse IgG1	Alexa 488	Biolegend	I
CD206	macrophage mannose receptor-1	mouse IgG1	Alexa 647	Biolegend	I
MRC-1	macrophage mannose receptor-1	mouse IgG1		Lifespan Bioscience	II
HLA-DR	human MHC-II	mouse IgG2a	Allophy-cocyanin	BD Pharmingen	II
174/2	PV-1	mouse IgG1		Niemela et al. (274)	
3G6	neg.control	mouse IgG1		Salmi et al. (275)	I,II
9B5	neg.control	rat IgG		Jalkanen et al. (276)	I
AK-1	neg.control	mouse IgG1		Karikoski et al. (204)	I
MEL-14	neg.control	rat IgG2a		Gallatin et al. (277)	I,II
MEL-14 F(ab)2	neg.control	rat IgG2a		Study I	I,II
Mouse IgG1	neg.control	mouse IgG1	Alexa 488	BD Pharmingen	I
Mouse IgG1	neg.control	mouse IgG1	Alexa 647	BD Pharmingen	I
Mouse IgG1	neg.control	mouse IgG1	PerCP cy5.5	BD Pharmingen	II
Mouse IgG2a	neg.control	mouse IgG2a	PE	BD Pharmingen	I
Mouse IgG2a	neg.control	mouse IgG2a	FITC	BD Pharmingen	I,II
Mouse IgG2a	neg.control	mouse IgG2a	Allophy-cocyanin	BD Pharmingen	II

Table 5 Secondary antibodies used in the studies

Antigen	*Isotype*	*Conjugate*	*Source*	*Study*
mouse IgG	goat F(ab)2	FITC	Sigma	I
mouse IgG1	goat IgG	PE	Southern Biotech	II
mouse IgG1	goat IgG	Alexa 546	Molecular Probes	I
rat IgG	goat IgG	Alexa 546	Molecular Probes	I
rat IgG F(ab)2	goat F(ab)2	FITC	Abcam	I
rat IgG F(ab)2	goat F(ab)2	PE	Abcam	II
rat IgG F(ab)2	goat F(ab)2	PE	Abcam	II

4.2 Cells, cell lines and transfectants

Table 6 Primary cells and cell lines used in the studies

Cell/Cell line	*Description*	*Source/Reference*	*Study*
Placental macrophages		human placenta	I
Monocytes		human blood	II
PBMC	Peripheral blood mononuclear cells	human blood	I
HEK and HEK stable transfectants	Human embryonic kidney cell line and HEK expressing stabilin-1 full length and 3kb fragment N-terminal part	Karikoski et al. (204), ATCC	I

HEK is the designation of the HEK-293 cell line

4.3 Methods

Table 7 Methods used in the studies

Method	*Study*
Cell isolation and culture	I,II
Capillary flow assay	I
ELISA	II
ELISPOT	II
Flow cytometry	I,II
In vitro stimulation	II
Immunofluorescence	I
Immunohistochemistry	I
Milliplex cytokine analysis	I
Microarray	II
RNA Sequencing	II
Polymerase chain reaction	II
Stamper-Woodruff adhesion assay	I
Transfection	I,II
Western blot	I

Selected methods are described briefly in the following sections.

4.3.1 Immunofluorescence staining

Frozen sections (6 μm) were cut on a Leica cryostat, fixed with ice cold acetone for 3-4 mins and air dried. These sections were then stained with primary antibodies for 30 minutes in a humid chamber at room temperature. After washing off the unbound antibodies with PBS, the sections were incubated with fluorophore labelled secondary antibodies for 30 min in the dark at room temperature. After a final wash with PBS, the slides were mounted using the Prolong gold antifade reagent and examined with an Olympus BX 60 microscope.

4.3.2 In vitro polarizations

Blood samples of healthy individuals were collected in the EDTA tubes, and then PBMCs were isolated using the Ficoll separation method. Monocytes were enriched from PBMCs using a MACS negative selection kit. For a non-polarized state, enriched monocytes ($1x10^6$ cells/well) were cultured in a growth medium Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS and 2mM L-glutamine. To polarize monocytes into M1 and M2 macrophages, monocytes were cultured in a growth medium containing TNFα (50 U/ml and LPS(10 ng/ml)) for M1, and containing IL4(10 ng/ml) and M-CSF(10 ng/ml), IL4(10 ng/ml) and M-CSF(10 ng/ml) and Dexamethasone (100 nM), Dexamethasone (100 nM) only or IL4(10 ng/ml) only for M2 for 48 hours.

4.3.3 ELISPOT

96 well ELISpot plates (MabTech, Stockholm, Sweden) were coated with anti- human IFNγ (1 μg/mL, MabTech) or IL-10 (1 μg/mL, MabTech) overnight at 4ºC or anti-human IL4 (15 μg/mL, MabTech) or IL5 (15 μg/mL, MabTech) for 48 hours at 4ºC, washed with PBS 5 times and blocked with culture medium (RPMI 1640 + 10% FCS) for 30 minutes at 37ºC in a CO_2 incubator. The purified monocytes and autologous T cells from tetanus vaccinated persons or persons allergic to timothy grass were co-cultured in pre-coated ELISpot wells in triplicates at a ratio of 1:10 (Monocytes 10 000:T cells 100 000) in a growth medium (RPMI-1640, 10% FCS, L-glutamine (2mM), 2β–mercapto ethanol (β-ME)(100μM) and gentamycin (50μg/ml)) with tetanus toxoid (20 μg/mL, from National Public Health Institute, Helsinki, Finland) or timothy extract (100 μg/mL, from GREER allergy immunotherapy, Lenoir, USA) for 3 days at 37ºC in a CO_2 incubator. Monocytes and T cells cultured in the growth medium without any stimulation served as background controls. On day 3, the wells were washed with PBS 5 times and incubated with biotinylated anti-IFNγ, IL4 or IL5 (all at 1μg/ml) antibodies in PBS with 0.5% FCS for 2hrs at room temperature. After wash-

ing, the wells were incubated with an alkaline phosphatase conjugated anti-biotin secondary antibody (1:1000) in PBS with 0.5% serum for 1 hour at room temperature in the dark. After washing, the spots were developed by using 5-bromo-4-chloro-3-indoyl phosphate nitro blue tetrazolium (BCIP/NBT) solution for 20-30 minutes at room temperature. After a final wash with water the spots were counted using an Olympus microscope.

5 RESULTS

5.1 Characterization of CLEVER-1$^+$ placental macrophages (I)

Stabilin-1 was reported to be present on placental macrophages (133, 186). However, it has not been extensively investigated, which subpopulation of macrophages expresses stabilin-1, and whether it is expressed on non-macrophage cell populations. Therefore, the focus of my research was to characterize CLEVER-1/stabilin-1 expression on placental macrophages.

Considering, the limitations of existing anti-stabilin-1 antibodies, including MS-1, 266 and 372 (185, 186), in certain immunological aspects, we generated new anti-CLEVER-1/stabilin-1 antibodies 9-11 (anti-human rat IgG2a) and 2-7 (anti-human rat IgG1) by immunizing rats with the immunoaffinity purified CLEVER-1/stabilin-1 from human placenta using the 372 antibody. The flow cytometry and immunoblotting data from CLEVER-1/stabilin-1 transfectants revealed that both these antibodies detect the full length and truncated recombinant 3kb fragment form of the CLEVER-1/stabilin-1 protein in the cells, and also recognized these molecules (270KDa and 110KDa with respective molecular weight) on the immunoblot. Moreover, the depletion of a placental lysate with 372 lead to a loss of signal in 9-11, likewise, depletion with 9-11 lead to no detectable signal from 372, which confirmed the specificity of these antibodies. Since monocytes and macrophages amply express Fc receptors, I generated $F(ab)_2$ from 9-11 and isotype matched negative control antibodies by a commercial source, and used 9-11 $F(ab)_2$ and isotype matched $F(ab)_2$ antibodies for all flow cytometric analyses.

To characterize the placental macrophages, formalin-fixed paraffin-embedded sections of human placenta were stained with the 2-7 CLEVER-1/stabilin-1 mAb. The results showed intense expression of CLEVER-1/stabilin-1 on placental leukocytes (and variable expression in vessels, whereas lymphocyte-like cells, trophoblasts and stromal components were CLEVER-1/stabilin-1 negative). To confirm whether these CLEVER-1/stabilin-1 positive placental leukocytes were macrophages, a two-color immunofluorescent staining with the macrophage pan marker CD68 and MRC-1(CD206), an established M2/type 2 macrophage marker, was used. These analyses demonstrated that all CD68 positive cells co-expressed CLEVER-1/stabilin-1, and that all of the CLEVER-1/stabilin-1 positive cells co-expressed MRC-1. However, when stained for macrophages of inflamed tonsil tissues, the anti-CLEVER-1/stabilin-1 antibody did not stain all of the macrophages. Instead, CD68 stained all macrophages and

most of the CD68 positive macrophages were devoid of CLEVER-1/stabilin-1 staining. Further, FACS analyses on leukocytes isolated from placenta (by mechanical teasing and Ficoll gradient centrifugation) confirmed that most macrophages co-expressed CD14, CD68, CD206 and CLEVER-1/stabilin-1. When the surface of placental macrophages from four individual placentas was analyzed, 97±1% of the $CD14^+$ macrophages co-expressed CD68, and 96±2% of the $CD14^+$ macrophages co-expressed CD206, and in all these macrophages CLEVER-1/stabilin-1 was expressed at a relatively low level and there was no clear detectable CLEVER-1/stabilin-1 negative population. Examination of CLEVER-1/stabilin-1 expression on the surface of these macrophages revealed that CLEVER-1/stabilin-1 is expressed at a similar level on all these macrophages: $CD14^+$ macrophages (mean fluorescence intensity (MFI), MFI=21±2), on $CD68^+$ macrophages (MFI=19±2) and $CD206^+$ macrophages (MFI=17±2), which demonstrated that CLEVER-1/stabilin-1 is present on the surface of placental macrophages. Collectively these results show that all macrophages in human placenta are CLEVER-1/stabilin-1 positive and they are of the type II (M2) phenotype.

5.2 Functional analysis of CLEVER-1 on placental macrophages (I)

To investigate whether CLEVER-1/stabilin-1 is just a marker for M2 or if it has any effect on polarization, CLEVER-1/stabilin-1 in isolated placental macrophages was silenced by siRNA, which resulted in an average knock-down efficacy of 60% in the CLEVER-1/stabilin-1 protein. When compared to medium of negative control siRNA transfected cells, an increase in pro-inflammatory cytokine TNFα was detected in culture medium of CLEVER-1/stabilin-1 silenced cells, when by analyzed by multiplex assay. However, there was also an increase in IL10 in the medium of CLEVER-1/stabilin-1 silenced cells. Moreover, the expression of MRC-1 was not altered in CLEVER-1/stabilin-1 silenced cells. Thus, CLEVER-1/stabilin-1 may not be needed for the production of cytokines, which regulate the polarization of M2 macrophages. Next, the function of CLEVER-1/stabilin-1 on placental macrophages was studied. The flow cytometry analyses showed that when control siRNA treated cells and CLEVER-1/stabilin-1 silenced cells were treated with a model antigen DQ-OVA (self-quenched model antigen BODIPY FL-labeled DQ-OVA), the uptake and/or processing of DQ-OVA by CLEVER-1/stabilin-1 silenced cells were significantly less at the 4 hr time point compared to control siRNA treated cells. Similarly, the uptake of Ac-LDL (a known ligand of stabilin-1) was reduced in CLEVER-1/stabilin-1 silenced cells. Thus, the results revealed that CLEVER-1/stabilin-1 acts as a scavenging receptor on placental macrophages and may participate in antigen presentation.

The role of endothelial CLEVER-1/stabilin-1 on the adhesion and transmigration of lymphocytes has been previously studied (194, 204), but its function in placental mac-

rophages regarding adhesion to the endothelium has not been studied. To identify a potential adhesive role, CLEVER-1/stabilin-1 on placental macrophages was blocked by pretreatment with an anti-CLEVER-1/stabilin-1 (9-11 mAb) or a control antibody, and then the macrophages were incubated on the placental sections. The number of cells adhered/bound to placental vessels was counted. These analyses revealed that the blocking of CLEVER-1/stabilin-1 on placental macrophages decreased the adhesion of macrophages to the placental blood vessels by 60%. Further, capillary flow assays were performed to study which step of the multistep adhesion cascade during the interaction between leukocytes and endothelial cells is inhibited by the antibody. In these assays placental macrophages blocked with the CLEVER-1/stabilin-1 antibody or cells treated with a control antibody were transfused over a confluent monolayer of human umbilical vein endothelial cells (HUVEC) under physiological shear stress. In these assays placental macrophages firmly adhered to endothelial cells, as was seen by real time imaging and some of the cells transmigrated. This was detected by the transformation of phase-bright cells into phase-dark cells during the transmigration step. These analyses showed that blocking CLEVER-1/stabilin-1 did not affect adhesion, but significantly reduced the transmigration of placental macrophages through the HUVECs. These data revealed a role for CLEVER-1/Stabiin-1 on placental macrophages as an adhesion molecule and its involvement in the trafficking of leukocytes.

5.3 Identification of CLEVER-1^{high} and CLEVER-1^{low} population on monocytes and their gene expression profiles (II)

Studies have reported stabilin-1 expression on M2 macrophages (166, 197, 278, 279), but the expression of CLEVER-1/stabilin-1 on normal monocytes has not been studied at the protein level. A study in hypercholesterolemia patients found that stabilin-1 was expressed on the monocytes of these patients, but was absent from normal monocytes (191). However, with our newly developed sensitive anti CLEVER-1/stabilin-1 antibody (9-11 F(ab)$_2$), I unexpectedly found CLEVER-1/stabilin-1 expression on the surface of monocytes. Therefore, I decided to analyze the expression of CLEVER-1/stabilin-1 in a heterogeneous monocyte populations by staining PBMCs for FACS analysis with the anti-CLEVER-1/stabilin-1, anti-HLA-DR, anti-CD14 and anti-CD16 antibodies, and then gated HLA-DR$^+$ cells to identify the three monocyte populations based on the expression of CD14 and CD16, as has been reported (161). These analyses revealed that CLEVER-1/stabilin-1 is expressed on CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ monocytes, but absent from CD14^{dim}CD16$^+$ monocytes. Moreover, CLEVER-1/stabilin-1 is absent from lymphocytes and granulocytes.

Since CLEVER-1/stabilin-1 is expressed on the surface of M2 macrophages, which are immunomodulatory, I studied the phenotype of CLEVER-1/stabilin-1 positive

monocytes and investigated whether the expression of CLEVER-1/stabilin-1 expression causes any change in the phenotype of monocytes. Therefore, I sorted $CD14^+$ monocytes into CLEVER-1/stabilin-1^{high} and CLEVER-1/stabilin-1^{low} positive cells using flow cytometry, and then analyzed the RNA of these populations by microarray, which revealed that there were many differentially expressed genes between these two populations. Further analyses of the data using IPA (Ingenuity Pathway analysis) revealed that many of the down-regulated genes in the CLEVER-1/stabilin-1^{high} population were involved in immune associated pathways and categories, and the top hits were mainly linked to the support of pro-inflammatory reactions. Moreover, a GESA (Gene Set Enrichment Analysis software) analysis in the CLEVER-1/stabilin-1^{low} population revealed significantly enriched gene sets (normalized enrichment scores (NES) with false discovery rates (FDR) <25%) for pro-inflammatory IL2/STAT5 and TNF/NFκB gene sets. Equally, the upstream regulator analysis of IPA using the differentially expressed genes in the CLEVER-1/stabilin-1^{high} population as an input dataset predicted that the prototype pro-inflammatory cytokine TNFα was inhibited in this population. Overall, these data suggest that the $CD14^+$ monocyte population with high CLEVER-1/stabilin-1 expression is less pro-inflammatory than monocyte population with low CLEVER-1/stabilin-1.

Furthermore, I wanted to study whether the pro-inflammatory potential of monocytes can be regulated by CLEVER-1/stabilin-1 itself. Therefore, I silenced CLEVER-1/stabilin-1 in monocytes using siRNA and analyzed the RNA of this population and a population treated with a negative control siRNA by RNA sequencing. Already on day 1 in CLEVER-1/stabilin-1 silenced monocytes many genes involved in the stimulation of the pro-inflammatory pathway were up-regulated compared to the monocytes transfected with the negative control siRNA. A further analysis of these differentially expressed genes in CLEVER-1/stabilin-1 silenced monocytes using IPA revealed that TNFα is involved in the regulation of many of these induced genes. Additionally, when the culture medium of CLEVER-1/stabilin-1 and control siRNA treated monocytes was analyzed by ELISA, the results indicated that CLEVER-1/stabilin-1 silenced monocytes secreted significantly more TNFα compared to monocytes treated with the control siRNA, which supported the prediction provided by the IPA. These data indicates that pro-inflammatory genes in human monocytes are directly or indirectly regulated by CLEVER-1/stabilin-1.

5.4 Functions of CLEVER-1 on monocytes (II)

CLEVER-1/stabilin-1 is expressed on the surface of normal monocytes and has an impact on controlling pro-inflammatory genes in monocytes and I decided to study the involvement of CLEVER-1/stabilin-1 in immune related functions. Therefore, I inves-

tigated the potential immune function of monocyte CLEVER-1/stabilin-1 in the activation of T cells and in the regulation of cytokine production.

I performed an antigen recall ELISPOT assay in which monocytes from individuals vaccinated against tetanus were sorted into CLEVER-1/stabilin-1^{high} and CLEVER-1/stabilin-1^{low} (by flow cytometry) sub-populations and were co-cultured with T cells isolated from the same individuals in the presence of the tetanus toxoid. In these assays T cells produced more IFNγ spots when co-cultured with CLEVER-1/stabilin-1^{low} monocytes compared to when they were co-cultured with CLEVER-1/stabilin-1^{high} monocytes. These results indicate that during antigen presentation CLEVER-1/stabilin-1^{high} monocytes diminish the Th1 cytokine profile. However, these assays did not reveal the direct involvement of CLEVER-1/stabilin-1 in dampening the Th1 cytokines. Therefore, the function of CLEVER-1/stabilin-1 during antigen presentation was analyzed using antigen recall assays.

In these assays, the direct effect of CLEVER-1/stabilin-1 function on diminishing the Th1 cytokine response was analyzed. Monocytes from individuals vaccinated against tetanus and treated with negative control siRNAs or siRNAs against CLEVER-1/stabilin-1 were co-cultured with T cells isolated from the same individuals in the presence of the tetanus toxoid. These assays revealed that the numbers of IFNγ spot forming T cells increased in the presence of CLEVER-1/stabilin-1 silenced cells when compared to co-cultures with monocytes treated with negative control siRNAs. Thus, low levels of CLEVER-1/stabilin-1 on monocytes favor high IFNγ production by T cells. CLEVER-1/stabilin-1 is involved either directly or indirectly in controlling IFNγ production in Th1 cells during antigen presentation, and thereby contributes to the regulation of the immune response. Further, I analyzed the influence of CLEVER-1/stabilin-1 on the production of the Th2 cytokine, but the tetanus toxoid recall assay did not lead to any countable numbers of Th2 cytokine forming spots. Hence, timothy grass extract was selected as a strong allergen for stimulating the production of Th2 type cytokines. To boost the sensitivity, both monocytes and autologous T cells were isolated from individuals with a known allergy against timothy. The results of the co-culture assays indicated that T cells produced fewer IL4 and IL5 spots in the presence of CLEVER-1/stabilin-1 silenced monocytes compared to those co-cultured with monocytes transfected with negative control siRNAs. Together, these data illustrate that monocyte CLEVER-1/stabilin-1 impairs Th1/pro-inflammatory immune responsiveness and favors Th2/immunosuppressive responses in humans.

Furthermore, it was studied whether CLEVER-1/stabilin-1 can be used as a potential target for the manipulation of immune responses. In these experiments PBMCs from tetanus-vaccinated persons were isolated and treated with anti-CLEVER-1/stabilin-1 antibodies and control antibodies. In these ELISPOT assays PBMCs produced high numbers of IFNγ spots when treated with anti-CLEVER-1/stabilin-1 antibodies, compared to PBMCs treated with negative (non-binding) control (3G6) or posi-

tive (binding) control (CD14) antibodies. Thus, the blocking of CLEVER-1/stabilin-1 with antibodies on monocytes shifts the antigen recall response to the Th1/pro-inflammatory direction.

The data from microarray and RNA seq experiments suggested that low expression of CLEVER-1/stabilin-1 on monocytes is associated with an upregulated pro-inflammatory transcriptome. Results from antigen recall assays showed that monocyte CLEVER-1/stabilin-1 dampens the production of the Th1/pro-inflammatory cytokine, IFNγ. Therefore, CLEVER-1/stabilin-1 expression on monocytes was examined after pro-inflammatory (M1) and an anti-inflammatory stimulus (M2) under *in vitro* culture conditions. Indeed, CLEVER-1/stabilin-1 expression on monocytes in pro-inflammatory conditions was significantly reduced, whereas in anti-inflammatory conditions no loss of expression was observed. Further, I wanted to investigate, whether the expression of CLEVER-1/stabilin-1 would be down-regulated under pro-inflammatory conditions *in vivo*, and whether there would be any change in the immunosuppressive condition. In this context, CLEVER-1/stabilin-1 expression on monocytes, placental macrophages and placental bed macrophages was analyzed by flow cytometry during normal pregnancy and in cases of pre-eclampsia. Strong immunosuppression and Th2 deviation are characteristic of normal pregnancy, whereas pre-eclampsia is a pro-inflammatory condition with abnormal placentation. The analyses showed that $CD14^+$ blood monocytes from pre-eclampsia patients expressed CLEVER-1/stabilin-1 significantly less when compared to those of normal pregnant women. Secondly, analyses of placental macrophages showed that CLEVER-1/stabilin-1 expression on $CD14^+$ macrophages from pre-eclampsia patients was significantly lower when compared to the situation in a normal pregnancy. Moreover, there were significantly fewer CLEVER-1/stabilin-1^+ placental bed macrophages in pre-eclampsia compared to placental bed samples derived from normal pregnancies. These data reveal that there is an association between CLEVER-1/stabilin-1 expression on monocytes and macrophages and the level of pro-inflammation and immunosuppression *in vivo*.

6 DISCUSSION

6.1 CLEVER-1 expression on monocytes/macrophages

This study elucidates the expression of CLEVER-1/stabilin-1 on placental macrophages and on normal monocyte subpopulation. The expression of CLEVER-1/stabilin-1 was reported on placental macrophages when it was identified as a MS-1 antigen on sinusoidal endothelial cells in the human spleen by Goerdt et al. in 1991 (186). However normal monocytes have been reported to lack stabilin-1 (191). Although the expression of CLEVER-1/stabilin-1 was reported in placental macrophages, it has not been clearly examined, which subpopulation of macrophages expresses CLEVER-1/stabilin-1, and the expression of CLEVER-1/stabilin-1 in a non-macrophage cell population in the placenta has remained an open question. The results of this thesis show that virtually all placental macrophages express CLEVER-1/stabilin-1 and most cells express it on their surface. This is important because the placenta is one of the few tissues, where all macrophages are positive for CLEVER-1/stabilin-1. For example, in cancer tissues, where type 2 macrophages contribute to the expansion of the tumor, not all type 2 macrophages are CLEVER-1/stabilin-1 positive (166, 199, 280). Moreover, the characterization of these CLEVER-1/stabilin-1 positive placental macrophages with different macrophage markers confirmed that these cells are type 2 macrophages. Furthermore, the multiplex cytokine analyses of the conditioned medium of macrophages showed that the silencing of CLEVER-1/stabilin-1 in macrophages increased the secretion of the pro-inflammatory cytokine TNFα, but also increased the secretion of IL10, an anti-inflammatory type 2 cytokine. This suggests that CLEVER-1/stabilin-1 is merely a type 2 macrophage marker and did not seem to alter macrophage polarization, at least by modulating cytokine expression. In addition, the expression of CLEVER-1/stabilin-1 on the placental macrophages of pre-eclampsia (a pro-inflammatory condition) was significantly lower compared to normal pregnancies. Similarly, the number of CLEVER-1/stabilin-1^{+} macrophages in the placental bed of pre-eclampsia was lower compared to those identified in normal pregnancy. Pre-eclampsia is a complex pathogenesis led by poor placentation, and second stage production of pro-inflammatory factors by the abnormal placenta, which subsequently results in the induction of a local systemic inflammatory response (222, 223). In normal pregnancy macrophages in the placental bed, along with other cell types, have been shown to contribute to the formation of proper spiral arteries, whereas their numbers were reduced in pre-eclampsia (281). This indicates that the surface expression of stabilin-1 on placental macrophages may be needed for key functions in the placenta.

This study demonstrates the expression of CLEVER-1/stabilin-1 on a heterogeneous population of normal monocyte surfaces. CLEVER-1/stabilin-1 was found to be expressed on monocyte surfaces in hypercholesterolemia patients, but it has been reported to be absent on normal monocytes (191). We detected CLEVER-1/stabilin-1 on the surface of normal monocytes with refined reagents and protocols to avoid the unspecific binding of Fc receptors. Further, the phenotypical analysis of a heterogeneous monocyte population showed that CLEVER-1/stabilin-1 is expressed on the surface of $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes, but not on $CD14^{dim}CD16^{+}$ cells. This is in line with the finding of the transcriptome of these three different monocyte population (161). Together, these data suggest that CLEVER-1/stabilin-1 may be functional in normal monocytes. Indeed, the microarray data from CLEVER-1/stabilin-1^{high} and CLEVER-1/stabilin-1^{low} monocytes revealed that they both have different transcriptomes, and implied that CLEVER-1/stabilin-1^{high} has a down-regulated pro-inflammatory transcriptome. Furthermore, the RNA sequencing data from CLEVER-1/stabilin-1 silenced monocytes verified that CLEVER-1/stabilin-1 is, directly or indirectly, involved in the upregulation of many pro-inflammatory genes, including oncostatin M and SAA2. These molecules play several roles, but they have been reported to share common key functions in regulating cytokine activity pathways, including the IFNγ pathway. Dendritic cells treated with oncostatin M have been shown to stimulate IFNγ secretion by T cells (282). Likewise, serum amyloid A (SAA) derived peptides have been reported to enhance the T cell inducing capability of antigen presenting cells (283), and induce the secretion of IFNγ by T cells in the human synovial fluid (284). Hence, the activity of these molecules in the regulation of cytokine pathways, including the IFNγ pathway may therefore be associated with the CLEVER-1/stabilin-1 dependent shift of polarization. Further, the upstream regulator analysis of IPA on differentially expressed genes in CLEVER-1/stabilin-1^{high} and CLEVER-1/stabilin-1 silenced monocytes predicted that TNFα could serve as a common up-stream regulator for several of these genes. Moreover, the ELISA analyses from the condition medium of CLEVER-1/stabilin-1 silenced monocytes indeed showed a higher concentration of the TNFα protein compared to the medium from negative control siRNA treated monocytes.

Additionally, the *in vitro* stimulation assays using blood monocytes and M1 and M2 cytokine conditions revealed that during the M1 stimulation the CLEVER-1/stabilin-1 expression is significantly down-regulated on monocytes, whereas the M2 stimulus not only prevented the loss of CLEVER-1/stabilin-1 on the surface, but also induced the intracellular levels of CLEVER-1/stabilin-1. Furthermore, the analyses on CLEVER-1/stabilin-1 expression on monocytes in pro-inflammatory and immunosuppressive *in vivo* conditions revealed that the expression of CLEVER-1/stabilin-1 is reduced on blood monocytes of pre-eclampsia (a pro-inflammatory condition with abnormal placentation) compared to the monocytes of normal pregnancy (a strong immunosuppressive condition).

Collectively, these results show that CLEVER-1/stabilin-1 is expressed by all placental macrophages, which are type 2 macrophages in phenotype, and that normal monocytes express CLEVER-1/stabilin-1 on their surface. Further, the gene expression analyses revealed that monocytes characterized by a high CLEVER-1/stabilin-1 expression have a transcriptome suggestive of a reduced pro-inflammatory potential. Further it suggests that CLEVER-1/stabilin-1 may directly or indirectly be involved in the regulation of these pro-inflammatory genes. Finally, CLEVER-1/stabilin-1 expression on monocytes and macrophages, and the number of CLEVER-1/stabilin-1$^+$ macrophages may contribute to the maintenance of an immunosuppressive state in normal pregnancy in humans.

6.2 New functions of CLEVER-1

Considering that CLEVER-1/stabilin-1 is known to be expressed on human placental macrophages, which are immune suppressive, and that CLEVER-1/stabilin-1 is expressed on normal monocytes with effects on regulating pro-inflammatory genes, my hypothesis was that CLEVER-1/stabilin-1 could be involved in immune regulation. Therefore, the immunological functions of CLEVER-1/stabilin-1 on human placental macrophages and on normal monocytes were investigated.

The flow cytometry data and immunofluorescence stainings demonstrated that CLEVER-1/stabilin-1 is expressed on human placental macrophages, which are type 2 (M2) macrophages. These M2 macrophages are known to play a role in immune modulations and to support anti-inflammatory functions in different pathophysiological conditions (34, 136, 184, 285). Furthermore, stabilin-1 on alternative macrophages has been shown to be involved in the uptake of placental lactogen, and stabilin-1 transfectants were reported to scavenge acLDL and to be involved in bacterial binding (172, 201, 205, 208). The flow cytometry data analyses revealed that placental macrophages treated with siRNAs against CLEVER-1/stabilin-1 indeed showed a decrease in the uptake of acLDL compared cells treated with control siRNAs. Further, the proteolytic processing of the model antigen DQ-OVA by CLEVER-1/stabilin-1 silenced cells was modestly, but significantly reduced compared to control siRNA treated cells. I assume that the reduced processing of DQ-OVA in CLEVER-1/stabilin-1 silenced cells is primarily due to a defective uptake of OVA that carries foreign modifications, rendering it susceptible to detection by scavenger receptors. Although, I succeeded in silencing the expression of CLEVER-1/stabilin-1 by 60% on human placental macrophages, the remaining 40 % can still play role in these processes. Thus, it is highly likely that in these assays the complete contribution of CLEVER-1/stabilin-1 remains underestimated. These findings suggest that placental CLEVER-1/stabilin-1 is a multifunctional scavenging receptor during pregnancy.

CLEVER-1/Stabilin-1 on endothelial cells was shown to mediate the transmigration of lymphocytes through blood and lymphatic vessels in both *in vitro* and *in vivo* experiments (194, 204). However, the role of CLEVER-1/stabilin-1 on leukocytes in adhesion or transmigration has not been studied. Further, many studies have reported monocyte recruitment to the placenta during pregnancy (211). I found that the functional blocking of CLEVER-1/stabilin-1 on placental macrophages reduced their interaction with/binding to the placental blood vessels. Furthermore, *in vitro* flow assays showed that CLEVER-1/stabilin-1 on placental macrophages is involved in the transmigration step of the adhesion cascade. Although the macrophages in the placenta are differentiated resident cells, these data suggest that CLEVER-1/stabilin-1 on the surface of blood monocytes in normal pregnancy may help in recruiting monocytes to the placenta and in maintaining the immunosuppressive condition in a pregnant woman.

Finally, I investigated CLEVER-1/stabilin-1 on normal monocytes. In ELISPOT antigen recall assays, T cells co-cultured with CLEVER-1/stabilin-1^{low} monocytes in the presence of the tetanus toxoid produced more IFNγ spots compared to those cultured with CLEVER-1/stabilin-1^{high} monocytes. Even more importantly, upon co-culture with CLEVER-1/stabilin-1 silenced monocytes in the presence of the tetanus toxoid the T cells produced more IFNγ spots compared to those cultured with monocytes treated with a negative control siRNA. In addition, CLEVER-1/stabilin-1 silenced monocytes supported the production of a lower number of IL4 and IL5 producing spots by T cells. Importantly, the functional blocking of CLEVER-1/stabilin-1 on PBMCs with mAb led to an increased number of IFNγ spots. These data suggest that CLEVER-1/stabilin-1^{low} monocytes are functionally different from CLEVER-1/stabilin-1^{high} monocytes, and that CLEVER-1/stabilin-1 itself either directly or indirectly regulates the capability of monocytes to polarize T cells into the Th1 vs Th2 direction upon an antigen challenge. Moreover, the ligation of CLEVER-1/stabilin-1 with antibodies can be therapeutically used to direct T cell polarization during an antigen challenge.

6.3 Potential therapeutic manipulation of CLEVER-1

In the following paragraphs I would like to speculate on the potential implications and future prospects of my studies.

CLEVER-1/stabilin-1 is expressed on M2 macrophages, tumor associated macrophages and sinusoidal endothelial cells, and CLEVER-1/stabilin-1 expression is known to be induced under inflammatory conditions (173, 196, 198). However, the existing monoclonal antibodies (mAbs) against human CLEVER-1/stabilin-1, like MS-1, 266 and 372 have certain limitations in many immunological aspects, such as in detecting

stabilin-1 expression in paraffin embedded tissues of pathophysiological conditions. Therefore, new anti-CLEVER-1/stabilin-1 antibodies, 2-7 and 9-11 were developed during these studies to extend the applications for analyzing CLEVER-1/stabilin-1.

Since the diminished expression of CLEVER-1/stabilin-1 on blood monocytes was found in pre-eclampsia, CLEVER-1/stabilin-1 may be used along with other biomarkers in research to diagnose the activation status of monocytes in pre-eclampsia studies. In addition, microarray analyses of CLEVER-1/stabilin-1^{high} and CLEVER-1/stabilin-1^{low} monocytes from normal healthy individuals revealed that many genes were differentially expressed between these two populations, and in particular, showed that CLEVER-1/stabilin-1^{high} monocytes carry a low pro-inflammatory gene signature. Moreover, the RNA sequencing analyses confirmed that the silencing of CLEVER-1stabilin-1 in normal monocytes directly or indirectly regulates pro-inflammatory genes. It might be useful to validate the gene hits of CLEVER-1/stabilin-1 silenced monocytes, by silencing or overexpressing these genes in normal monocytes to interpret whether these genes have any direct effects on the regulation of the expression and function of CLEVER-1/stabilin-1.

The level of immune suppression is one of the key concepts in the field of pathophysiological conditions, such as cancer, inflammatory diseases and autoimmune disorders. Our group has shown the expression of CLEVER-1/stabilin-1 in tumor associated macrophages in human cancer, and that blocking CLEVER-1/stabilin-1 reduces metastasis and tumor growth in mouse models (197, 198). My studies investigating the role of CLEVER-1/stabilin-1 on normal monocytes in antigen recall assays revealed that CLEVER-1/stabilin-1^{low} monocytes and CLEVER-1/stabilin-1 silenced monocytes increased the formation of IFNγ spots by T cells. IFNγ is a signature cytokine of Th1 (8). Moreover, the ligation of CLEVER-1/stabilin-1 on PBMCs with a function blocking anti-CLEVER-1/stabilin-1 antibody led to an increase in IFNγ synthesis. Hence, anti-CLEVER-1/stabilin-1 antibodies can be used to promote the Th1/pro-inflammatory response, which could be useful for targeting cancer induced immunosuppression.

However, it would be important to study the mechanisms, which CLEVER-1/stabilin-1 uses to regulate IFNγ production by T cells before considering anti-CLEVER-1/stabilin-1 antibodies for therapeutic purposes. This could be addressed by analyzing the signaling pathways and cytokines from the culture medium of CLEVER-1/stabilin-1 silenced monocytes or anti-CLEVER-1/stabilin-1 antibody treated cells in the presence or absence of antigens. Moreover, an additional option is to investigate the possible mechanisms of CLEVER-1/stabilin-1 in immune suppressive conditions more efficiently *in vivo*, using CLEVER-1/stabilin-1 knockout mice (which have been generated by our group) upon an antigen challenge and in tumor models.

7 SUMMARY

Immune response and immune suppression are the two fundamental mechanisms of the immune system which guard the host from infection and disease. When the host encounters an antigen or any foreign substance it defends itself using its immune system, known as an immune response. Soon after the elimination of pathogens, the immune system has to return to its normal state to protect normal healthy cells, which is attained by the process called immune suppression. Both these mechanisms are equally crucial for the immune system to perform its functions, which is to eliminate pathogens and to protect self-molecules. However, there are situations in which the immune system is compromised in performing both functions simultaneously. Pregnancy is one of these conditions, in which the maternal immune system remains resistant against pathogens and yet attains tolerance to protect the fetus, which has partially different genetic material. Immune cells, including monocytes and macrophages undergo functional characteristic changes to control immune suppression. However, if the immune suppression is not balanced properly, it can lead to different pathophysiological conditions, such as pre-eclampsia, cancer and autoimmune disorders.

In these studies the expression and functions of CLEVER-1/stabilin-1 (a multifunctional protein expressed on subsets of endothelial cells and type II macrophages) on human placental macrophages was elucidated. The results demonstrate that CLEVER-1/stabilin-1 is expressed on all human placental macrophages which are M2 type and the CLEVER-1/stabilin-1 on these cells functions as a scavenging molecule. Moreover, it plays a role in adhesion and transmigration. The difference in the expression of CLEVER-1/stabilin-1 on monocytes and placental macrophages of pre-eclampsia (pathophysiological condition) and normal pregnancy was investigated. The analyses revealed that CLEVER-1/stabilin-1 expression was strikingly reduced on monocytes and placental macrophages of pre-eclampsia in comparison to normal pregnancy.

Further the expression of CLEVER-1/stabilin-1 was investigated on the monocytes of healthy individuals, which was reported to be absent on normal monocytes (191). The results also revealed differential expression of CLEVER-1/stabilin-1 on heterogeneous monocyte populations. Moreover, the investigation of gene expression between CLEVER-1/stabilin-1^{low} and CLEVER-1/stabilin-1^{high} monocytes disclose that CLEVER-1/stabilin-1^{low} monocytes carry pro-inflammatory gene signature, and that the silencing of CLEVER-1/stabilin-1 directly or indirectly controls the pro-inflammatory genes. Finally, the immunological function of CLEVER-1/stabilin-1 on normal monocytes was elucidated by showing its importance in dampening the IFNγ production by Th1 cells.

In conclusion, these studies characterized the expression of CLEVER-1/stabilin-1 profiles on monocytes and human placental macrophages in normal pregnancy and pre-eclampsia. I have identified the potential immunological functions of CLEVER-1/stabilin-1 on normal monocytes. Collectively, these findings suggest that CLEVER-1/stabilin-1 is an immune suppressive molecule, which can be used to promote Th1 immune responsiveness and can act as a valid target for cancer therapy. Additional research has to be done to support this data, which might open a new arena in the field of tumor immunology.

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Senthil Palani.

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