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CHARACTERIZATION OF A NOVEL M-CSF INDUCED EFFECTOR MACROPHAGE

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Abbreviations

ADCC	Antibody dependent cell cytotoxicity
APC	Antigen presenting cell
CD	Cluster of differentiation
cDC	Classical spleen DC
CDP	Common DC precursor
cpm	Counts per minute
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
DNA	Desoxyribonucleinacid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent-activated cell sorter
FcγR	Receptor for the Fc fragment of IgG
FITC	Flouresceinisocyanat
FSC	Forward scanner
GM-CSF	Granulocyte-monocyte colony-stimulating factor
ICAM	Intercellular adhesion molecule
Ig	Immunoglobuline

IL	Interleukin
INF	Interferon
LPB	LPS binding protein
LPS	Lipopolysaccheride
MAC	Adherently differentiated macrophages
MAC + M-CSF	Adherently differentiated macrophages with extra M-CSF
M-CSF	Macrophage colony-stimulating factor
MDP	Macrophage and DC precursor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMAC	Non-adherently differentiated macrophages
MNC	Mononuclear cells
MPS	Mononuclear phagocyte system
ND	Not detected
NF	Nuclear factor
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid DC
PE	Phycoerythrin
PI	Propidium iodid
PMT	Photo multiplyer tube
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RES	Reticulo-endothelial system
SSC	Sideward scanner
$T_H1/2$	T helper cell type 1/2
TCR	T cell receptor

TLR	Toll like receptor
TNF	Tumor necrosis factor

1 Introduction

1.1 Innate and adaptive immune system

The purpose of this study was to characterize the viability and the differentiation of human peripheral monocytes cultured in suspension. To identify our novel cell type we have tested its capacity to perform some of the classical functions of monocytes, macrophages and dendritic cells (DCs). The diverse functions of monocytes and its more differentiated forms macrophages and DCs are integral to and interconnect the innate and adaptive immune systems. Below is a short review of the innate and adaptive immune system and how the effector functions of these cells contribute to resolve various immunological challenges.

1.1.1 Innate immune system

The immune system is based on the very ancient innate immune system that vertebrates share with invertebrates and plants. It relies on epithelial barriers, microbicidal environments and cell surface receptors for conserved pathogen-associated molecular patterns (PAMPs) (Gordon, 2002). This germ-line encoded pattern recognition receptors (PRRs) allow a perfect self/non-self discrimination, selected over evolutionary time (Taylor et al., 2005). The cells of the innate immune system survey the organism for pathologic antigen and have the power to decide, relying on their PRRs, whether or not to react to a certain stimulus (Janeway and Medzhitov, 2002). The inflammatory response of macrophages,

DCs, polymorphonuclear leukocytes, natural killer cells, and mast cells resolve the attack of pathogenic microbes through a wide variety of methods. But, as microbes have developed mechanisms to evade the innate immune system, the means of the innate immune response are not always sufficient to entirely clear the situation.

1.1.2 Adaptive immune system

Only vertebrates have developed an adaptive immune system in addition to the innate system. It consists of two lymphocytes species, the T and the B cells. The diversity of their receptors, T cell receptor (TCR) and antibodies, respectively, is the result of random receptor gene rearrangement and somatic hypermutation leading to myriad possible clonal T and B cells. As the random nature of this receptor generation cannot exclude self-reacting properties, the T cells undergo positive and negative selection in the thymus during ontogenesis.

1.1.3 Activation of the adaptive immune system

The adaptive immune system can only be activated by the innate immune system. The TCR and the major histocompatibility complex (MHC) molecules link the two apparently distinct systems. T cells cannot react to any native antigen; rather, the antigen has to be presented by a surface MHC molecule (Unanue, 2002). There are two classes of MHC molecules. Class I molecules are expressed on all nucleated cells and present fragments of peptides circulating in the cell cytoplasm and interact with cluster of differentiation (CD)8^+ T cells. Class II molecules are restricted to professional antigen presenting cells (APCs), including macrophages, DCs and B cells. These class II molecules present peptide fragments of phagocytized particles and interact with $CD4^+$ T cells. To activate the adaptive immune system, a peptide-bound MHC molecule must be

identified by a specific TCR. For T cell proliferation and to avoid T cell anergy, the co-stimulatory molecules CD80 and/or CD86 must be expressed on the very same cell as the MHC-peptide complex. In APCs this co-stimulatory molecules are up-regulated upon stimulation following PRR engagement (Taylor et al., 2005). So, the stimulation of the innate system precedes the activation of the adaptive immune system.

1.1.4 Effector functions of the adaptive immune system

The effector functions of the adaptive immune system are, with exception of the cytotoxic $CD8^+$ killer cells, mediated by the innate immune system. The link therefore are the $CD4^+$ cells, also called T helper cells, which are divided into two major groups, the type 1 (T_H1) and type 2 (T_H2) cells. A T_H1 cell can maximize the killing efficacy of a macrophage presenting the same MHC-peptide complex with which the T cell has been activated. They also induce the proliferation of $CD8^+$ killer cells. A T_H2 cell on the other hand can stimulate a B cell that recognized the antigen in question by its membrane bound antibody, inducing proliferation, antibody class switch and higher antibody production. This secreted specific antibody now enables enhanced phagocytosis of antibody-opsonized antigen and antibody dependent cell cytotoxicity (ADCC), both performed by macrophages.

The development of these cells is influenced in an autocrine/paracrine fashion mediated through the impact of numerous cytokines, chemokines and growth factors. The T_H1 cells develop under the influence of interferon- (INF)-γ, tumor necrosis factor- (TNF)-α, and interleukine (IL)-2. They, themselves, produce INF-γ, which stimulates DCs and macrophages to produce IL-12, which in turn up-regulates INF-γ production by T cells, resulting in a positive feedback loop. The T_H2 cells develop under the influence of IL-4, IL-5, IL-6, IL-10 and IL-13.

T_H2 cells produce IL-4, which is auto-regulatory, and IL-10, which inhibits INF-γ and IL-2 production by T cells and IL-12 production by DCs and macrophages. These positive feedback loops ensure progress in the direction, whether T_H1 or T_H2, which was initially chosen. The factors leading to this decision are not entirely clear. They depend on the phenotype of the activating APC and the factors present within the microenvironment.

1.1.5 DC and macrophage activation

Recognizing and subsequently binding an antigen are sufficient stimuli for the immature DC to maturate. The mature DC expresses high levels of MHC class II and costimulatory molecules, and migrates to the next lymph node. This anatomic architecture of the adaptive immune system offers the greatest possibility for the APC to meet a specific, naïve T cell. After a mature DC activates a T cell, the T cell needs no further activation signals.

For macrophage activation, on the other hand, three distinct activation modi have been described: the 'classical' macrophage activation, the 'alternative' macrophage activation and the more recently proposed 'type 2' macrophage activation (Edwards et al., 2006)

Classical macrophage activation The classical activation pathway is the longest known and by far best characterized (Adams and Hamilton, 1984; Gordon, 1998). It is a two-step process in which the macrophage is primed prior to being activated. For example, the macrophage is primed by INF-γ before it is exposed to TNF-α or a stimulus inducing TNF-α. A very potent stimulator of TNF-α secretion is lipopolysaccharide (LPS), a component of the cell wall of Gram-negativ bacteria (Morrison and Ryan, 1979). Although there are many variants of LPS, all of them have a constant region termed lipid A, which alone

can reproduce all the biological effects of LPS. This activation process takes three steps: first a secreted LPS binding protein (LPB) binds to LPS. This complex is recognized by membrane bound CD14 (Wright et al., 1990), which cannot be activated by lipid A alone (Tobias et al., 1999). Because CD14 does not have a cytoplasmatic domain, this complex must be delivered to a signal protein of the Toll like receptor (TLR) family as the last step. Experiments in mice have shown that deletion of the genes encoding for LPB or CD14 does only reduce the responsiveness to LPS. In contrast, mice without the TLR4 gene show a total loss of LPS reactivity (Poltorak et al., 1998). This indicates the great importance of this third step (Janeway and Medzhitov, 2002). This classical macrophage activation induces the production of reactive oxygen and nitrogen radicals, which enable the macrophage to kill intracellular microorganisms. Further, classically activated macrophages produce IL-12, directing the T cell response towards the T helper type 1 (T_H1) response.

Alternative macrophage activation About 17 years ago, Stein et al. (Stein et al., 1992) described a functionally and biochemically distinct activated macrophage phenotype resulting from stimulation with the T_H2 cytokine IL-4. This alternatively activated macrophage is characterized by the production of IL-10 and IL-1 receptor antagonist, and low antigen presenting capacity towards T cells (Gordon, 2003). Furthermore, production of matrix-associated proteins and promotion of fibrogenesis by these cells was suggested to be possible contributing factors in wound healing and tissue repair (Albina et al., 1990; Song et al., 2000). In the mouse model, it could be shown that the differentiation of monocytes into fibrocytes is regulated by $CD4^+$ T cells (Niedermeier et al., 2009). This phenotype change in alternative macrophage activation is epigenetically regulated (Ishii et al., 2009).

Type 2 macrophage activation A third type of activated macrophage population can be generated by stimulation of macrophages with classical activation signals in the presence of immunoglobuline (Ig)G containing immune complexes (Sutterwala et al., 1998). TLR as well as CD40 activation after ligation of receptors for the Fc fragment of IgG (FcγR) resulted in suppression of IL-12 production and a strong induction of IL-10 (Mosser, 2003). This turns these type 2 activated macrophages into potent anti-inflammatory cells (Gerber and Mosser, 2001). They also stimulate the production of T_H2 cells, which produce high levels of IL-4 (Anderson and Mosser, 2002).

Not activated macrophages Macrophages very important in the removal of apoptotic cells but do not activate T cells that recognize apoptotic body- associated antigens (Henson and Hume, 2006). It has been shown that macrophages, in contrast to DCs, mediate anergy in T cells. This results suggest that, during non-inflammatory conditions, macrophages which phagocytose mainly self-peptides, mediate anergy in T cells that recognize the self-peptides presented on a MHC class II molecules, thus helping in maintaining peripheral tolerance (Hoves et al., 2006). Brem-Exner et al. (Brem-Exner et al., 2008) identified INF-γ-stimulated monocyte derived cells in the mouse model which have certain T cell-supressive effects with potential therapeutic benefit in the treatment of autoimmune inflammations.

1.2 The macrophage: origin and differentiation

Elie Mechnikoff described more than 100 years ago a large mononuclear phagocytic cell and named it 'macrophage' (Karnovsky, 1981). After the discovery of different phagocytic cells in various tissues of the body, the idea of a reticuloendothelal system (RES) was postulated in 1924 by Aschoff (Aschoff, 1924).

Because the RES contained cells not belonging to the mononuclear lineage, van Furth proposed the mononuclear phagocyte system (MPS) (van Furth, 1982). The MPS comprises myeloid progenitor cells that differentiate into blood monocytes, which circulate and extravasate to become tissue macrophages. The different macrophage subtypes in various tissues are listed in table 1.1. In the classical sense, cell divisions in this lineage occur in the monoblast and promonocyte stages and the pool of macrophages in the periphery is renewed by circulating monocytes rather than by local cell division. This paradigm has been challenged as recent findings show at least a small percentage of cell renewal by local cell division under steady state conditions (Tacke and Randolph, 2006) and evidence exists, that M-CSF can induce proliferation in monocytes (Clanchy et al., 2006). Recently, several authors proposed, that the model of the MPS might have outlived its usefulness, as there might be as many different macrophage subtypes as markers applied for their description (Hume, 2006, 2008). They claim that beside differentiated DC and osteoclasts, all macrophages can change as a consequence of their microenviroment, continuosly adapting their functional pattern in response to the progressive inflammatory response (Stout and Suttles, 2004; Stout et al., 2005, 2009).

The origin of the DCs and the connections to the monocyte lineage are still not entirely clear (Ardavin et al., 2001; Leon et al., 2005; Fogg et al., 2006). There is evidence that DCs can be generated *in vitro* from both, myeloid and lymphoid progenitors, but it's not sure, whether there are physiologic counterparts for this *in vitro* generated subtypes. A common precursor for macrophage and DC was proclaimed by Fogg et al. (Fogg et al., 2006). If there are distinct myeloid and lymphoid DC subsets or if there is just a continuum between two extremes is still point of discussion (Hume et al., 2002; Hume, 2006, 2008).

Tissue	Cell
Bone marrow	Monoblasts
	Promonocytes
	Monocytes
	Macrophages
Peripheral blood	Monocytes
Liver	Kupffer cells
Lung	Alveolar macrophages
Connective tissue	Histiocytes
Spleen	Red Pulp Macrophages
Lymph node	Macrophages
Thymus	Macrophages
Bone	Osteoclasts
Synovium	Type A Cells
Mucosa-associatied lymphoid tissue	Macrophages
Gastrointestinal tract	Macrophages
Central nervous system	Microglia
Skin	Histiocytes/ Langerhans cells
Serous cavities	Pleura/Peritoneal macrophages
Inflammatory tissues	Epitheloid cells
	Exudative macrophages
Granuloma	Multinucleated giant cells

Table 1.1: Mononuclear phagocytes in different tissues, adapted from (Ross and Auger, 2002)

1.2.1 Differentiation from stem cell to monocyte

The origin of the blood monocyte is a pluripotent stem cell in the bone marrow, dependent on stem cell factor (or c-Kit ligand). The next step in differentiation is the common myeloid progenitor for erythrocytes, neutrophiles and monocytes, followed by the granulocyte-monocyte colony-forming unit, which can still differentiate into neutrophiles and monocytes. Under the influence of granulocyte-monocyte colony-stimulating factor (GM-CSF) and macrophage CSF (M-CSF) the monoblast differentiates past the premonocyte point to monocytes, the first cell population that enters the blood.

1.2.2 Monocyte classification

Blood monocytes are not a homogeneous population, but show at least two distinct subtypes, a $CD14^{high}$ $CD16^{-}$ 'inflammatory' monocyte and a $CD14^{low}$ $CD16^{+}$ 'resident' monocyte (Passlick et al., 1989; Gordon and Taylor, 2005). The minor $CD16^{+}$ monocyte subgroup seems to contain an 'intermediate' $CD14^{+}$ $CD16^{+}$ $CD64^{+}$ monocyte subgroup (Grage-Griebenow et al., 2001b), which has a high phagocytic activity and produces large amounts of cytokines comparable to the 'classical' monocytes, but show high expression of CD86 and HLA-DR and a stimulatory activity in mixed leukocyte reactions comparable to DCs (Grage-Griebenow et al., 2001a). Both human monocyte subtypes can differentiate *in vitro* in the presence of GM-CSF and IL-4 into DCs (Sanchez-Torres et al., 2001). In a mouse model of transendothelial trafficking the $CD14^{+}$ $CD16^{+}$ monocyte subset differentiated to DC more likely than the $CD14^{high}$ $CD16^{-}$ monocyte subset (Randolph et al., 1998). The transcriptional profile of the $CD16^{+}$ monocytes suggests a more advanced stage of differentiation compared to $CD16^{-}$ monocytes (Ancuta et al., 2009). The physiological role of this monocyte subtypes remains unclear, as the performed *in vitro* experiments do not allow a direct transfer to the *in vivo* situation.

The identification of mouse counterparts of the human monocyte subsets have contributed to the understanding of their function *in vivo*. Geissmann et al. (Geissmann et al., 2003) have described two monocyte subsets with distinct homing potential in the mouse. The mouse Gr-1^{+} $CCR2^{+}$ CX_3 $CR1^{lo}$ monocyte subset, correspond to the human $CD14^{high}$ $CD16^{-}$ monocyte subset, and a Gr-1^{-} $CCR2^{-}$ CX_3 $CR1^{hi}$ monocyte subset, correspond to the human $CD14^{lo}$ $CD16^{+}$ monocyte subset. They showed, that the Gr-1^{+} CX_3 $CR1^{lo}$ subset is recruited preferentially to inflammatory sites, and that the Gr-1^{-} CX_3 $CR1^{hi}$ subset serves as a precursor of resident myeloid cells. Sunderkötter et

al. (Sunderkotter et al., 2004) have claimed that the different monocyte subsets in mouse are developmentally connected. They hypothesized that monocytes leave the bone marrow as Gr-1^{hi} cells and mature over an intermediate Gr-1^{med} stage to the Gr-1^{lo} monocytes. They showed that after complete depletion only Gr-1^{hi} monocytes repopulate the blood, and Gr-1^{lo} monocytes are seen on day 7 first. They confirmed the findings of Geissmann et al. (Geissmann et al., 2003), in that Gr-1^{hi} monocytes are recruited preferentially to inflammatory sites, and showed furthermore that during inflammation Gr-1^{hi} monocytes are released into the blood, analogous to the left shift seen also with granulocytes. Ginhoux et al. (Ginhoux et al., 2006) further showed that Gr-1^{hi} monocytes could give rise to Langerhans cells (LCs) and macrophages in inflamed skin *in vivo*, confirming the *in vitro* findings that monocytes can differentiate into both, macrophages and DCs, at least under inflammatory conditions. In the lung the two distinct monocyte populations Gr-1^{hi} and Gr-1^{lo} give raise to two distinct tissue DC populations (Jakubzick et al., 2008). Using this mouse model, Liu et al. (Liu et al., 2009) depict a procursor-progeny relationship of monocytes, classical spleen (c)DCs and plasmacytoid (p)DCs. Starting with a myeloid progenitor that gives rise to macrophage and DC progenitors (MDPs) which can differentiate to monocytes or common DC progenitors (CDPs). This CDPs generate pre-cDCs and pDCs. They point out that monocytes do not develop into cDCs and contribute little to lymphoid-organ DC network in steady state conditions. They define the point of divergence between multipotential precursors and pre-cDCs in the bone marrow, from where the latter migrate through the blood to lymphoid tissues, where they divide and fill the DC compartment. For the DC network in the lamina propria of the gut this findings lead to the idea of a dual origin of DCs in the steady state. It consists of DCs derived from monocytes and pre-cDCs. The DCs that arise from pre-cDCs are addressed as

the key sentinels of the gut immune system (Bogunovic et al., 2009; Varol et al., 2009).

Reports that the human $CD14^{lo}$ $CD16^{+}$ monocyte subset is elevated in sepsis (Skinner et al., 2005), rheumatoid arthritis (Kawanaka et al., 2002b) chronic renal failure (Kawanaka et al., 2002a; Saionji and Ohsaka, 2001), cancer (Saleh et al., 1995), tuberculosis and HIV infection (Grage-Griebenow et al., 2001a) lead to the idea that this subtype is 'inflammatory'. Taken into account that the mouse counterparts, the Gr-1^{lo} monocytes do not migrate to sites of inflammation *in vivo* (Geissmann et al., 2003; Sunderkotter et al., 2004), this term has to be re-evaluated.

1.2.3 Experimental models of monocyte differentiation

Since macrophages and DCs are difficultly to obtain from human donors in the required amounts, these cells have to be generated *in vitro* for further analyses. Several *in vitro* systems to differentiate them from blood monocytes have been developed. When the first researchers started to work with human monocytes, they soon realized that adhesion is a very important keystone for monocyte survival and differentiation, but still not sufficient without human serum (Becker et al., 1987). Investigators then tried to define the biochemical factors present in human serum that lead to monocyte maturation. Many chemicals have been identified, either by using purified or recombinant proteins, or by inhibition of known molecules with specific monoclonal inhibitory antibodies. It could, for example, be shown that M-CSF is a critical factor for monocyte survival as the treatment of adherent cultures with human serum in the presence of anti-M-CSF antibodies inhibited monocyte maturation (Andreesen et al., 1990; Brugger et al., 1991). On the other hand, M-CSF alone is not a sufficient replacement for human serum. Recently Way et al. (Way et al., 2009) described two serum-free

M-CSF containing cytokine cocktails to generate monocytes and macrophages from human $CD34^+$ hemopoietic stem cells.

1.2.4 The impact of monocyte adhesion

Before a blood monocyte becomes a differentiated macrophage, it leaves the bone marrow and circulates in the blood, adheres to the endothelium of a blood vessel and transmigrates into the surrounding tissue. This involves an initial selectin-glycoprotein interaction, resulting in monocyte rolling. Then, the monocyte integrins have to be activated by chemokines, allowing a firm integrin-protein adhesion. Following cell polarisation, the monocyte can migrate by diapedesis between the epithelial cells into the subendothelial extracellular matrix (ECM) (Imhof and Aurrand-Lions, 2004). Although there are many chemokines known to induce leucocyte adhesion under inflammatory conditions (Cravens and Lipsky, 2002), little is known about the constitutively expressed chemokines leading to monocyte adhesion in non-inflammatory conditions (Imhof and Aurrand-Lions, 2004).

The recent findings about different monocyte sub-populations in mice suggest an inflammatory and a non-inflammatory monocyte subset, and confirm the idea that macrophages in the periphery are replenished by circulating blood monocytes (see 1.2.2). The origins of the many diverse forms of macrophages seen in various tissues are not mature forms of different monocyte sub-populations but rather multiple phenotypic manifestations of monocytes reflecting tissue-specific ECM and the local biochemical microenvironment. Once a monocyte enters a tissue, it soon becomes indistinguishable from the resident macrophages (Hume, 2006).

So, the cell-cell and cell-matrix contacts can be suspected to have an impact on monocyte differentiation. Integrin ligation causes an "outside-in" signal

involving many transcription factors controlling complex functions like cell polarisation and migration. Some of these transcription factors induced by integrin engagement are also involved in monocyte survival and differentiation (Shi and Simon, 2006). In vitro experiments using various ECM molecules showed that fibronectin seems to induce stronger monocyte to macrophage differentiation than collagen I or collagen IV (Jacob et al., 2002).

Recent results from this group have clearly demonstrated that monocyte adhesion could inhibit apoptosis. This is most likely due to adhesion-induced secretion of M-CSF, which acts subsequently in a autocrine manner (Mondal et. al, unpublished data). Inhibition of apoptosis following monocyte transmigration through epithelia was confirmed by Williams et al. (Williams et al., 2009). Similar results were found for polymorphonuclear leukocytes, where transmigration inhibited apoptosis (Hu et al., 2004).

Monocyte adhesion and subsequent integrin engagement results in "outside-in" integrin signaling, causing tyrosine phosphorylation of certain intracellular proteins, namely ERK, p38 and JNK. This results in rapidly activated transcription, stabilization of the produced mRNA and organization of the cytoskeleton (Mondal et al., 2000). To what extent this tyrosine phosphorylation cascade is affecting the monocyte differentiation is incompletely defined. Recently, Shi et al. (Shi and Simon, 2006) pointed out the role of the forkhead transcription factor Foxp1 in monocyte differentiation. On the other hand, Himes et al. (Himes et al., 2006) displayed the interaction of the JNK and M-CSF, thus indicating a synergic effect of both, M-CSF and adhesion, on monocyte differentiation. M-CSF in this context is also known to promote macrophage motility in addition to adhesion to extracellular matrix (Rovida et al., 2005).

The firm adhesion to plastic could prevent monocytes in serum free cultures from cell death, through the production of autocrine survival factors like M-

CSF and TNF-α (Haskill et al., 1988). The not-so-firmly-adherent moncytes on Teflon foils could be rescued from apoptosis in serum free medium only with additional M-CSF. Under both conditions and similar on other surface coatings, monocyte survival was never accompanied with differentiation under serum free conditions (Andreesen et al., 1990).

1.2.5 The role of M-CSF and its receptor

Since the first description of M-CSF and its receptor many effort has been made to elucidate the biochemical structures and the intra-cellular signal cascades that in the end lead to morphologic changes in the monocyte.

M-CSF is a disulfide-linked homodimer and is expressed by many cells in three isoforms: secreted glycopeptide and peptidoglycan that are synthesized by endothelial cells and enter the circulation, and a membrane bound cell-surface glycopeptide which is involved in local regulation. The secreted peptidoglycan can also act locally by binding to extracellular matrices (Chitu and Stanley, 2006). Macrophages derived from monocytes after 5 days in culture constitutively produce M-CSF (about 15 ng/ml) for at least 28 day (Scheibenbogen and Andreesen, 1991).

The M-CSF receptor seems to be restricted to monocytes and their precursors, macrophages, DCs and osteoclasts and the female reproductive tract (Sasmono et al., 2003). The M-CSF receptor is a receptor tyrosin kinase and closely related to the c-Kit receptor. Binding of M-CSF to its receptor results in non-covalent dimerization of the M-CSF receptor, activation of its kinase and a first wave of M-CSF receptor tyrosine phosphorylation. After covalent dimerization and a second wave of tyrosine phosphorylation, affecting about 0.02 % of the total cellular protein, the ligand-receptor complex gets ubiquitinated and lysosomal degraded (Stanley et al., 1997; Pixley and Stanley, 2004).

In vitro results

In early experiments to generate macrophages *in vitro*, M-CSF, at least in physiological serum concentrations, was found to be obligatory for monocyte survival and differentiation, as monocytes die in serum free media or if M-CSF was depleted from serum by neutralizing antibodies (Becker et al., 1987; Andreesen et al., 1990; Brugger et al., 1991). Macrophages need continuos M-CSF, as the removal of this cytokine results in apoptosis (Komuro et al., 2005). Several factors related to monocyte or macrophage survival, for example adhesion (Mondal, unpublished data) induce autocrine M-CSF production, but also M-CSF itself induces the production of survival and differentiation factors (Komuro et al., 2005). M-CSF could also induce proliferation in a monocyte subset (Finnin et al., 1999).

In vivo results

Many insights to the *in vivo* function of M-CSF were gained using a osteopetrotic mouse model, which is deficient for M-CSF ($Csf1^{op/op}$). These mice show normal blood monocyte counts but a reduced number of almost all tissue macrophages populations, which could be restored by daily intracutaneous M-CSF injection (Stanley et al., 1997; Chitu and Stanley, 2006) or by using M-CSF transgenes for the different isoforms (Ryan et al., 2001; Dai et al., 2004; Nandi et al., 2006). In contrast, $Csf1^{op/op}$ mice have fully differentiated DCs and LCs, which lead to the hypothesis that these cells develop independently from mononuclear phagocytes (Wiktor-Jedrzejczak and Gordon, 1996).

However, using a mouse model deficient for the M-CSF receptor ($Csf1r^{-/-}$), Ginhoux (Ginhoux et al., 2006) showed, that LCs development in the epidermis is dependent on M-CSF and that under inflammatory conditions LCs can arise form monocytes *in vivo*. Furthermore, MacDonald (MacDonald et al.,

2005) showed that the M-CSF receptor is upregulated in all DC subsets during differentiation, supporting the idea that most tissue DCs are of myeloid origin. Recently could be shown that the DC favoring cytokine GM-CSF and IL-4 in combination up-regulate the TNF-α converting enzyme expression and activity, causing ectodomain shedding of the membrane-bound M-CSF receptor and thereby directing the monocyte towards a DC like phenotype (Hiasa et al., 2009).

M-CSF in diseases

Blood levels of M-CSF are elevated in several diseases. High levels were found in patients undergoing dialysis (Saionji and Ohsaka, 2001) and during acute renal allograft rejection in human (Le Meur et al., 2004) and mouse (Le Meur et al., 2002). The latter proposed using M-CSF as a specific marker of acute allograft rejection. M-CSF seems also to be elevated in severely ill trauma patients, where it possibly impairs the immunological defense (De et al., 2003). In cancer, especially of the female reproductive tract, increased circulating M-CSF can be found (Kacinski, 1997). In this and other neoplasias, so called tumor-associated macrophages seem to have a two edged role: promotion of tumor invasion via macrophage recruitment by the secreted M-CSF versus the stimulation of direct killing and antigen-processing by the cell-surface M-CSF (Chitu and Stanley, 2006). This offers also therapeutic options, as M-CSF blockade by antisense oligonucleotides and small interfering RNAs can suppress the growth of human mammary tumor xenografts in mice (Aharinejad et al., 2004). In rheumatoid arthritis, where macrophages play an important role (Hamilton and Tak, 2009) elevated levels of M-CSF are correlated with an increased number of $CD14^{lo}CD16^{+}$ monocytes (see above), thus indicating that M-CSF may contribute to this more mature monocyte subset in the blood (Kawanaka et al.,

2002b). Monocytes of severely injured trauma patients do not differentiate into DC under appropriate stimuli as a sign of previous differentiation into a more macrophage-like cell type caused by enhanced M-CSF responsiveness (De et al., 2003). M-CSF release by endothelial vascular cells is up-regulated by C reactive protein inducing macrophage proliferation causing atherosclerosis (Devaraj et al., 2009; Irvine et al., 2009). Several therapeutic strategies targeting M-CSF are now in preclinical studies or phase I clinical trials (Hamilton, 2008).

2 Prework and aims

Various ways have been described to cultivate and differentiate human peripheral blood monocytes, for example into macrophages or DCs. All of them share an adherent culture condition. As cells of the monocytic lineage exist also in body fluids (see table 1.1), differentiation may occur physiologically also under non-adherent conditions. Although a lot of research has been published concerning the intracellular changes caused by monocyte adhesion, up to now little is known about the impact of adhesion on monocyte differentiation. To approach this, we used an experimental setting developed by members of this group and first published by Arndt (Arndt et al., 2007) to culture monocytes non-adherently. It could be shown that monocytes undergo apoptosis within 3 days if culturing them in a continuously rotating culture system that inhibits cell adhesion. This apoptosis could be inhibited by a one-time addition of exogenous M-CSF at the beginning of the culture period. As adhesion is inducting the secretion of M-CSF, this led to the hypothesis that adhesion inhibits apoptosis through the autocrine action of M-CSF (Mondal et al., unpublished data).

First aim of this study was to quantify the survival rate of this M-CSF induced non-adherently cultured monocytes (MMAC) and to compare it to that of adherently generated macrophages (MAC). Second aim was to validate whether these MMAC had differentiated. To this end, their phenotype was characterized in detail using monoclonal antibodies and flow cytometry. Their functional

properties were determined in terms of phagocytosis, antigen presentation and cytokine production. The results were compared to those of MAC and immature monocyte derived dendritic cells (DC). To determine the influence of M-CSF, they were also compared to monocytes that were cultivated adherently with additional M-CSF (MAC + M-CSF). The experiments were performed under donor matched conditions, in that all DC, MMAC, MAC + M-CSF and MACs were generated in parallel from freshly isolated monocytes from the same donor to minimize donor dependent variation of survivability or differentiation characteristics.

3 Material and methods

3.1 Basic techniques

3.1.1 Media and solutions

The compositions of the frequently used media and solutions are provided in the appendix, table 7.1. A list of all companies is also given there in table 7.2.

3.1.2 Sterile protocols

All work with cell cultures and functional analyses were carried out under sterile conditions, using a laminar air flow work bench (Hareus) and sterile single use pipettes. If not otherwise declared, cell centrifugation was performed at 300 g using a table centrifuge (Hareus) and centrifuge tubes (Falcon) with 15 ml or 50 ml. Strictly sterile protocols were used to prevent any nonspecific activation of the cells.

3.1.3 Cell culture conditions

All cells for long term culturing and for short term functional analyses were incubated in an incubator (Hareus) at a constant temperature of 37°C, with a 95 % humidity and 5% CO_2 concentration.

3.1.4 Viable cell count using trypan blue

To determine the number of viable and dead cells, the vital dye trypan blue was used. Trypan blue is actively excluded by living cells and stains dead cells with damaged membranes blue. For this, 50 µl of cell suspension were added to 50 µl of trypan blue solution (table 7.1). Cells were counted under a microscope (Axiovert 25, Zeiss) at 100× magnification, using a Neubauer counting chamber as hemacytometer. For accuracy all four 1 mm^2 quadrants were counted and the cell number was calculated according to the following formula:

$$Cellcount = \frac{N}{4} \cdot D \cdot 10^4\,\mathrm{ml}^{-1}$$

N = cell number, D = dilution factor

3.2 Monocyte isolation through counter-current elutriation

Leukocyte enriched blood was obtained via leukapheresis (Graw et al., 1971) form healthy volunteers. Mononuclear cells (MNC) were isolated from this leukapheresis concentrates by density gradient centrifugation over Ficoll-Hypaque (Pharmacia) (Johnson et al., 1977). Monocytes were separated from MNC by counter-current elutriation (Sanderson et al., 1977) in a J6M-E centrifuge (Beckman) with a 50 ml chamber and a JE-5 rotor. Initially, the centrifuge was sterilized with 6% H_2O_2 in H_2O for 20 minutes and washed two times with PBS (PAN).

The variable pump was calibrated with Hanks' balanced salt solution (PAN) at constant 1100 g and 4°C. MNCs were given into the system at a flow rate

fraction	flow rate (ml/min)	main cells in the fraction
1a	52	thrombocytes
1b	57	small lymphocytes
2a	64	intermediate lymphocytes
2b	74	intermediate lymphocytes
2c	92	big lymphocytes
3	111	monocytes

Table 3.1: Elutriation parameters and corresponding cell fractions

of 52 ml/min, followed by continuous infusion of Hanks' solution supplemented with 6 % autologous plasma. The flow rate was increased step by step following the scheme displayed in table 3.2 and monocytes as the largest cells in the MNC fraction were collected in the last fraction 3. These elutriated monocytes were more than 80% pure as determined by morphology and by the expression of the CD14 antigen (table 3.2), measured by flow cytometry (3.5).

3.3 Monocyte culture conditions

Because monocytes show a great donor dependent variability, MMAC, MAC, DC and MAC + M-CSF were prepared in parallel cultures from each donor.

3.3.1 Classical monocyte derived macrophages (MAC)

Monocytes were cultured at a cell density of $1{\cdot}10^6$ cells/ml in hydrophobic Teflon bags for 7 days in macrophage media (table 7.1) (Andreesen et al., 1983). To harvest the adherent macrophages from the Teflon bags, they were cooled for 15 minutes at 4°C and then loosened by gently tapping the cells off the Teflon while leaving the plasma membrane intact.

3.3.2 Monocyte derived dendritic cells (DC)

Monocytes were cultured at a cell density of $1{\cdot}10^6$ cells/ml in vented flasks (Costar) for 7 days in DC media (table 7.1) with 6 pg/ml IL-4 (Immunotools) and 50 pg/ml GM-CSF (Essex). Only the non-adherent cells were harvested (Sallusto and Lanzavecchia, 1994).

3.3.3 Non-adherently cultured monocytes (MMAC)

In order to establish an *in vitro* system to culture monocytes under absolute non-adherent conditions, we used a system described by Arndt et al. (Arndt et al., 2007) in which monocytes were cultured in a continuously rotating (12 U/min) flask (250 ml centrifuge tubes, Falcon) with a vented cab (Costar), using a Universal Turning Device (Greiner Bio-one). The cells were seeded at a density of $1{\cdot}10^6$ cells/ml in macrophage medium (table 7.1) supplemented with 100 ng/ml recombinant human M-CSF (Cetus) and cultured for 7 days. For further processing, these cells had to be handled on ice to avoid adhesion to the plastic containers.

3.3.4 Adherently cultured monocytes with additional M-CSF (MAC + M-CSF)

Monocytes were cultured and harvested according to the same protocol as for MAC (see 3.3.1) with additional 100 ng/ml recombinant human M-CSF (Cetus) analogous to MMAC (see 3.3.3.

3.4 Light microscopy and photographs

For photographs, unstained cells were prepared seperately. To demonstrate cell morphology under non-adherent conditions, pictures of MMAC, MAC and MAC

+ M-CSF were taken immediately after harvesting, by suspending large droplets of cells on a small Teflon foil to prevent cell adhesion. Pictures of MAC and MAC + M-CSF were also taken adherent on Teflon prior to harvesting. Light-microscopic examinations were done using an Axiovert 25 microscope (Zeiss) and a Finepix S1 pro camera (Fujifilm) for photographs. The photographs were edited for printing with the freely available software "The GIMP".

3.5 Flow cytometry

Antigen	**Label**	**Isotype**	**Clone**	**Company**
primary antibodies (all from mouse):				
Isotype	FITC	IgG1	X40	BD Biosciences
Isotype	FITC	IgG		Beckman Coulter
Isotype	PE	IgG1	X40	BD Biosciences
Isotype	PE	$IgG2_b$	MPC-11	BD Biosciences
Isotype	none	IgG1		
CD 1a	PE	IgG1	BL6	Beckman Coulter
CD 14	FITC	$IgG2_a$	Mμ4	Beckman Coulter
CD 16	FITC	$IgG2_a$	5D2	PeliCluster
CD 40	PE	IgG1	5C3	BD Pharmingen
CD 71	FITC	IgG1	YDL.1.2.2	Immunotech
CD 80	PE	IgG1	L307.4	BD Pharmingen
CD 84	PE	IgG1	2G7	BD Pharmingen
CD 86	FITC	IgG1	2331 (FUN-1)	BD Pharmingen
HLA-DR	FITC	IgG1	B-F1	Diaclone
HLA-ABC	PE	IgG1	G46-2.6	BD Pharmingen
DC-SIGN	PE	$IgG2_b$	DCN46	BD Pharmingen
MAX.11	none	IgG1		own lab (Rehli et al., 1995)
secondary antibody (from goat):				
anti-mouse	FITC	IgG+M		Jackson ImmunoResearch

Table 3.2: Antibodies used for flow cytometry. Conjugated fluorochromes: flouresceinisocyanat (FITC) or phycoerythrin (PE).

The phenotype of the cells was determined by flow cytometry using a *fluorescent-activated cell sorter* (FACS). Flow cytometry allows the measurement of the

fluorescence of a single cell or particle, in contrast to related techniques like spectrometry, where only the absorption and transmission of the whole sample can be determined. In short, a stream of fluid containing cells flows through the machine such that a single cell at a time passes by a light source. Here the cells intercept with light of a certain wavelenght, usually from a laser source. As the beam hits a cell, only a part of the light passes unaffected, which is detected as forward scatter (FSC) and corresponds to the size of the cell. The part that is reflected 90° off is detected as sideward scatter (SSC) and corresponds to cell's complexity and granularity. If the cell or particle has fluorescent properties, the light beam can excite these fluorochrome molecules to emit photons of the wavelength characteristic for the fluorochrome. For example, fluorescein isocyanat (FITC) emits light between 500-550 nm wavelength and phycoerythrin (PE) emits from 550-650 nm wavelenght. By using detectors with different wavelength filters, fluorochromes with different wavelenghts can be detected simultaneously. The sensitivity of the detectors, which most commonly are photo multiplier tubes (PMT), can be adjusted by changing their voltage. The data of many events (usually 20,000) is stored in a computer and can be visualized logarithmically as histogram or dot plot. To compare the results of different experiments, the mean fluorescent intensity (MFI) is calculated in every histogram.

To prepare the cells for flow cytometry, they were centrifuged at 300 g for 5 minutes at 4°C and washed 2 times with FACS washing buffer (table 7.1). The human immunoglobulins present in the FACS washing buffer serves to block the cells' endogenous Fc-receptors and thus to minimize the non-specific binding of the subsequently used antibodies. After the first washing step, the cells were resuspended to $5{\cdot}10^5$ cells/ml and about $2.5{\cdot}10^5$ cells each were evenly distributed to 5 ml polystyrene tubes (Falcon) for FACS analysis. After another centrifuga-

tion, the supernatant was decanted and the pellet loosened. The cells were then incubated with saturating amounts of specific antibody or an isotype control (see table 3.2) for 30 min at 4°C in the dark, followed by two washing steps with FACS washing buffer. In case of an un-conjugated antibody, the cells were incubated with 50 µl secondary antibody (FITC conjugated goat-anti-mouse) for another 30 minutes. After two final washing steps all cells were fixed in 200 µl FACS fixative (table 7.1). The cells were single stained only. Instrument settings were set so that the isotype control fell in the first decade. The results were displayed logarithmically as histograms. The cells were analyzed between 24-48hrs using a FACSCalibur flow cytometer (Becton-Dickinson). All data were analyzed with the CellQuest software (Becton-Dickinson), using the forward scatter (FSC) and sideward scatter (SSC) to gate cell debris out.

3.6 Short description of the detected antigens

Freshly isolated monocytes, MAC and MMAC after 3 days in culture and MAC, MMAC, DC and MAC + M-CSF after 7 days were stained for the following markers: antigens typically associated with either macrophage or DC differentiation and antigens that are expressed on both macrophage and DC, reflecting their function as antigen presenting cells and therefore involved in antigen uptake and T cell activation.

3.6.1 Antigens of macrophage differentiation: CD14, CD16, CD71, CD84 and MAX.11

CD14 is important for the reaction of the organism to LPS. It acts as a receptor for LPS bound to LBP (Wright et al., 1990) and is expressed on monocytes and macrophages (Todd et al., 1981). CD14 is membrane bound but lacks a cytoplasmic domain. Thus it needs the Toll like receptor 4 (TLR4) as a

coreceptor to trigger the signal cascade leading to TNF-α release (Poltorak et al., 1998).

CD16 or FcγIII is a low-affinity receptor for monomeric IgG. It is developmentally up-regulated in the *in vitro* differentiation from monocyte to macrophage (Clarkson and Ory, 1988; Andreesen et al., 1990). Although it was initially thought to be restricted to differentiated macrophages, a monocyte subpopulation expressing CD16 has been described (Passlick et al., 1989).

CD71 or transferrin receptor is a differentiation marker for macrophages and not present on monocytes (Andreesen et al., 1990). It is also expressed on intestinal DC and on monocyte derived DC where it acts as a receptor for IgA (Pasquier et al., 2004).

CD84 is a member of the CD2 subgroup of Ig super-family (de la Fuente et al., 1997). It is identical to the antigen recognized by the MAX.3 antibody and is a glycoprotein expressed on mature macrophages (Krause et al., 2000). Although MAX.3 bound only to mature macrophages, other anti-CD84 monoclonal antibodies detecting distinct epitopes showed a weak expression on monocytes and DC (Zaiss et al., 2003). It is a co-stimulatory molecule and binds to itself, thus enabling contact between APC and T cell. The CD84-CD84 connection inducts INF-γ secretion (Martin et al., 2001).

The MAX.11 antibody recognizes Carboxypeptidase M (Rehli et al., 1995; Krause et al., 1998), a surface molecule characteristic for monocyte to macrophage differentiation (Andreesen et al., 1986).

3.6.2 Cell surface antigen of DC differentiation: CD1a

CD1a is an antigen-presenting molecule distantly related to the MHC class I molecules (Martin et al., 1986; Blumberg et al., 1995; Banchereau et al., 2000). It binds non-peptic self and foreign molecules and presents them to T cells (Dutronc and Porcelli, 2002). Thus it contributes not only to host immunity but also to autoimmune and anti-tumor reactions (Hunger et al., 2004). CD1a is a good marker for *in vitro* generated blood-monocyte derived DCs (Sallusto and Lanzavecchia, 1994) and epidermal Langerhans cells (Fithian et al., 1981).

3.6.3 Functional antigenes of antigen presenting cells: CD40, DC-SIGN, CD80/CD86, HLA-ABC and HLA-DR

CD40 is a member of the TNF-receptor super-family and is present on all APCs, particularly on DCs (Rogers et al., 2003). It is critical for presentation to and induction of cytotoxic T cells. CD40 is known to be induced after activation of PRRs like the TLRs. Activation of CD40 by its ligand CD40L results in up-regulation of the co-stimulatory CD80 and CD86 and MHC class II via the transcription factor nuclear factor (NF)-κB (O'Sullivan and Thomas, 2003).

CD209 or DC-SIGN (DC-specific ICAM3-grabbing non-integrin) is a C-type lectin receptor for intercellular adhesion molecule (ICAM) 3 and ICAM2 (Geijtenbeek et al., 2000). Furthermore it can act as a PRR and provide a mechanism for pathogens, particularly HIV, to evade the immune surveillance (van Kooyk and Geijtenbeek, 2003). It is constitutionally expressed on DCs and macrophages (Soilleux et al., 2002).

CD80 and CD86 or B7-1 and B7-2 are co-stimulatory molecules associated with

APC - T cell interaction. Their counter ligands on T cells are CD28 and cytotoxic T lymphocyte-associated antigen (CTLA)-4. It has been reported that CD28 triggers T cell response whereas CTLA-4 seems to suppress or terminate T cell response. CD86 is constitutively expressed at a low levels and is upregulated upon stimulation, whereas CD80 is only expressed following stimulation (Greenwald et al., 2005).

HLA-ABC and HLA-DR: For the two groups of MHC receptors, HLA-ABC was chosen for class I, which is expressed on virtually every cell surface, and HLA-DR for class II, which is restricted to APC.

3.7 Apoptosis assay

An early event in apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer side of the plasma membrane. In viable cells PS is located exclusively on the inner layer of the cell membrane, whereas in apoptotic cells the PS translocates to the cell surface. Annexin V is a molecule that binds specifically to PS. Using flow cytometry, the location of PS can be detected using FITC-labeled Annexin V (Vermes et al., 1995). In necrotic cells the cytoplasmatic membrane is damaged, cells become permeable and allow Annexin V to enter the cytoplasma and bind to PS on the inner side of the membrane. To discriminate between early apoptotic cells with intact membrane and late necrotic cells by flow cytometry, a DNA-staining dye like Propidium iodide (PI) was used. In necrotic cells the dye enters through the damaged membrane and binds to the cellular DNA. By plotting the fluorescence of Annexin V-FITC versus PI, three cell populations can be discriminated: Annexin V-FITC$^-$/PI$^-$ viable cells, Annexin V-FITC$^+$/PI$^-$ early apoptotic cells and Annexin V-FITC$^+$/PI$^+$ late apoptotic or necrotic cells. Since apoptosis is a biochemically active process,

requiring energy, the experiments were performed strictly on ice to halt cellular activity. About $2.5 \cdot 10^5$ cells were washed twice with ice cold PBS in 5 ml polystyrene tubes and re-suspended in 200 µl staining solution, containing 2.5 µl Annexin V and 2.5 µl PI in 195 µl Annexin binding buffer (table 7.1). After 20 minutes incubation, cells were analyzed within one hour on a FACSCalibur flow cytometer. To allow for the best compensation parameters samples labeled with each dye singly were also analyzed.

3.8 Allogenic T cell stimulation assay

The T cell stimulation capacity of APC can be assessed in a model of allogenic transplantation, the mixed leucocyte reaction (MLR), where APC are co-cultured with allogenic T cells. The T cells recognize the MHC molecules as non-self and get activated depending on the stimulatory or inhibitory molecules presented by the APC. Proliferation is a good indicator for T cell activation upon stimulation. To measure proliferation, ^{3}H-methyl-thymidine (Hartmann Analytica) was added in excess to replace the cells' own thymidine pool. Thus ^{3}H-methyl-thymidine is incorporated into the newly synthesized DNA of proliferating cells. After cell lysis with deionized water, the DNA was collected on fiberglass filters. The radioactivity, measured in counts per minute (cpm), represents ^{3}H-methyl-thymidine incorporated into the cellular DNA and thus cell proliferation.

The different stimulator cells, MAC, MMAC or DC were examined for their capacity to stimulate allogenic T cells in an allogenic MLR. The T cells were separated from the MNC by counter-current elutriation (3.2) and frozen immediately in freezing medium containing 10 % DMSO (Sigma) and 90 % fetal calf serum (FCS)(PAA). For MLR, T cells were rapidly thawed, washed and resuspended in MLR medium (table 7.1). For MLR, $5{\cdot}10^4$ T cells were incubated at

stimulator: responder ratios from 1:2 till 1:1250. The cells were cultured in 96-well round bottom tissue culture plates (Falcon) in a total volume of 200µl MLR medium (table 7.1). As negative controls, T cells and stimulator cells were also incubated alone. After 6 days, 1 µCi ^{3}H-methyl-thymidine (0.037 MBq specific activity) was added to each well. Following a total incubation of 7 days, cells were harvested onto glass fiber filters (Printed Filtermat B, Wallac Oy) using a Vacusafe IH280 harvester (Innotech) and subsequently lysed. Scintillation fluid (Betaplate Scint, Wallac) was added to the filters and the radioactivity was determined by a liquid scintillation counter (1450 MicroBeta, Wallac). All samples were tested in quadruplicates and values indicate means ± SD.

3.9 Phagocytosis assay

Endocytosis is a basic feature of all eucaryote cells. The most common way is pinocytosis, where the plasma membrane builds small vesicles containing liquids or single molecules. Phagocytosis is more restricted to phagocytic cells like macrophages. Here the cell can engulf big particles like bacteria or virus, forming phagosomes which later fuse with lysosomes that contain enzymes to degrade the engulfed content. The phagocytic capacity was assessed by the cells' ability to ingest small fluorescently labeled latex. The phagocytized fluorescent beads could be monitored by flow cytometry.

Therefore, the harvested cells were washed and resuspended in polypropylene tubes (to prevent adhesion to the tubes) at 10^6 cells/ml with fresh medium. The cells were incubated with 0.5 µl FITC-labeled Flouresbrite Microspheres latex particles with 1.0 micron diameter (Polyscience) at 37°C in an oscillating water bath to insure continuous exposure of the cells to the beads. To control for random sticking of the beads to the cell surface, cells treated with beads were incubated on ice with additional 6 mM EDTA (Sigma) to totally inhibit

phagocytic uptake. After 1h incubation was stopped by placing the cells on ice and adding 6 mM EDTA. Following 3 washes an average of 30,000 cells was analyzed in a FACSCalibur (Beckton-Dickinson).

3.10 Cytokine production following stimulation with LPS

One main feature of macrophage or DC activation is increased cytokine secretion. To determine how the different cells react to an activation stimulus in terms of cytokine production, they were incubated with LPS, and the supernatant was analyzed for secreted cytokines. To this end, monocytes or cells after 7 days in culture were harvested, washed and $2{\cdot}10^6$ cells were cultured in 2 ml fresh medium for additional 24h with or without 10 ng/ml LPS. The supernatants were collected and further centrifuged for 30 minutes at 13,000 rpm (ultra centrifuge, Beckmann) to remove cellular debris, and stored at $-20°C$ in micro test tubes (Eppendorf) for later enzyme-linked immunosorbent assay (ELISA) analyses. The following ELISAs were performed according to the manufacturer's instructions: IL-10 (Becton-Dickinson), IL-12p70 (eBioscience), and TNF-α (eBioscience).

3.11 Statistics

Statistic analyses were performed with SPSS (SPSS). For descriptive statistics mean and standard deviation (SD) were calculated. All data was continuous and metric but not normally distributed, which was tested with the Kolmogorov-Smirnov test. Therefor tests for non-parametric data were applied on further statistic analyses. All experiments from one donor were treated as related data, the results of different donors treated as independent data. To evaluate a difference between multiple paired data, Friedman-test and post-hoc Wilcoxon Signed

Ranks Test were used to evaluate if a difference between a set of non-parametric data was statistically significant. For $p \leq 0.05$ the difference between two values was considered to be statistically significant.

4 Results

4.1 Survival of monocytes under non-adherent conditions

condition	mean of vital cells (%)	SD	p	n
MAC	50.1	8.9	> 0.05	(12)
MMAC	50.5	13.4		(13)
MAC + M-CSF	40.8	9.4	> 0.05	(3)
DC	40.2	10.7	> 0.05	(8)

Table 4.1: Survival rates of different culture conditions. The viability of MAC, MMAC, MAC + M-CSF and DC after 7 culture days was estimated by trypan blue staining. The % vital cells refers to the percentages of the initial seeded monocytes alive after 7 days in culture. Statistical analyses showed no significant differences, p refers to the difference to MMAC. Mean values of n independent experiments, SD: standard deviation.

In short, we wanted to study whether monocytes in suspension, such as in peripheral blood can survive and eventually differentiate. We thus used a culture system developed earlier by members of this workgroup (Arndt et al., 2007), whereby monocytes are grown completely adhesion-free and compared these cells with conventionally differentiated MAC. The viability of cells under all different culture conditions was determined by trypan blue staining (3.1.4) at the end of any culture period before further processing. The number of viable cells was set in ratio to the number of cells at the beginning of the culture period.

Table 4.1 shows the results of all experiments on day 7. There was no significant difference between the survival rate of adherent MAC (50.1%) and non-

adherent MMAC (50.5%). The poorer values of MAC + M-CSF (40.8%) were statistically not significant lower than MAC's or MMAC's.

4.2 Detection of apoptotic cells

Condition	n	% vital cells		% apoptotic cells		% dead cells		p
		mean	SD	mean	SD	mean	SD	
Mono	3	84	16	1	1	13	14	
MAC d3	4	77	15	5	7	12	3	> 0.05
MMAC d3	4	65	24	5	5	25	21	
MAC d7	4	81	8	5	5	11	4	> 0.05
MMAC d7	4	75	7	6	5	16	4	
DC d7	2	68	3	13	1	17	2	> 0.05

Table 4.2: Distribution of vital, apoptotic and dead cells. Monocytes and freshly harvested MAC, MMAC and DC at different timepoints were stained with Annexin V FITC/PI and analyzed with a flow cytometer. Results of quadrant analyses, the table shows mean values and SD of n independent experiments. No significant differences were found between MMAC and MAC or DC.

In the Annexin V FITC/PI assay (3.7) freshly isolated monocytes showed a viability of 84 % and showed almost no apoptotic cells. After a culture period of 3 days, the harvested MAC showed a viability of 77 % with 5 % apoptotic cells and 12 % dead cells, the corresponding MMAC had a viability of 65 % with 5 % apoptotic cells and 25 % dead cells, the difference was not significant for vital, apoptotic or dead cells. After a culture period of 7 days MAC showed a viablity of 81% with 5 % apoptotic and 11 % dead cells, MMAC were viable to 75% with 6 % apoptotic and 16 % dead cells. DC on day 7 were viable to 68 % with 13 % apoptotic and 17 % dead cells, also with no significant differences (figure 4.2).

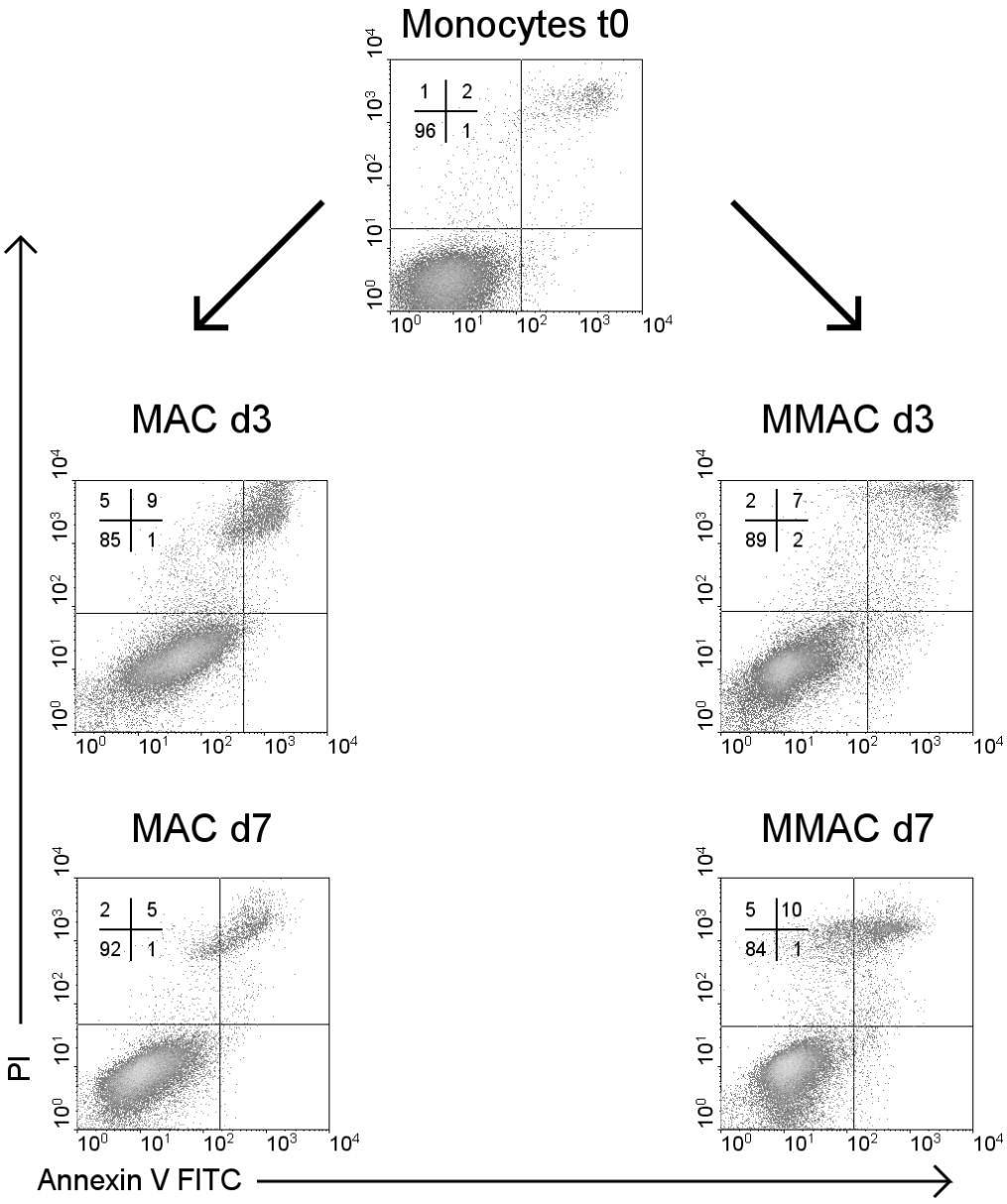

Figure 4.1: Detection of apoptotic cells in FACS-Analysis. Monocytes and freshly harvested MAC and MMAC at different timepoints were stained with Annexin V FITC/PI and analyzed with a flow cytometer. The figure shows dotplots of one representative two color analysis, results of the quadrant analyses are represented by the numbers in the small cross.

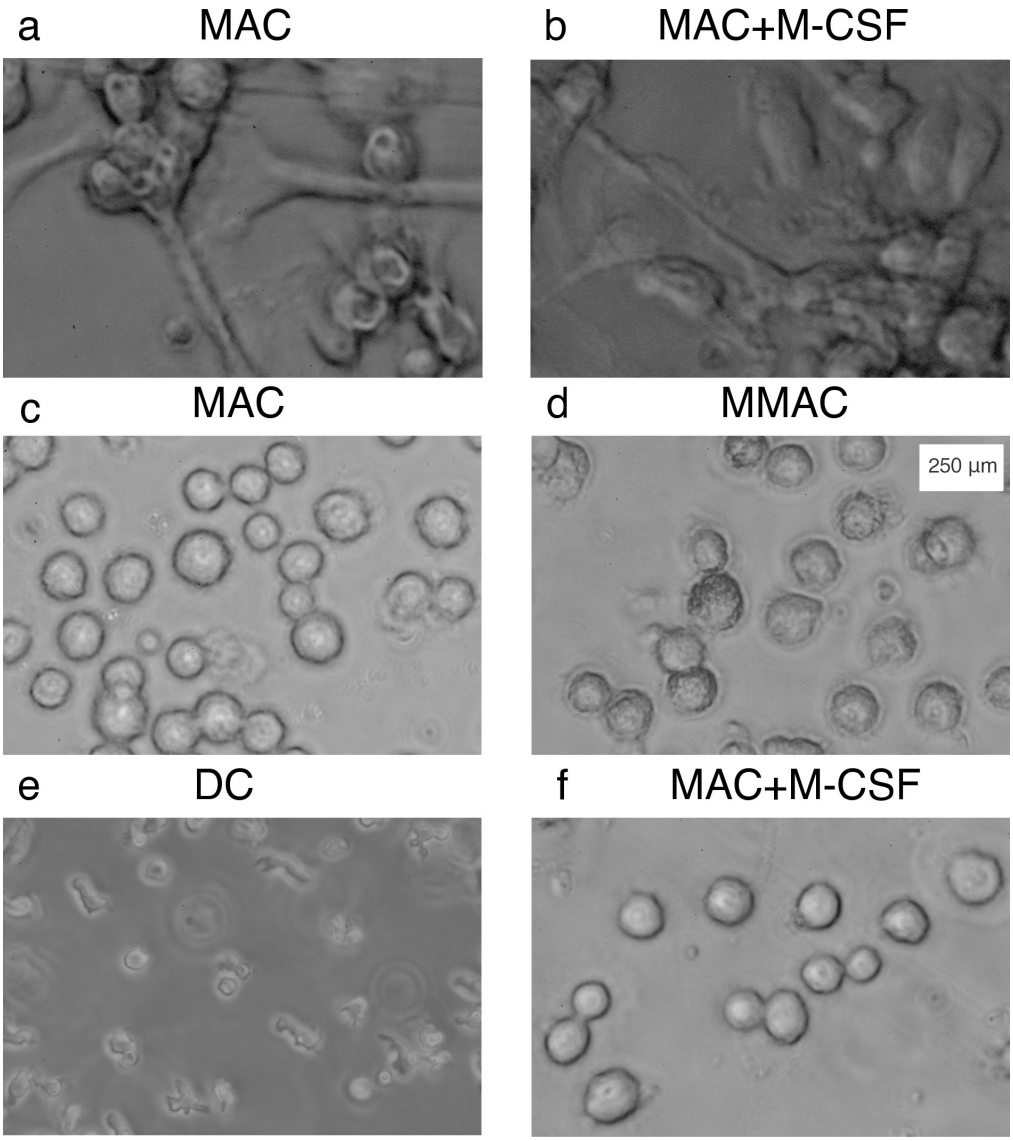

Figure 4.2: Morphology in light microscopy. Light microscopic images of MAC and MAC + M-CSF adherent on teflon (a & b) and of loosened MAC, MMAC, DC and loosened MAC + M-CSF (c, d, e & f) in suspension. Magnification 100x, native cells without staining.

4.3 Morphology in light microscopy

The morphology of the cells was also evaluated in parallel with the viability by light microscopy (3.4). The MAC showed spindle shaped bodies when attached to the Teflon membrane (figure 4.2 a). The extra M-CSF in the MAC + M-CSF culture did not cause any morphological changes (figure 4.2 b) different from those observed in MAC. In the DC culture, the non-adherent cells were smaller in size and had typical dendrites (figure 4.2 e). In suspension, MMAC presented a highly granular round body with small cell plasma protrusions (figure 4.2 d). The loosened MAC + M-CSF looked similar to the loosened MAC (figure 4.2 c & f). All three macrophage populations, MAC, MMAC and MAC + M-CSF attached rapidly to the usual glass microscope slides displaying a classic 'fried egg' form.

4.4 Phenotype characterization with monoclonal antibodies

After establishing the similar survival rates of non-adherent MMAC and adherent MAC, the question occured whether the surviving cells actually underwent differentiation. As cell differentiation is associated with changes in surface antigen expression, the phenotype of these cells was determined using fluorescently tagged antibodies against differentiation-associated antigens which were identified with flow cytometry.

MMAC had higher values in FSC and SSC, corresponding to higher granularity and cell size compared to MAC (figure 4.3 a). Figure 4.3 depicts data from one representative experiment for MAC, MMAC and DC after 7 days. In general, MMAC had a higher auto-fluorescence, mainly in the FITC-channel, which made adjustment of the instrument settings necessary to bring the background

fluorescent reading (isotype control) into a minimum. No significant differences could be found for the adherently cultured MAC + M-CSF, which had extra M-CSF, compared to MAC, which lacked extra M-CSF. Thus, MAC + M-CSF are present on the bar graphs for illustration, but are not further mentioned in the following paragraphs.

4.4.1 Antigens of macrophage differentiation: CD14, CD16, CD71, CD84 and MAX.11

All isolated monocytes (610 ± 109 mean ± SD MFI), MAC (429 ± 172) and MMAC (861 ± 323) expressed CD14 at a high level in comparison to the down-regulation seen in monocyte to DC (52 ± 39) differentiation. Still, the CD14 expression on MMAC was higher than on MAC, which was already significant after 3 days. MAC (73 ± 37) and MMAC (169 ± 98) expressed higher levels of CD16 than did the freshly isolated monocytes (38 ± 27). But the CD16 expression on MMAC was even higher than on MAC, a difference that was even more pronounced on day 3 (17 ± 14 vs. 197 ± 116). Expression on DC was very weak (7 ± 8). Freshly isolated monocytes did not express CD71, but it was detected on all differentiated cells (MAC 77 ± 51, MMAC 33 ± 9, DC 35 ± 31). Low CD84 expression was found on monocytes (27 ± 6) and DC (43 ± 25), slightly, but not significant higher levels were seen on both MAC (366 ± 151) and MMAC (285 ± 128), which expressed equivalent levels of CD84 already on day 3 (263 ± 22 vs. 257 ± 26). MAX.11 was absent on freshly isolated monocytes, but expressed by all differentiated cells. A high level was seen on MAC (518 ± 172), compared to significantly lower amounts on MMAC (133 ± 94) and DC (186 ± 108). Already by day 3, the level of MAX.11 apparent on MAC (429 ± 136) exceed that apparent on MMAC (76 ± 65).

For details see figures 4.4 and 4.5.

4.4.2 Cell surface antigen of DC differentiation: CD1a

Beside the high expression of CD1a on DC (1544 ± 1397), only low levels were observed on the other cells. Notably, CD1a expression on MMAC was not constant: MMAC of some donors expressed low levels of CD1a while those of others didn't, resulting in the slightly, but not significant higher levels seen on MMAC (22 ± 28) compared to MAC (6 ± 6).

See also figure 4.5.

4.4.3 Functional antigenes of antigen presenting cells: CD40, DC-SIGN, CD80/CD86, HLA-ABC and HLA-DR

CD40 was not detected on monocytes. The highest level was found for DC (62 ± 20), significantly lower levels for MAC (27 ± 8) and even lower amounts were present on MMAC (15 ± 7). DC-SIGN expression was low on monocytes (28 ± 21) and MAC (11 ± 9). DC (337 ± 185) expressed the highest levels whereas levels on MMAC (116 ± 118) were moderate, significantly distinct from that of MAC and DC. The changes in DC-SIGN expression seemed to be a late event in differentiation pathway, as no difference could be noticed at day 3 (MAC 62 ± 27, MMAC 56 ± 5). No expression of CD80 was detected on monocytes, MAC or MMAC, and only a very low expression was seen on DC (9 ± 13). Compared to freshly isolated monocytes (24 ± 10), CD86 was equally more intensely expressed on all cell types (MAC 56 ± 22, MMAC 75 ± 29, DC 83 ± 106). Freshly isolated monocytes expressed the highest levels of HLA-ABC (412 ± 409) and modest levels of HLA-DR (81 ± 57). All cells down-regulated HLA-ABC upon differentiation (MAC 193 ± 321, MMAC 200 ± 249, DC 48 ± 134). The level of HLA-DR expression was stable on MAC (60 ± 51), but up-regulated on MMAC (174 ± 132) and even more on DC (434 ± 294) respectively.

See also figures 4.6 and 4.7.

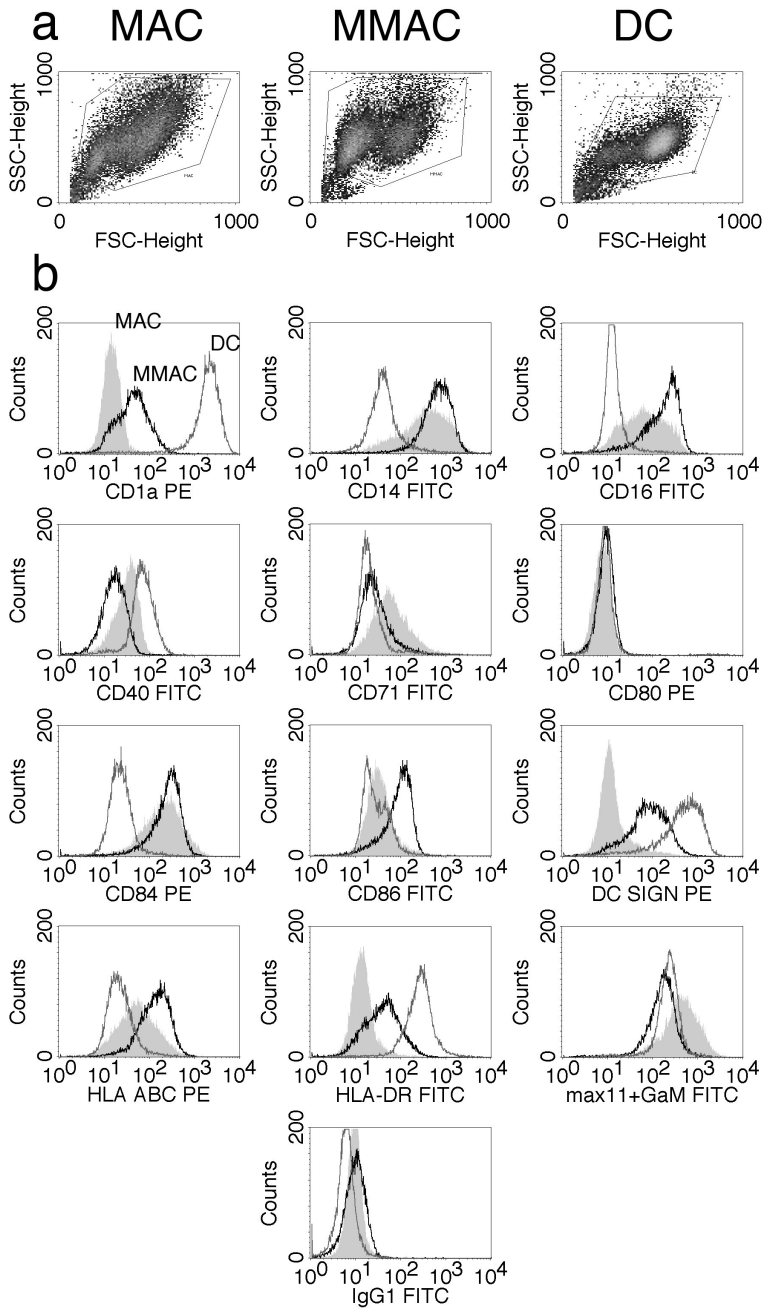

Figure 4.3: Flowcytometric analysis of differentiation antigens expressed by one representative donor after 7 days in culture. **(a)** FSC/SSC dot plots with gate settings; **(b)** overlaid histograms of MAC (filled light grey), MMAC (unfilled black) and DC (unfilled grey). IgG1: Isotype control, GaM: second antibody goat-anti-mouse.

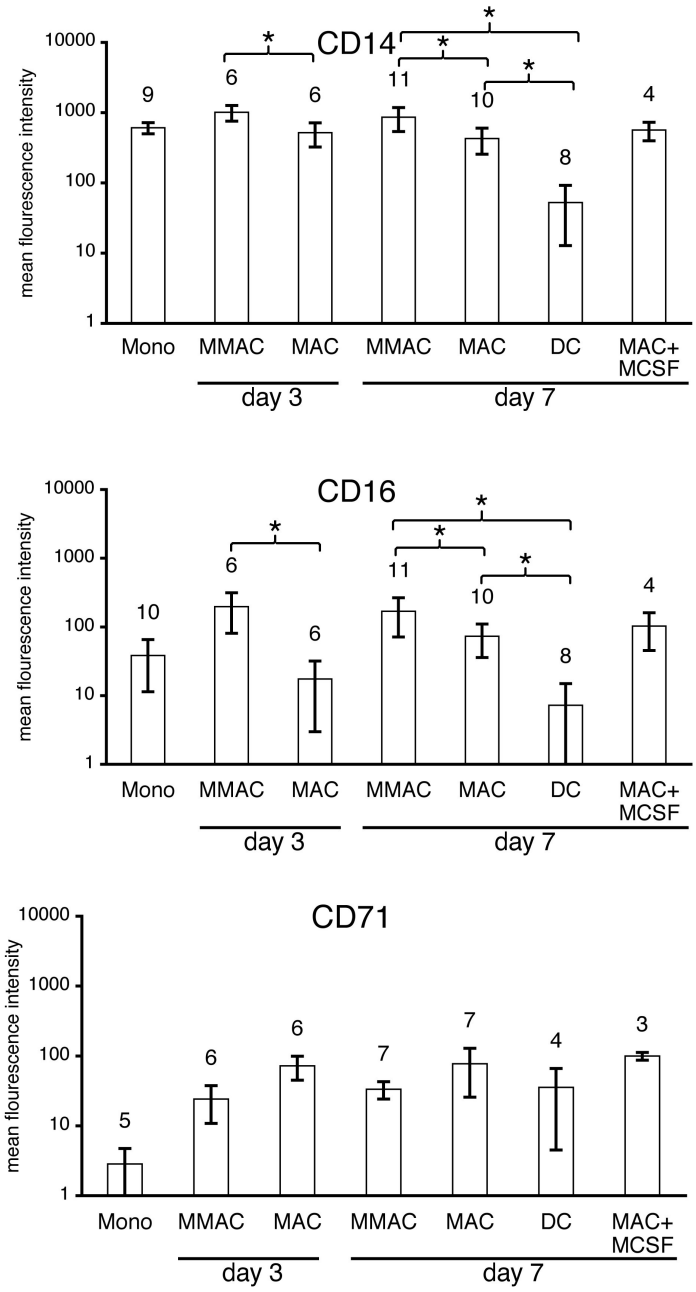

Figure 4.4: Expression pattern of macrophage antigens during different days of differentiation - bars: mean values of mean fluorescence intensity; error bars: SD; small numbers above the bars: number of experiments; Mono: untreated monocytes, t0; $\star$ indicates a difference with $p < 0.05$.

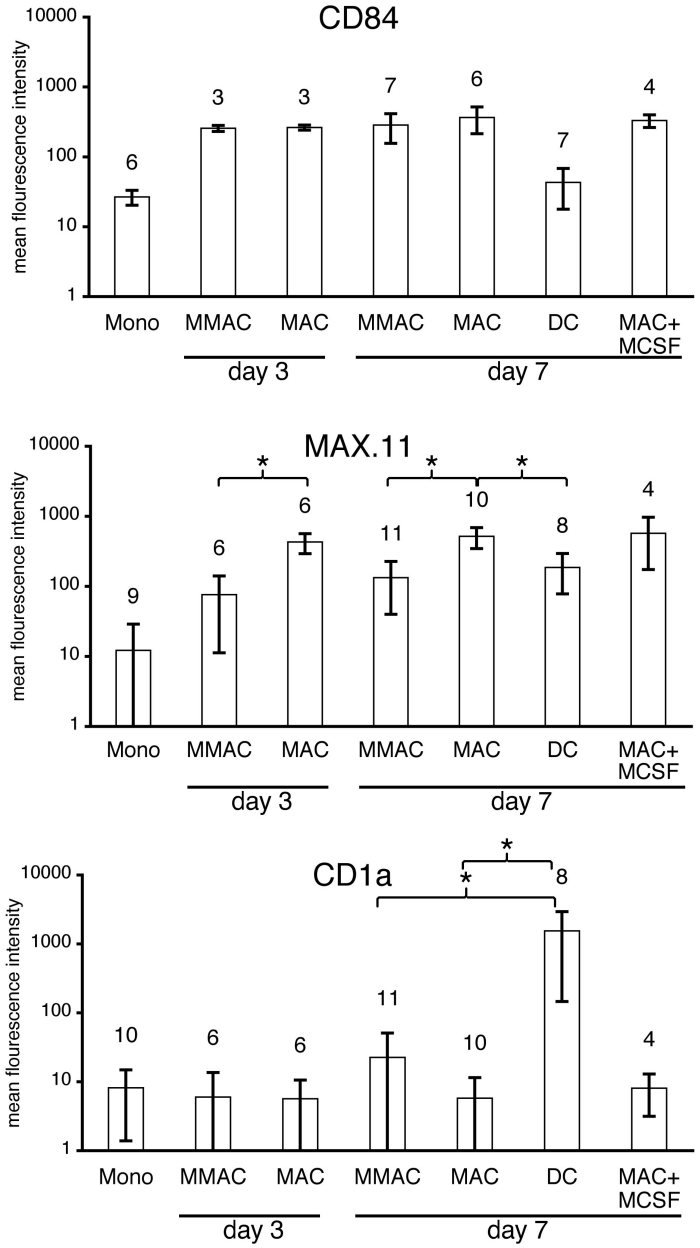

Figure 4.5: Expression pattern of macrophage antigens and CD1a during different days of differentiation - bars: mean values of mean fluorescence intensity; error bars: SD; small numbers above the bars: number of experiments; Mono: untreated monocytes, t0; $\star$ indicates a difference with $p < 0.05$.

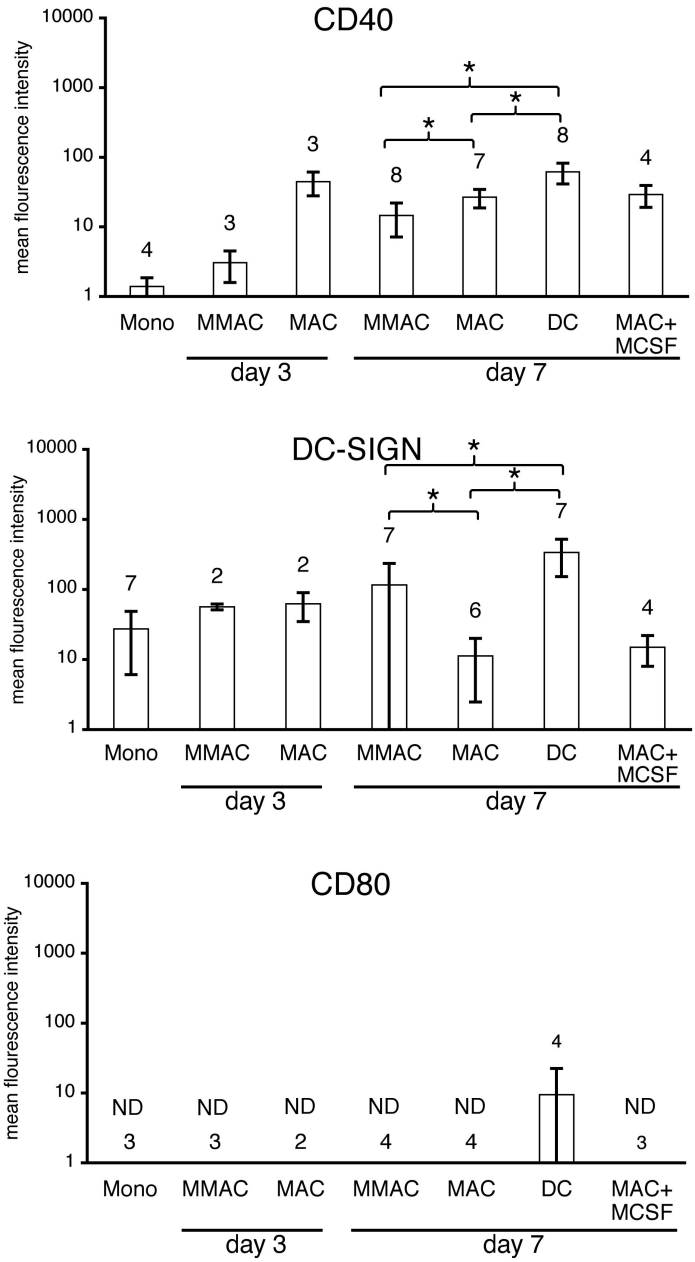

Figure 4.6: Presence of surface molecules associated with APC during different stages of differentiation - bars: mean values of mean fluorescence intensity; error bars: SD; small numbers above the bars: number of experiments; Mono: untreated monocytes, t0; $\star$ indicates a difference with $p < 0.05$; ND: not detected;

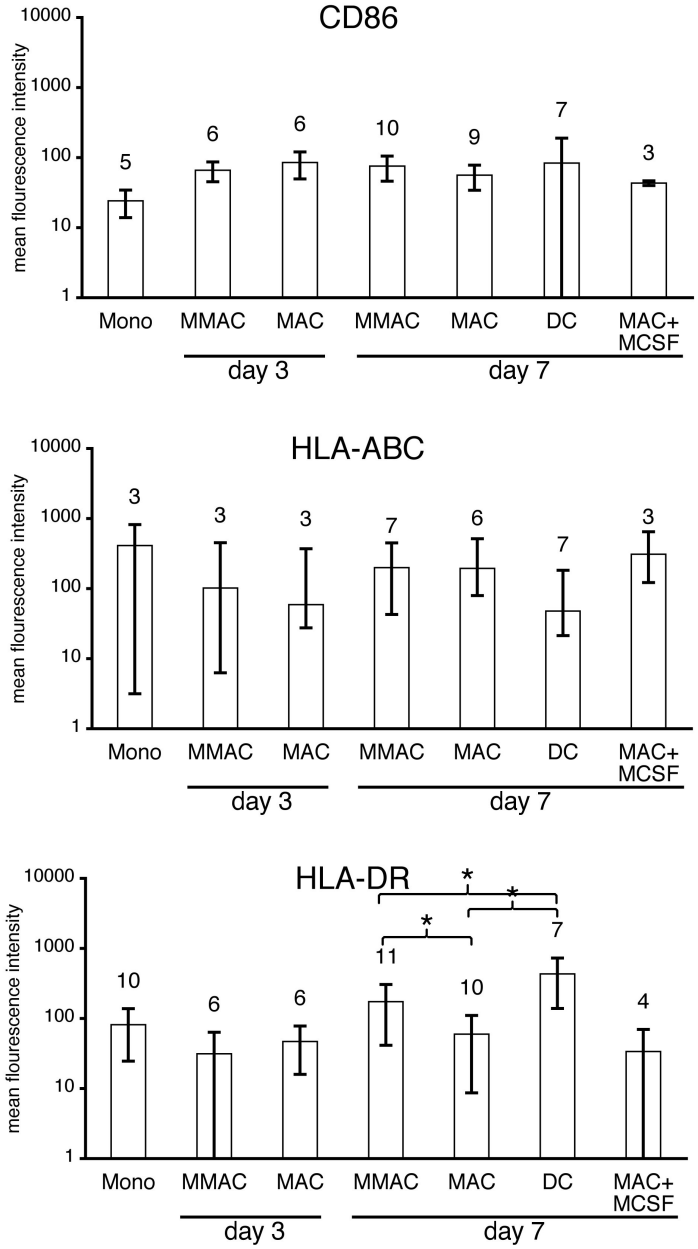

Figure 4.7: Expression profile of CD86, HLA-ABC and HLA-DR during cell differentiation along the monocytic lineage - bars: mean values of mean fluorescence intensity; error bars: SD; small numbers above the bars: number of experiments; Mono: untreated monocytes, t0; $\star$ indicates a difference with $p < 0.05$;

4.5 Phagocytosis assay

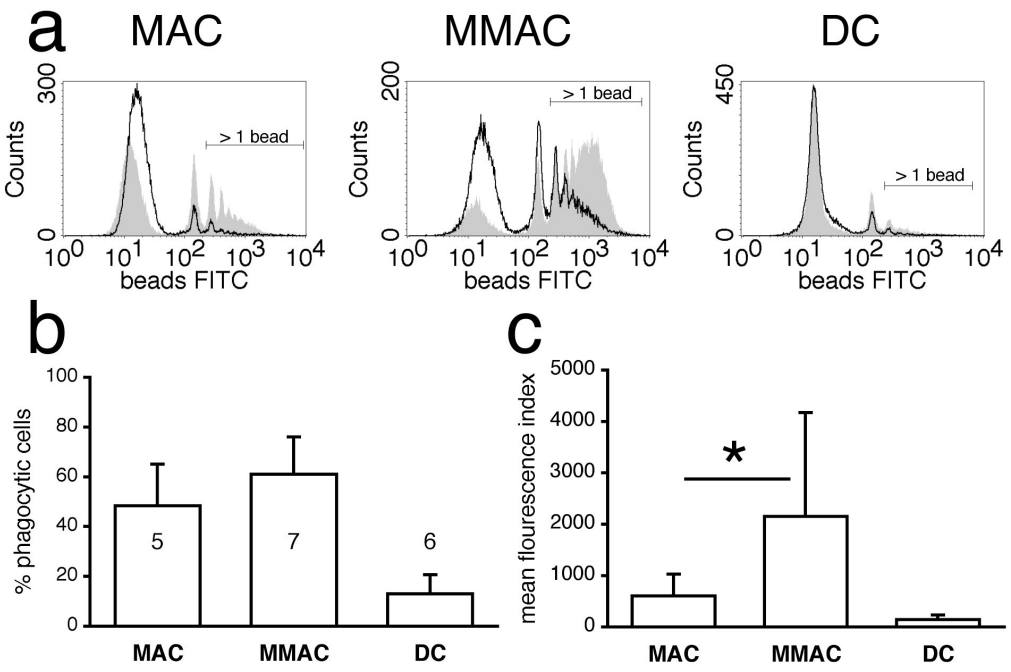

Figure 4.8: Phagocytosis assay with fluorescent latex beads. **a** - histograms of a representative experiment, cells after 1h incubation with latex beads at 37°C (filled graphs) or on ice (unfilled graphs); **b** - percentage of phagocytic cells, corresponding to the marker in a; **c** - MFI values of the cells under the marker; bars - mean values of n independent experiments; error bars: SD; $\star$ indicates a difference with $p < 0.05$;

The phagocytic capacity was examined using the cells' ability to engulf fluorescent latex beads. The analyzed cells had the fluorescence distribution shown in figure 4.8 a. The first big peak can be attributed to autofluorescence of the cell. The following distinct peaks result from the uptake of one, two and more beads by a single cell. Because in the control samples some cells seemed to bind at least one bead, a marker was set for more than one bead. DC (13% ± 8) did hardly take up latex beads. The number of cells that ingested more than one bead did not differ significantly between MAC (48% ± 17) and MMAC (61% ± 15) (see figure 4.8 b). But on average MMAC did incorporate significantly more

beads per cells than MAC. This difference is represented by a higher MFI for MMAC (2152 ± 2022) compared to MAC (610 ± 420) under the marker (see figure 4.8 c).

4.6 Activation of APC with bacterial endotoxin

The stimulation and subsequent cytokine secretion of APC is an important step for the activation of the adaptive immune system. Several macrophage activation patterns have been described, leading either to propagation or inhibition of the inflammatory process. Here, the cells were activated with the 'classical' macrophage stimulus LPS without prior priming.

The supernatants of the unstimulated MMAC contained more IL-10 than MAC, more pronounced on day 3 (253 pg/ml ± 266 vs. 17 pg/ml ± 20) compared to day 7 (133 pg/ml ± 220 vs. 27 pg/ml ± 35). The contents of IL-10 in DC (46 pg/ml ± 80) cultures were comparable to MMAC at day 7. Upon stimulation, all cells produced significantly more IL-10. MMAC (3212 pg/ml ± 2459) on day 7 exceeding the similar values of MAC (1071 pg/ml ± 909) and DC (1670 pg/ml ± 1913). The IL-12 secretion was low for all culture conditions before and after stimulation, the only significant rise was seen after the stimulation of monocytes (22 pg/ml ± 15 to 52 pg/ml ±29). The testing for TNF-α in unstimulated cultures after 7 days showed a tendency to higher levels in MMAC (703 pg/ml ± 642) and DC (1183 pg/ml ± 195) compared to monocytes (90 pg/ml ± 52) or MAC (91 pg/ml ± 46). However, these differences were not statistically significant. Stimulation with LPS resulted in higher TNF-α values for MMAC (4885 pg/ml ± 2448), MAC (2702 pg/ml ± 2828) and DC (3131 pg/ml ± 4368), also with no significant difference.

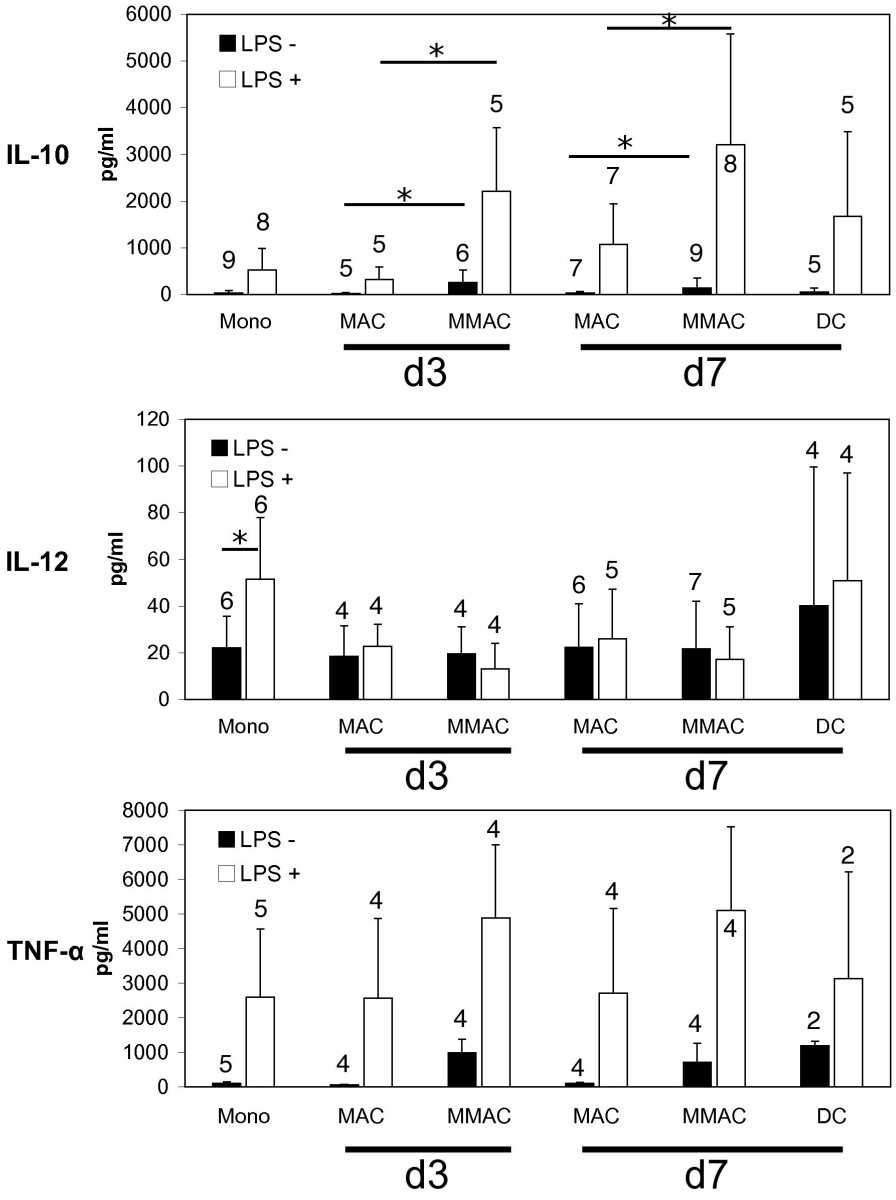

Figure 4.9: Cytokine production upon stimulation with LPS. Monocytes (Mono) or freshly harvested MAC, MMAC and DC after 3 and 7 culture days were incubated with or without 10 ng/ml LPS for 24h and the supernatants analyzed with specific ELISAs for IL-10, IL-12p70 and TNF-α. Bars - mean values of n independent experiments, error bars: SD, small numbers on the bars: number of independent experiments, $\star$ indicates a difference with $p < 0.05$.

4.7 Allo-reactiv T cell response

The ativation of T cells by APC is critical for the function of the adaptive immune system. The stimulatory capacity of the different APCs was tested in a MLR (3.8). Proliferative capacity of allogenic T cells in cocultures with MMAC (ratio 1:1 15347 cpm ± 22343 mean ± SD, ratio 1:2 8545 cpm ± 13339) was stronger than in cocultures with MAC (ratio 1:1 2389 cpm ± 1493, ratio 1:2 2347 cpm ± 1528). However, MMAC did not induce the high proliferation activity seen in cocultures with DC (ratio 1:1 32463 cpm ± 31481, ratio 1:2 18792 cpm ± 8996). The difference between MAC, MMAC and DC were significant for both the 1:1 and the 1:2 ratio. Proliferation of the stimulator or responder cells cultured alone was not seen.

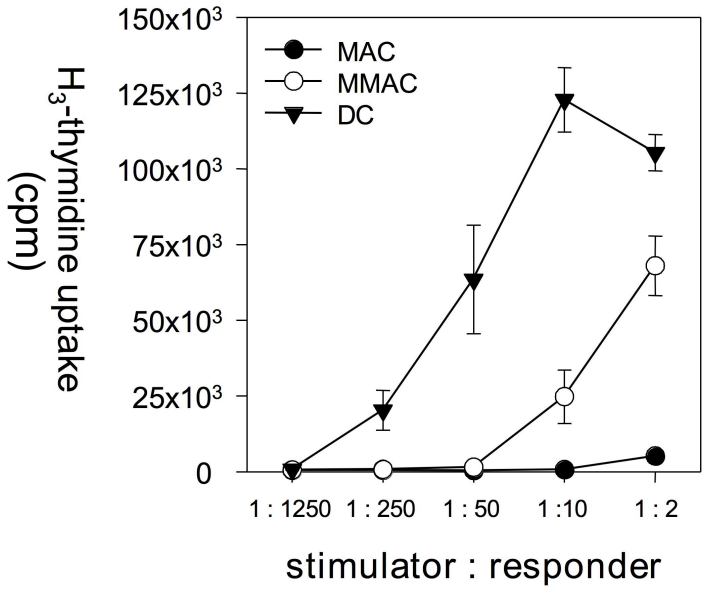

Figure 4.10: Allogenic activation of T cells by MAC, MMAC and DC. Allogenic T cells were incubated with either macrophages, MMAC or DC at the different stimulator responder ratios indicated. On day 6, 3H-methyl-thymindine was added. After 24 hours the cocultures were harvested. Values indicate means of cell-associated radioactivity +/- SD of quadruplicates. One representative out of 6 experiments is shown.

5 Discussion

5.1 Survival

The *in vitro* differentiation of monocytes to macrophages usually implicates an adherent culture condition, which is caused particularly by the nature of monocytes to adhere to the commonly used culture plates. One disadvantage of this culture method is that such differentiated cells firmly stick to their tissue plates, demanding quite a force to harvest them for further analyses. Thus, Andreesen et al. (Andreesen et al., 1983) cultured monocytes semi-adherent on hydrophobic Teflon foils from where they could be harvested without damaging the cells' integrity. However, in the absence of human serum, these Teflon grown monocytes required exogenous M-CSF for survival (Andreesen et al., 1990) in contrast to monocytes cultured firmly adherent on plastic (Haskill et al., 1988). It could later be shown that the quantity of M-CSF physiologically present in human serum is enough to assure monocyte survival on Teflon foils (Brugger et al., 1991). To elucidate the impact of adhesion on monocyte survival or apoptosis, a non-adherent culture system developed by members of this group and published by Arndt et al. (Arndt et al., 2007) was used for this study (3.3.3). It has been showed by Mondal (unpublished data) that non-adherently cultured monocytes undergo apoptosis even in the presence of human serum as a result of the rapid loss of the anti-apoptotic protein Mcl-1 which could be inhibited by addition of exogenous M-CSF.

The similar survival rates of MMAC and MAC (4.1) underline the proposed anti-apoptotic effect of M-CSF on monocytes and demonstrate that adhesion is not an absolute condition for monocyte survival. The additional Annexin V/PI staining confirmed the results of the trypan blue staining in that no early apoptotic cells were counted as vital. Focusing on apoptosis, responsible for the loss of about 50% of seeded cells under all conditions, it was remarkable that only about 10% dead cells were found at the end of the culture period. It cannot be ruled out that the remaining ones, in both, adherent and non-adherent conditions, ingested the apoptotic cells. Human blood monocytes have been shown to phagocytise apoptotic cells already after their third day in circulation (Mikolajczyk et al., 2009).

5.2 Differentiation

Monocytes give rise to all different kind of tissue macrophages, DC subsets and osteoclasts. Although specific monocyte subsets have been described to differentiate preferably to DC (Randolph et al., 2002), no specific monocyte characteristics have been found that lead to the various macrophage types in the different tissues (Hume, 2006, 2008). It seems more likely that the monocytes enter the tissues randomly, but retain a certain plasticity to react to the local microenviroment rather than exhibiting multiple but distinct subpopulations (Stout and Suttles, 2004). As an inflammatory process is never static, macrophages could change their phenotype according to the stage of the process (Stout et al., 2005, 2009). DCs and osteoclasts might be terminally differentiated exceptions (Palucka et al., 1998). The micro-enviroment is defined by stromal and lymphoid cells, ECM, and soluble factors. This implicates the very important role for cell-cell and cell-substrate interactions, influencing a broad range of cellular characteristics, as reviewed by Shi et al. (Shi and Simon, 2006).

On the other hand, monocytes and differentiated macrophages are also found in body fluids like blood, peritoneal, pleural and synovial fluid. After having establishing that monocytes can survive in suspension culture, it remained to be proven that these cells had also differentiated. In fact, the surviving cells in the suspension culture did not remain static, rather they underwent tremendous morphological changes.

By light microscopy, MMAC in suspension did not show pseudopodia as did the adherent MAC and MAC + M-CSF. Compared to MAC and MAC + M-CSF in suspension, the only distinguishing feature of MMAC were little cytoplasmic protrusions. As this characterized only MMAC and not MAC + M-CSF, this could be caused by the non-adherent culture condition. Further demonstrated MMAC a strong capacity to attach to the usual glass slides, as did MAC in suspension.

By flow cytometry, MMAC were observed as large, highly granular cells with high values in the FSC and SSC, according to the light microscopic observations. The two different populations shown in the MMAC FSC/SSC dot plot did not reveal any distinguishing features in further analyses. Moreover, all analyzed cells were fixed in paraformaldehyde. In the vital staining with Annexin V/PI, where cells were processed immediately without fixation, this feature could not be reproduced and MMAC showed an unique population in the FSC/SSC dot plot. Thus this was most likely an artifact due to the fixation procedure. MMAC also were more auto-fluorescent compared to MAC. Likewise, Njoroge (Njoroge et al., 2001) describes a non-adherent cell population in an adherent monocyte culture, resembling MMAC with high auto-fluorescence and an ability for re-attachment.

Flow cytometric analysis with monoclonal antobodies demonstrated that MMAC did not only survive, but clearly had differentiated into a macrophage-like phe-

notype. Notably, the additional M-CSF in the MAC + M-CSF culture did not cause any significant difference to MAC. MMAC upregulated CD14, CD16, CD71, CD84 and MAX.11 compared to monocytes, as did MAC. All these markers are known to be related to monocyte to macrophage differentiation (Andreesen et al., 1990).

However, MMAC showed a remarkable elevation of CD16 expression as an early event after 3 days, at a time-point when adherent MAC showed still low CD16 expression. A similar human $CD14^{+}CD16^{+}$ monocyte sub-population was described in several diseases, such as AIDS (Cassol et al., 2006), rheumatoid arthritis (Kawanaka et al., 2002b) and chronic renal failure (Kawanaka et al., 2002a). This $CD16^{+}$ subset is also expanded after treatment with M-CSF in both humans (Saleh et al., 1995) and primates (Munn et al., 1996). These cells have been described as a more mature type of blood monocytes, differentiated in the blood under the influence of elevated levels of serum M-CSF and other inflammatory cytokines (Kawanaka et al., 2002b; Saionji and Ohsaka, 2001). Since the culture conditions of MMAC resembled in part the situation in the blood, these *in vitro* results suggest that monocytes in a non-adherent condition can differentiate into a macrophage-like effector cell.

Even though adhesion is not an absolute condition to monocyte differentiation, it undoubtedly has an influence on the cells' characteristics. Adherence, for example to collagen molecules, induces CD14 down-regulation (Jacob et al., 2002). As MMAC lack adhesion, these findings stand in agreement with the results that CD14 expression in MMAC remained at a high level in contrast to the down-regulation in adherent MAC.

Taken into consideration that blood monocytes can give rise also to DCs, MMAC were compared not only to MAC but also to DC. Considering the light microscopic phenotype and the markers of macrophage differentiation discussed

above, MMAC showed clearly a macrophage-like phenotype. In addition to that, no expression of the DC-marker CD1a was detected in any culture condition except DC.

To account for the antigen presenting function of the evaluated cells, the expression pattern of antigen presenting and costimulatory molecules that correlate with their function as APCs were determined. Upon differentiation from the monocyte, the expression of MHC class I molecules diminished equally in MMAC and MAC. In contrast, the level of MHC class II molecules was stable in MAC, but increased in MMAC and to an even higher degree in DC. No significant difference was found for the costimulatory molecules of the B7 family. CD80 was very weakly expressed on DC and not detected on any of the other cells. CD86 was equally upregulated on all cells in the differentiation process from monocytes. However, significant difference was found in the expression of the T cell adhesion molecules CD40 and DC-SIGN. Both molecules were present mostly on DC. But whereas CD40 expression was lower in MMAC than on MAC, the opposite was found for DC-SIGN, where the expression was higher on MMAC than on MAC. The characterization of DC-SIGN as a DC marker has been challenged by the discovery that in patients with leprosy, the macrophages express DC-SIGN and CD16, whereas the DC, characterized by CD1 lack fDC-SIGN (Krutzik et al., 2005). More recently, $CD14^{+}$ DC-SIGN $^{+}$ macrophages in the lamina propria were described as potent antigen presentig cells and are supposed to play an important role in maintaining the immunological balance in the gut (Kamada et al., 2009).

Conclusively, the phenotype of MMAC is clearly macrophage-like, with some APC-related markers more accentuated. In part, MMAC resemble the phenotype of peritoneal macrophages, which was reported for other M-CSF differentiated monocytes before (Xu et al., 2007; Akagawa et al., 2006). In patients un-

dergoing peritoneal dialysis, the monocytes invading the peritoneum expressed high CD14, CD16, CD71 and HLA-DR (Andreesen et al., 1990; Brauner et al., 1998). The M-CSF levels in the peritoneal fluid are higher even in healthy individuals (Weinberg et al., 1991), but rise in accordance to high M-CSF blood levels, for example in patients undergoing dialysis (Saionji and Ohsaka, 2001).

5.3 Phagocytosis

Endocytosis is performed by all eucaryotic cells in form of uptake of liquids and single molecules into small plasma membrane enclosed vesicles, which is referred to as pinocytosis. The engulfment of big particles like bacteria is restricted to professional phagocytes. All antigen-presenting cells are also phagocytes but the various subtypes differ considerably in their phagocytic capacity. DCs are very good antigen presenting cells but poor phagocytes, macrophages instead are very good phagocytes but weak antigen presenting cells. Macrophages are distinguished by their ability to ingest large particles. They even can merge together and form multinuclear giant cells in order to digest a larger foreign body.

Functionally, MMAC appear to be a more efficient MAC. Its phagocytic capacity is more robust than that of an adherently-generated MAC. Again this could be due to the extra M-CSF in the culture media, as M-CSF is known to enhance phagocytosis (Akagawa, 2002; Nemunaitis, 1998). Moreover, the strong expression of CD14, CD16, and DC-SIGN on MMAC, which all are involved in antigen uptake, could also account for this caracteristic. There was no uptake of beads in DC as expected.

5.4 Cytokine production

Another keystone to the activation of the immune system is cell-cell interaction via cytokines. Upon stimulation with LPS, all cells secreted high amounts of IL-10 and TNF-α, confirming previous results of this group for MAC and DC (Ammon et al., 2000). MMAC however produced more IL-10 and TNF-α compared to MAC upon stimulation. In contrast to monocytes, which secreted IL-12 following stimulation, no increase of this cytokine was found in MAC and MMAC.

MMAC constitutively produced IL-10. Accordingly, Smith et al. (Smith et al., 1998) found the same cytokine secretion pattern with high IL-10 and low IL-12 secretion for MAC, DC and MAC + M-CSF. They found that constitutive IL-10 secretion of M-CSF-induced macrophages did not account for low IL-12 levels, as it occurred also in the presence of inhibitory anti-IL-10-antibodies. Constitutive IL-10 secretion was also found for M-CSF induced macrophages that also were highly active in phagocytosis (Xu et al., 2006), although in this report, these macrophages did not produce TNF-α as did MMAC. Conclusively the $CD14^+$ $CD16^+$ monocytes were identified as main producers of IL-10 in the blood (Skrzeczyńska-Moncznik et al., 2008).

5.5 T cell stimulatory capacity

Cocultures of allogenic T cells with MMAC caused a T cell proliferation much more pronounced than with MAC, but not as potent as with DC. This is in accordance with higher expression of MHC class II molecules and DC-SIGN on MMAC. DC-SIGN is important for the early T cell - APC interaction and results in profound T cell stimulation (Geijtenbeek et al., 2002; Gijzen et al., 2007). DC-SIGN can be induced by cyclic nucleotides, which inhibit DC generation

and favor a cell type expressing high MHC class II and CD86 molecules, and a high proliferative T cell response in MLR (Giordano et al., 2003). The weak proliferation of T cells in coculture with MAC is in line with the inhibitory proprieties of non-activated MAC, which have recently been stated (Hoves et al., 2006). The M-CSF in MMAC cultures could be responsible for a more activated cell type, as M-CSF seems to favor the alternative activation pathway (Martinez et al., 2006). Also IL-10 could have an influence on the T cell activation, as it inhibits T_H1 response, favoring the generation of T_H2 cells (Conti et al., 2003).

Interestingly, MMAC showed higher phagocytosis rates and higher stimulatory capacity toward T cells, two items that generally are divergent. Monocytes loose their phagocytic capacity and gain better antigen presenting upon differentiation towards DC.

6 Conclusion

The results of this study establish that monocyte adhesion, which undoubtedly is responsible for many substantial changes in the monocyte, is not essential for monocyte survival and differentiation to a macrophage-like cell. As monocytes and their differentiated forms are found, not only in the extra cellular matrix, but also in suspension in the blood and the peritoneal fluid, this can help to understand their function under these circumstances. Many details have been revealed for the different monocyte subsets. This study gives now further substance for the hypothesis that the $CD16^+$ monocytes are a more mature subset, differentiated in the blood under the influence of M-CSF. Moreover, MMAC have as a unique feature the upregulation of both, phagocytosis and T cell stimulatory capacity, two functions that usually are not up-regulated in parallel. Whether this new macrophage type has its own place in the MPS, or it stands for another possible phenotype of a chameleon like cell type, known as the macrophage, remains to be elucidated.

7 Zusammenfassung

Monozyten und die aus ihnen hervorgehenden Makrophagen und dendritischen Zellen (DC) haben im Immunsystem des Menschen eine besondere Rolle als Bindeglied zwischen dem angeborenen und dem adaptiven Immunsystem. In vielen Experimenten zur Differenzierung von Makrophagen aus Monozyten erwies sich der Wachstumsfaktor M-CSF als überlebensnotwendig. Dabei wurden jedoch die Monozyten stets adhärent kultiviert, sodass der Einfluss von M-CSF auf die Differenzierung nie unabhängig von den Auswirkungen der Adhäsion betrachtet werden konnte. M-CSF kommt auch in nennenswerten Mengen im Blut gesunder Individuen vor und kann bei bestimmten Krankheiten in deutlich höheren Konzentrationen nachgewiesen werden. Da Monozyten auf ihrem Weg vom Knochenmark zum Gewebe einige Zeit im Blut zirkulieren und sich dort unter dem Einfluss von M-CSF verändern könnten, wurde versucht diese Umgebung *in vitro* nachzuformen. Dafür wurden humane Blutmonozyten in einer Suspension zur Vermeidung von Zell-Zell oder Zell-Substrat-Kontakten und unter Zugabe von rekombinantem M-CSF in einer rotierenden Flasche kultiviert.

Diese durch M-CSF induzierten Makrophagen (MMAC) exprimierten signifikant höhere Werte der Oberflächenantigene CD14, CD16, HLA-DR und DC-SIGN im Vergleich zu adhärent generierten Makrophagen (MAC). Im Vergleich zu DC zeigten sie niedrigere Werte der Marker CD1a und DC-SIGN, jedoch höhere Werte der Marker CD84 und HLA-ABC. Nach Stimulation mit Lipopo-

lysaccharid (LPS) produzierten MMAC, analog zu den DC, Interleukin (IL-)10 und Tumornekrosefaktor (TNF-)alpha. Im Vergleich zu MAC zeigten MMAC eine deutlich erhöhte Phagozytoserate und T Zell stimulierende Eigenschaften.

Zusammenfassend zeigten sich MMAC als Makrophagen-ähnliche Zellen mit ausgeprägten Effektoreigenschaften, die jedoch nicht an die der DC heranreichten.

Appendix

7.1 Solutions and media

Annexin V-	10 ml	1M HEPES (Sigma)
binding buffer:	8.12 g	NaCl (Merck)
	0.28 g	$CaCl_2$ (Merck)
	ad 1000 ml	ultra pure water
DC medium:	500 ml	RPMI 1640 (Biochrom)
	5 ml	L-Glutamine (Biochrom)
	5 ml	Sodium pyruvate (100 mM, Gibco)
	5 ml	Non-essential amino acids (100 x, Gibco)
	2 ml	Vitamins (100 x, Gibco)
	2.5 ml	Penicillin-Streptomycin (10^4 U/ml, Gibco)
	0,5 ml	2-Mercaptoethanol (50 mM, Gibco)
	add 10 % *fetal calf serum* (FCS, PAA) right before use	
FACS fixative:	500 ml	PBS (PAN)
	50 ml	Paraformaldehyde (10 % w/v in PBS, Sigma)
FACS washing buffer:	500 ml	PBS (PAA)
	5 ml	Immunoglobulin (60 mg/ml, Sandoz Pharma)
	5 ml	Sodium acide (10 % w/v in PBS, Sigma)

Macrophage medium:	500 ml	RPMI 1640 (Biochrom)
	5 ml	L-Glutamine (Biochrom)
	2.5 ml	Penicillin-Streptomycin (10^4 U/ml, Gibco)
	add 2 % pooled AB-group serum (Cambrex) right before use	
MLR medium:	500 ml	RPMI 1640 (Biochrom)
	5 ml	L-Glutamine (Biochrom)
	5 ml	Sodium pyruvate (100 mM, PAN)
	5 ml	Non-essential amino acids (100 x, PAN)
	2 ml	Vitamins (100 x, PAN)
	2.5 ml	Penicillin-Streptomycin (10^4 U/ml, Gibco)
	0,5 ml	2-Mercaptoethanol (50 mM, Gibco)
	add 10 % pooled male human AB group serum (PAN) right before use	
Trypan blue solution:	0.4 % (w/v)	Trypan blue (Sigma)
	in 0.9 % (w/v)	NaCl (Merck) in ultra pure water

7.2 List of all companies

BD Biosciences	San Jose, CA, USA
BD Pharmingen	San Diego, CA, USA
Beckman Coulter	Fullerton, CA, USA
Beckmann	Munich, Germany
Becton-Dickinson	San Jose, CA, USA
Biochrom	Berlin, Germany
Cambrex	East Rutherford, NJ, USA
Cetus Corp.	Emeryville, CA, USA
Costar	Cambridge, UK
Diaclone	Besançon, France
eBioscience	San Diego, CA, USA
Eppendorf	Hamburg, Germany
Essex	Munich, Germany
BD Falcon	Heidelberg, Germany
Fujifilm	Düsseldorf, Germany
Gibco	Karlsruhe, Germany
Greiner Bio-one	Frickenhausen, Germany
Hareus	Hanau, Germany
Hartmann Analytica	Munich, Germany
Immunotech	Marseille, France
Immunotools	Friesoythe, Germany
Innotech	Dottikon, Switzerland
Jackson ImmunoResearch	Suffolk, UK
Merck	Darmstadt, Germany
PAA	Linz, Austria

PAN	Aidenbach, Germany
PeliCluster	Amsterdam, Netherlands
Pharmacia	Freiburg, Germany
Polyscience	Warrington, PA,USA
Sandoz Pharma AG	Basel, Swizzerland
Sigma	Deisenhof, Germany
SPSS	Chicago, USA
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Bibliography

Adams, D. O. and Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annu Rev Immunol*, 2:283–318.

Aharinejad, S., Paulus, P., Sioud, M., Hofmann, M., Zins, K., Schafer, R., Stanley, E. R., and Abraham, D. (2004). Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. *Cancer Res*, 64(15):5378–5384.

Akagawa, K. S. (2002). Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Int J Hematol*, 76(1):27–34.

Akagawa, K. S., Komuro, I., Kanazawa, H., Yamazaki, T., Mochida, K., and Kishi, F. (2006). Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Respirology*, 11 Suppl:32–36.

Albina, J. E., Mills, C. D., Henry, W. L., and Caldwell, M. D. (1990). Temporal expression of different pathways of l-arginine metabolism in healing wounds. *J Immunol*, 144(10):3877–3880.

Ammon, C., Meyer, S. P., Schwarzfischer, L., Krause, S. W., Andreesen, R., and Kreutz, M. (2000). Comparative analysis of integrin expression on monocyte-derived macrophages and monocyte-derived dendritic cells. *Immunology*, 100(3):364–9.

Ancuta, P., Liu, K. Y., Misra, V., Wacleche, V. S., Gosselin, A., Zhou, X., and Gabuzda, D. (2009). Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. *BMC Genomics*, 10:403–403.

Anderson, C. F. and Mosser, D. M. (2002). A novel phenotype for an activated macrophage: the type 2 activated macrophage. *J Leukoc Biol*, 72(1):101–106.

Andreesen, R., Bross, K. J., Osterholz, J., and Emmrich, F. (1986). Human macrophage maturation and heterogeneity: analysis with a newly generated set of monoclonal antibodies to differentiation antigens. *Blood*, 67(5):1257–1264.

Andreesen, R., Brugger, W., Scheibenbogen, C., Kreutz, M., Leser, H. G., Rehm, A., and Lohr, G. W. (1990). Surface phenotype analysis of human monocyte to macrophage maturation. *J Leukoc Biol*, 47(6):490–7.

Andreesen, R., Picht, J., and Löhr, G. W. (1983). Primary cultures of human blood-born macrophages grown on hydrophobic teflon membranes. *J Immunol Methods*, 56(3):295–304.

Ardavin, C., Martinez del Hoyo, G., Martin, P., Anjuere, F., Arias, C. F., Marin, A. R., Ruiz, S., Parrillas, V., and Hernandez, H. (2001). Origin and differentiation of dendritic cells. *Trends Immunol*, 22(12):691–700.

Arndt, S., Melle, C., Mondal, K., Klein, G., Eggeling, v. F., and Bosserhoff, A. K. (2007). Interactions of TANGO and leukocyte integrin CD11c/CD18 regulate the migration of human monocytes. *J Leukoc Biol*, 82:1466–1472.

Aschoff, L. (1924). Das retikulo-endotheliale System. *Ergeb Inn Med Kinderheilkunde*, 26:1178–80.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol*, 18:767–811.

Becker, S., Warren, M. K., and Haskill, S. (1987). Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *J Immunol*, 139(11):3703–9.

Blumberg, R. S., Gerdes, D., Chott, A., Porcelli, S. A., and Balk, S. P. (1995). Structure and function of the CD1 family of MHC-like cell surface proteins. *Immunol Rev*, 147:5–29.

Bogunovic, M., Ginhoux, F., Helft, J., Shang, L., Hashimoto, D., Greter, M., Liu, K., Jakubzick, C., Ingersoll, M. A., Leboeuf, M., Stanley, E. R., Nussenzweig, M., Lira, S. A., Randolph, G. J., and Merad, M. (2009). Origin of the lamina propria dendritic cell network. *Immunity*, 31(3):513–525.

Brauner, A., Lu, Y., Halldén, G., Hylander, B., and Lundahl, J. (1998). Difference in the blood monocyte phenotype between uremic patients and healthy controls: its relation to monocyte differentiation into macrophages in the peritoneal cavity. *Inflammation*, 22(1):55–66.

Brem-Exner, B. G., Sattler, C., Hutchinson, J. A., Koehl, G. E., Kronenberg, K., Farkas, S., Inoue, S., Blank, C., Knechtle, S. J., Schlitt, H. J., Fändrich, F., and Geissler, E. K. (2008). Macrophages driven to a novel state of activation have anti-inflammatory properties in mice. *J Immunol*, 180(1):335–349.

Brugger, W., Kreutz, M., and Andreesen, R. (1991). Macrophage colony-stimulating factor is required for human monocyte survival and acts as a cofactor for their terminal differentiation to macrophages in vitro. *J Leukoc Biol*, 49(5):483–8.

Cassol, E., Alfano, M., Biswas, P., and Poli, G. (2006). Monocyte-derived macrophages and myeloid cell lines as targets of HIV-1 replication and persistence. *J Leukoc Biol*, 80(5):1018–1030.

Chitu, V. and Stanley, E. R. (2006). Colony-stimulating factor-1 in immunity and inflammation. *Curr Opin Immunol*, 18(1):39–48.

Clanchy, F. I. L., Holloway, A. C., Lari, R., Cameron, P. U., and Hamilton, J. A. (2006). Detection and properties of the human proliferative monocyte subpopulation. *J Leukoc Biol*, 79(4):757–766.

Clarkson, S. B. and Ory, P. A. (1988). CD16. developmentally regulated IgG Fc receptors on cultured human monocytes. *J Exp Med*, 167(2):408–420.

Conti, P., Kempuraj, D., Kandere, K., Di Gioacchino, M., Barbacane, R. C., Castellani, M. L., Felaco, M., Boucher, W., Letourneau, R., and Theoharides, T. C. (2003). Il-10, an inflammatory/inhibitory cytokine, but not always. *Immunol Lett*, 86(2):123–129.

Cravens, P. D. and Lipsky, P. E. (2002). Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. *Immunol Cell Biol*, 80(5):497–505.

Dai, X. M., Zong, X. H., Sylvestre, V., and Stanley, E. R. (2004). Incomplete restoration of colony-stimulating factor 1 (CSF-1) function in CSF-1-deficient CSF1op/CSF1op mice by transgenic expression of cell surface CSF-1. *Blood*, 103(3):1114–1123.

De, A. K., Laudanski, K., and Miller-Graziano, C. L. (2003). Failure of monocytes of trauma patients to convert to immature dendritic cells is related to preferential macrophage-colony-stimulating factor-driven macrophage differentiation. *J Immunol*, 170(12):6355–62.

de la Fuente, M. A., Pizcueta, P., Nadal, M., Bosch, J., and Engel, P. (1997). CD84 leukocyte antigen is a new member of the Ig superfamily. *Blood*, 90(6):2398–2405.

Devaraj, S., Yun, J. M., Duncan-Staley, C., and Jialal, I. (2009). C-reactive protein induces M-CSF release and macrophage proliferation. *J Leukoc Biol*, 85(2):262–267.

Dutronc, Y. and Porcelli, S. A. (2002). The CD1 family and T cell recognition of lipid antigens. *Tissue Antigens*, 60(5):337–353.

Edwards, J. P., Zhang, X., Frauwirth, K. A., and Mosser, D. M. (2006). Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol*, 80(6):1298–1307.

Finnin, M., Hamilton, J. A., and Moss, S. T. (1999). Characterization of a CSF-induced proliferating subpopulation of human peripheral blood monocytes by surface marker expression and cytokine production. *J Leukoc Biol*, 66(6):953–960.

Fithian, E., Kung, P., Goldstein, G., Rubenfeld, M., Fenoglio, C., and Edelson, R. (1981). Reactivity of langerhans cells with hybridoma antibody. *Proc Natl Acad Sci U S A*, 78(4):2541–2544.

Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., Cumano, A., and Geissmann, F. (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*, 311(5757):83–87.

Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van Kooyk, Y., and Figdor, C. G. (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*, 100(5):575–585.

Geijtenbeek, T. B., van Duijnhoven, G. C., van Vliet, S. J., Krieger, E., Vriend, G., Figdor, C. G., and van Kooyk, Y. (2002). Identification of different binding sites in the dendritic cell-specific receptor DC-SIGN for intercellular adhesion molecule 3 and HIV-1. *J Biol Chem*, 277(13):11314–11320.

Geissmann, F., Jung, S., and Littman, D. R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19(1):71–82.

Gerber, J. S. and Mosser, D. M. (2001). Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol*, 166(11):6861–6868.

Gijzen, K., Tacken, P. J., Zimmerman, A., Joosten, B., de Vries, I. J., Figdor, C. G., and Torensma, R. (2007). Relevance of DC-SIGN in DC-induced T cell proliferation. *J Leukoc Biol*, 81(3):729–740.

Ginhoux, F., Tacke, F., Angeli, V., Bogunovic, M., Loubeau, M., Dai, X.-M., Stanley, E. R., Randolph, G. J., and Merad, M. (2006). Langerhans cells arise from monocytes in vivo. *Nat Immunol*, 7(3):265–273.

Giordano, D., Magaletti, D. M., Clark, E. A., and Beavo, J. A. (2003). Cyclic nucleotides promote monocyte differentiation toward a DC-SIGN+ (CD209) intermediate cell and impair differentiation into dendritic cells. *J Immunol*, 171(12):6421–6430.

Gordon, S. (1998). The role of the macrophage in immune regulation. *Res Immunol*, 149(7-8):685–688.

Gordon, S. (2002). Pattern recognition receptors: doubling up for the innate immune response. *Cell*, 111(7):927–930.

Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol*, 3(1):23–35.

Gordon, S. and Taylor, P. R. (2005). Monocyte and macrophage heterogeneity. *Nat Rev Immunol*, 5(12):953–964.

Grage-Griebenow, E., Flad, H. D., and Ernst, M. (2001a). Heterogeneity of human peripheral blood monocyte subsets. *J Leukoc Biol*, 69(1):11–20.

Grage-Griebenow, E., Zawatzky, R., Kahlert, H., Brade, L., Flad, H., and Ernst, M. (2001b). Identification of a novel dendritic cell-like subset of CD64(+) / CD16(+) blood monocytes. *Eur J Immunol*, 31(1):48–56.

Graw, R. G., Herzig, G. P., Eisel, R. J., and Perry, S. (1971). Leukocyte and platelet collection from normal donors with the continuous flow blood cell separator. *Transfusion*, 11(2):94–101.

Greenwald, R. J., Freeman, G. J., and Sharpe, A. H. (2005). The B7 family revisited. *Annu Rev Immunol*, 23:515–548.

Hamilton, J. A. (2008). Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*, 8(7):533–544.

Hamilton, J. A. and Tak, P. P. (2009). The dynamics of macrophage lineage populations in inflammatory and autoimmune diseases. *Arthritis Rheum*, 60(5):1210–1221.

Haskill, S., Johnson, C., Eierman, D., Becker, S., and Warren, K. (1988). Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J Immunol*, 140(5):1690–1694.

Henson, P. M. and Hume, D. A. (2006). Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol*, 27(5):244–250.

Hiasa, M., Abe, M., Nakano, A., Oda, A., Amou, H., Kido, S., Takeuchi, K., Kagawa, K., Yata, K., Hashimoto, T., Ozaki, S., Asaoka, K., Tanaka, E.,

Moriyama, K., and Matsumoto, T. (2009). GM-CSF and IL-4 induce dendritic cell differentiation and disrupt osteoclastogenesis through M-CSF receptor shedding by up-regulation of TNF-alpha converting enzyme (TACE). *Blood*.

Himes, S. R., Sester, D. P., Ravasi, T., Cronau, S. L., Sasmono, T., and Hume, D. A. (2006). The JNK are important for development and survival of macrophages. *J Immunol*, 176(4):2219–28.

Hoves, S., Krause, S., Schütz, C., Halbritter, D., Schölmerich, J., Herfarth, H., and Fleck, M. (2006). Monocyte-derived human macrophages mediate anergy in allogenic T cells and induce regulatory T cells. *Journal of immunology*, 177:2691–2698.

Hu, M., Miller, E. J., Lin, X., and Simms, H. H. (2004). Transmigration across a lung epithelial monolayer delays apoptosis of polymorphonuclear leukocytes. *Surgery*, 135(1):87–98.

Hume, D. A. (2006). The mononuclear phagocyte system. *Curr Opin Immunol*, 18(1):49–53.

Hume, D. A. (2008). Macrophages as apc and the dendritic cell myth. *J Immunol*, 181(9):5829–5835.

Hume, D. A., Ross, I. L., Himes, S. R., Sasmono, R. T., Wells, C. A., and Ravasi, T. (2002). The mononuclear phagocyte system revisited. *J Leukoc Biol*, 72(4):621–627.

Hunger, R. E., Sieling, P. A., Ochoa, M. T., Sugaya, M., Burdick, A. E., Rea, T. H., Brennan, P. J., Belisle, J. T., Blauvelt, A., Porcelli, S. A., and Modlin, R. L. (2004). Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *J Clin Invest*, 113(5):701–708.

Imhof, B. A. and Aurrand-Lions, M. (2004). Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol*, 4(6):432–444.

Irvine, K. M., Andrews, M. R., Fernandez-Rojo, M. A., Schroder, K., Burns, C. J., Su, S., Wilks, A. F., Parton, R. G., Hume, D. A., and Sweet, M. J. (2009). Colony-stimulating factor-1 (CSF-1) delivers a proatherogenic signal to human macrophages. *J Leukoc Biol*, 85(2):278–288.

Ishii, M., Wen, H., Corsa, C. A., Liu, T., Coelho, A. L., Allen, R. M., Carson, W. F., Cavassani, K. A., Li, X., Lukacs, N. W., Hogaboam, C. M., Dou, Y., and Kunkel, S. L. (2009). Epigenetic regulation of the alternatively activated macrophage phenotype. *Blood*, 114(15):3244–3254.

Jacob, S. S., Shastry, P., and Sudhakaran, P. R. (2002). Monocyte-macrophage differentiation in vitro: modulation by extracellular matrix protein substratum. *Mol Cell Biochem*, 233(1-2):9–17.

Jakubzick, C., Tacke, F., Ginhoux, F., Wagers, A. J., van Rooijen, N., Mack, M., Merad, M., and Randolph, G. J. (2008). Blood monocyte subsets differentially give rise to CD103+ and CD103- pulmonary dendritic cell populations. *J Immunol*, 180(5):3019–3027.

Janeway, C. A. and Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol*, 20:197–216.

Johnson, W. D., Mei, B., and Cohn, Z. A. (1977). The separation, long-term cultivation, and maturation of the human monocyte. *J Exp Med*, 146(6):1613–1626.

Kacinski, B. M. (1997). CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract. *Mol Reprod Dev*, 46(1):71–74.

Kamada, N., Hisamatsu, T., Honda, H., Kobayashi, T., Chinen, H., Kitazume, M. T., Takayama, T., Okamoto, S., Koganei, K., Sugita, A., Kanai, T., and Hibi, T. (2009). Human CD14+ macrophages in intestinal lamina propria exhibit potent antigen-presenting ability. *J Immunol*, 183(3):1724–1731.

Karnovsky, M. L. (1981). Metchnikoff in messina: a century of studies on phagocytosis. *N Engl J Med*, 304(19):1178–1180.

Kawanaka, N., Nagake, Y., Yamamura, M., and Makino, H. (2002a). Expression of Fc gamma receptor III (CD16) on monocytes during hemodialysis in patients with chronic renal failure. *Nephron*, 90(1):64–71.

Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y., and Makino, H. (2002b). CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum*, 46(10):2578–2586.

Komuro, I., Yasuda, T., Iwamoto, A., and Akagawa, K. S. (2005). Catalase plays a critical role in the CSF-independent survival of human macrophages via regulation of the expression of bcl-2 family. *J Biol Chem*, 280(50):41137–41145.

Krause, S. W., Rehli, M., and Andreesen, R. (1998). Carboxypeptidase M as a marker of macrophage maturation. *Immunol Rev*, 161:119–127.

Krause, S. W., Rehli, M., Heinz, S., Ebner, R., and Andreesen, R. (2000). Characterization of MAX.3 antigen, a glycoprotein expressed on mature macrophages, dendritic cells and blood platelets: identity with CD84. *Biochem J*, 346 Pt 3:729–736.

Krutzik, S. R., Tan, B., Li, H., Ochoa, M. T., Liu, P. T., Sharfstein, S. E., Graeber, T. G., Sieling, P. A., Liu, Y.-J., Rea, T. H., Bloom, B. R., and

Modlin, R. L. (2005). TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. *Nat Med*, 11(6):653–660.

Le Meur, Y., Jose, M. D., Mu, W., Atkins, R. C., and Chadban, S. J. (2002). Macrophage colony-stimulating factor expression and macrophage accumulation in renal allograft rejection. *Transplantation*, 73(8):1318–1324.

Le Meur, Y., Leprivey-Lorgeot, V., Mons, S., José, M., Dantal, J., Lemauff, B., Aldigier, J. C., Leroux-Robert, C., and Praloran, V. (2004). Serum levels of macrophage-colony stimulating factor (M-CSF): a marker of kidney allograft rejection. *Nephrol Dial Transplant*, 19(7):1862–1865.

Leon, B., Lopez-Bravo, M., and Ardavin, C. (2005). Monocyte-derived dendritic cells. *Seminars in Immunology*, 17(4):313–318.

Liu, K., Victora, G. D., Schwickert, T. A., Guermonprez, P., Meredith, M. M., Yao, K., Chu, F. F., Randolph, G. J., Rudensky, A. Y., and Nussenzweig, M. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science*, 324(5925):392–397.

MacDonald, K. P. A., Rowe, V., Bofinger, H. M., Thomas, R., Sasmono, T., Hume, D. A., and Hill, G. R. (2005). The colony-stimulating factor 1 receptor is expressed on dendritic cells during differentiation and regulates their expansion. *J Immunol*, 175(3):1399–1405.

Martin, L. H., Calabi, F., and Milstein, C. (1986). Isolation of CD1 genes: a family of major histocompatibility complex-related differentiation antigens. *Proc Natl Acad Sci U S A*, 83(23):9154–9158.

Martin, M., Romero, X., de la Fuente, M. A., Tovar, V., Zapater, N., Esplugues, E., Pizcueta, P., Bosch, J., and Engel, P. (2001). CD84 functions as a ho-

mophilic adhesion molecule and enhances IFN-gamma secretion: adhesion is mediated by Ig-like domain 1. *J Immunol*, 167(7):3668–3676.

Martinez, F. O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: New molecules and patterns of gene expression. *J Immunol*, 177(10):7303–7311.

Mikolajczyk, T. P., Skrzeczynska-Moncznik, J. E., Zarebski, M. A., Marewicz, E. A., Wisniewska, A. M., Dzieba, M., Dobrucki, J. W., and Pryjma, J. R. (2009). Interaction of human peripheral blood monocytes with apoptotic polymorphonuclear cells. *Immunology*, 128(1):103–113.

Mondal, K., Sirenko, O. I., Lofquist, A. K., Morris, J. S., Haskill, J. S., and Watson, J. M. (2000). Differential role of tyrosine phosphorylation in adhesion-induced transcription, mRNA stability, and cytoskeletal organization in human monocytes. *J Leukoc Biol*, 67(2):216–225.

Morrison, D. C. and Ryan, J. L. (1979). Bacterial endotoxins and host immune responses. *Adv Immunol*, 28:293–450.

Mosser, D. M. (2003). The many faces of macrophage activation. *J Leukoc Biol*, 73(2):209–212.

Munn, D. H., Bree, A. G., Beall, A. C., Kaviani, M. D., Sabio, H., Schaub, R. G., Alpaugh, R. K., Weiner, L. M., and Goldman, S. J. (1996). Recombinant human macrophage colony-stimulating factor in nonhuman primates: selective expansion of a CD16+ monocyte subset with phenotypic similarity to primate natural killer cells. *Blood*, 88(4):1215–1224.

Nandi, S., Akhter, M. P., Seifert, M. F., Dai, X. M., and Stanley, E. R. (2006). Developmental and functional significance of the CSF-1 proteoglycan chondroitin sulfate chain. *Blood*, 107(2):786–795.

Nemunaitis, J. (1998). Use of macrophage colony-stimulating factor in the treatment of fungal infections. *Clin Infect Dis*, 26(6):1279–1281.

Niedermeier, M., Reich, B., Gomez, M. R., Denzel, A., Schmidbauer, K., Göbel, N., Talke, Y., Schweda, F., and Mack, M. (2009). CD4+ t cells control the differentiation of Gr1+ monocytes into fibrocytes. *Proc Natl Acad Sci U S A*, 106(42):17892–17897.

Njoroge, J. M., Mitchell, L. B., Centola, M., Kastner, D., Raffeld, M., and Miller, J. L. (2001). Characterization of viable autofluorescent macrophages among cultured peripheral blood mononuclear cells. *Cytometry*, 44(1):38–44.

O'Sullivan, B. and Thomas, R. (2003). Recent advances on the role of CD40 and dendritic cells in immunity and tolerance. *Curr Opin Hematol*, 10(4):272–278.

Palucka, K. A., Taquet, N., Sanchez-Chapuis, F., and Gluckman, J. C. (1998). Dendritic cells as the terminal stage of monocyte differentiation. *J Immunol*, 160(9):4587–95.

Pasquier, B., Lepelletier, Y., Baude, C., Hermine, O., and Monteiro, R. C. (2004). Differential expression and function of IgA receptors (CD89 and CD71) during maturation of dendritic cells. *J Leukoc Biol*, 76(6):1134–1141.

Passlick, B., Flieger, D., and Ziegler-Heitbrock, H. W. (1989). Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*, 74(7):2527–2534.

Pixley, F. J. and Stanley, E. R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol*, 14(11):628–638.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR4 gene. *Science*, 282(5396):2085–2088.

Randolph, G. J., Beaulieu, S., Lebecque, S., Steinman, R. M., and Muller, W. A. (1998). Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science*, 282(5388):480–483.

Randolph, G. J., Sanchez-Schmitz, G., Liebman, R. M., and Schäkel, K. (2002). The CD16(+) (Fc gamma RIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J Exp Med*, 196(4):517–527.

Rehli, M., Krause, S. W., Kreutz, M., and Andreesen, R. (1995). Carboxypeptidase M is identical to the MAX.1 antigen and its expression is associated with monocyte to macrophage differentiation. *J Biol Chem*, 270(26):15644–15649.

Rogers, N. J., Jackson, I. M., Jordan, W. J., Lombardi, G., Delikouras, A., and Lechler, R. I. (2003). CD40 can costimulate human memory T cells and favors IL-10 secretion. *Eur J Immunol*, 33(4):1094–1104.

Ross, J. and Auger, M. (2002). *The macrophage*, chapter The biology of the macrophage. Oxford University Press, second edition.

Rovida, E., Lugli, B., Barbetti, V., Giuntoli, S., Olivotto, M., and Dello Sbarba, P. (2005). Focal adhesion kinase is redistributed to focal complexes and mediates cell spreading in macrophages in response to M-CSF. *Biol Chem*, 386(9):919–929.

Ryan, G. R., Dai, X. M., Dominguez, M. G., Tong, W., Chuan, F., Chisholm, O., Russell, R. G., Pollard, J. W., and Stanley, E. R. (2001). Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood*, 98(1):74–84.

Saionji, K. and Ohsaka, A. (2001). Expansion of CD14+CD16+ blood monocytes in patients with chronic renal failure undergoing dialysis: possible involvement of macrophage colony-stimulating factor. *Acta Haematol*, 105(1):21–26.

Saleh, M. N., Goldman, S. J., LoBuglio, A. F., Beall, A. C., Sabio, H., McCord, M. C., Minasian, L., Alpaugh, R. K., Weiner, L. M., and Munn, D. H. (1995). CD16+ monocytes in patients with cancer: spontaneous elevation and pharmacologic induction by recombinant human macrophage colony-stimulating factor. *Blood*, 85(10):2910–2917.

Sallusto, F. and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*, 179(4):1109–1118.

Sanchez-Torres, C., Garcia-Romo, G. S., Cornejo-Cortes, M. A., Rivas-Carvalho, A., and Sanchez-Schmitz, G. (2001). CD16+ and CD16- human blood monocyte subsets differentiate in vitro to dendritic cells with different abilities to stimulate CD4+ T cells. *International Immunology*, 13(12):1571–1581.

Sanderson, R. J., Shepperdson, R. T., Vatter, A. E., and Talmage, D. W.

(1977). Isolation and enumeration of peripheral blood monocytes. *J Immunol*, 118(4):1409–1414.

Sasmono, R. T., Oceandy, D., Pollard, J. W., Tong, W., Pavli, P., Wainwright, B. J., Ostrowski, M. C., Himes, S. R., and Hume, D. A. (2003). A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood*, 101(3):1155–1163.

Scheibenbogen, C. and Andreesen, R. (1991). Developmental regulation of the cytokine repertoire in human macrophages: IL-1, IL-6, TNF-alpha, and M-CSF. *J Leukoc Biol*, 50(1):35–42.

Shi, C. and Simon, D. I. (2006). Integrin signals, transcription factors, and monocyte differentiationignals, transcription factors, and monocyte differentiationals, transcription factors, and monocyte differentiation. *Trends in Cardiovascular Medicine*, 16(5):146–152.

Skinner, N. A., MacIsaac, C. M., Hamilton, J. A., and Visvanathan, K. (2005). Regulation of Toll-like receptor (TLR)2 and TLR4 on CD14dimCD16+ monocytes in response to sepsis-related antigens. *Clin Exp Immunol*, 141(2):270–278.

Skrzeczyńska-Moncznik, J., Bzowska, M., Loseke, S., Grage-Griebenow, E., Zembala, M., and Pryjma, J. (2008). Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. *Scand J Immunol*, 67(2):152–159.

Smith, W., Feldmann, M., and Londei, M. (1998). Human macrophages induced in vitro by macrophage colony-stimulating factor are deficient in IL-12 production. *Eur J Immunol*, 28(8):2498–2507.

Soilleux, E. J., Morris, L. S., Leslie, G., Chehimi, J., Luo, Q., Levroney, E., Trowsdale, J., Montaner, L. J., Doms, R. W., Weissman, D., Coleman, N., and Lee, B. (2002). Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. *J Leukoc Biol*, 71(3):445–457.

Song, E., Ouyang, N., Hörbelt, M., Antus, B., Wang, M., and Exton, M. S. (2000). Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol*, 204(1):19–28.

Stanley, E. R., Berg, K. L., Einstein, D. B., Lee, P. S., Pixley, F. J., Wang, Y., and Yeung, Y. G. (1997). Biology and action of colony-stimulating factor-1. *Mol Reprod Dev*, 46(1):4–10.

Stein, M., Keshav, S., Harris, N., and Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*, 176(1):287–292.

Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K., and Suttles, J. (2005). Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol*, 175(1):342–349.

Stout, R. D. and Suttles, J. (2004). Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol*, 76(3):509–513.

Stout, R. D., Watkins, S. K., and Suttles, J. (2009). Functional plasticity of macrophages: in situ reprogramming of tumor-associated macrophages. *J Leukoc Biol*.

Sunderkotter, C., Nikolic, T., Dillon, M. J., van Rooijen, N., Stehling, M., Drevets, D. A., and Leenen, P. J. M. (2004). Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *The Journal of Immunology*, 172(7):4410–4417.

Sutterwala, F. S., Noel, G. J., Salgame, P., and Mosser, D. M. (1998). Reversal of proinflammatory responses by ligating the macrophage Fc gamma receptor type I. *J Exp Med*, 188(1):217–222.

Tacke, F. and Randolph, G. J. (2006). Migratory fate and differentiation of blood monocyte subsets. *Immunobiology*, 211(6-8):609–618.

Taylor, P. R., Martinez-Pomares, L., Stacey, M., Lin, H. H., Brown, G. D., and Gordon, S. (2005). Macrophage receptors and immune recognition. *Annu Rev Immunol*, 23:901–944.

Tobias, P. S., Tapping, R. I., and Gegner, J. A. (1999). Endotoxin interactions with lipopolysaccharide-responsive cells. *Clin Infect Dis*, 28(3):476–481.

Todd, R. F., Nadler, L. M., and Schlossman, S. F. (1981). Antigens on human monocytes identified by monoclonal antibodies. *J Immunol*, 126(4):1435–1442.

Unanue, E. R. (2002). Perspective on antigen processing and presentation. *Immunol Rev*, 185:86–102.

van Furth, R. (1982). Current view on the mononuclear phagocyte system. *Immunobiology*, 161(3-4):178–185.

van Kooyk, Y. and Geijtenbeek, T. B. (2003). DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol*, 3(9):697–709.

Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., Fehling, H. J., Hardt, W. D., Shakhar, G., and Jung, S. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity*, 31(3):502–512.

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*, 184(1):39–51.

Way, K. J., Dinh, H., Keene, M. R., White, K. E., Clanchy, F. I., Lusby, P., Roiniotis, J., Cook, A. D., Cassady, A. I., Curtis, D. J., and Hamilton, J. A. (2009). The generation and properties of human macrophage populations from hemopoietic stem cells. *J Leukoc Biol*, 85(5):766–778.

Weinberg, J. B., Haney, A. F., Xu, F. J., and Ramakrishnan, S. (1991). Peritoneal fluid and plasma levels of human macrophage colony-stimulating factor in relation to peritoneal fluid macrophage content. *Blood*, 78(2):513–516.

Wiktor-Jedrzejczak, W. and Gordon, S. (1996). Cytokine regulation of the macrophage (m phi) system studied using the colony stimulating factor-1-deficient op/op mouse. *Physiological Reviews*, 76(4):927–947.

Williams, M. R., Sakurai, Y., Zughaier, S. M., Eskin, S. G., and McIntire, L. V. (2009). Transmigration across activated endothelium induces transcriptional changes, inhibits apoptosis, and decreases antimicrobial protein expression in human monocytes. *J Leukoc Biol*.

Wright, S., Ramos, R., Tobias, P., Ulevitch, R., and Mathison, J. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, 249(4975):1431–1433.

Xu, W., Roos, A., Schlagwein, N., Woltman, A. M., Daha, M. R., and van Kooten, C. (2006). Il-10-producing macrophages preferentially clear early apoptotic cells. *Blood*, 107(12):4930–4937.

Xu, W., Schlagwein, N., Roos, A., van den Berg, T. K., Daha, M. R., and van Kooten, C. (2007). Human peritoneal macrophages show functional characteristics of M-CSF-driven anti-inflammatory type 2 macrophages. *Eur J Immunol*, 37(6):1594–1599.

Zaiss, M., Hirtreiter, C., Rehli, M., Rehm, A., Kunz-Schughart, L. A., Andreesen, R., and Hennemann, B. (2003). CD84 expression on human hematopoietic progenitor cells. *Exp Hematol*, 31(9):798–805.

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