1 Introduction

According to the view of modern behavioral and psychosomatic medicine, autonomous and hormonal changes which result from interaction between burdensome life events and individual factors (e.g. coping strategies) can lead to adverse health consequences with involvement of the immune system (Ehlert & von Känel, 2010). Research findings in the field of psychoneuroimmunology (PNI) partly constitute the empirical basis of this view. PNI is an interdisciplinary science that studies the interactions between the nervous, immune and endocrine systems, and particularly how these interactions contribute to health.

A large amount of research interest in the field of PNI is directed towards wound healing (Godbout & Glaser, 2006). It has repeatedly been shown that psychological stress delays skin wound healing (Walburn, Vedhara, Hankins, Rixon, & Weinman, 2009). An efficient immune reaction is essential, especially in early phases of wound healing. Only then can microbes which have penetrated into the wound area be killed and wound healing can progress normally (Rojas, Padgett, Sheridan, & Marucha, 2002). Although current findings suggest that the stress-induced delay of wound healing is associated with a suppressed immune reaction, the exact mechanisms underplaying this stress effect remain to be elucidated (Gouin & Kiecolt-Glaser, 2011).

A special type of immune cell, which is found in peripheral tissues and plays a major role in the wound healing process, is called the macrophage (Mahdavian Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011). Macrophages, recruited in early phases of wound healing, are characterized by high microbicidal activity, through which they kill potential microbes and prevent colonization of the wound (Mahdavian Delavary et al.,

2011). Moreover, they also promote inflammatory processes and hence generally increase the efficiency of the immune reaction locally (Martinez, Sica, Mantovani, & Locati, 2008).

Given that psychological stress can impair wound healing (Altemus, Rao, Dhabhar, Ding, & Granstein, 2001; Robles, 2007; Robles, Brooks, & Pressman, 2009; Walburn et al., 2009), and given the role of microbicidally active macrophages in early wound healing phases (Mahdavian Delavary et al., 2011), it might be speculated that psychological stress exerts at least part of its wound healing impairment by inhibiting the microbicidal potential of macrophages. However, the effect of psychological stress on the microbicidal potential of human macrophages has not yet been investigated. One reason for this lack of research may be related to both the absence of and familiarity with simple and cost-efficient methods for analyzing the macrophages' microbicidal potential.

Therefore, the aim of this thesis was to establish an appropriate method for the investigation of the microbicidal potential of human macrophages in a first step. In a second step, we set out to examine the influence of a psychological stressor on the microbicidal potential of human macrophages within a wound paradigm. It was hypothesized that this research project would shed more light on the mechanisms by which psychological stress delays skin wound healing.

The presentation is organized as follows: First the theoretical background briefly reviews the subject areas of macrophages, psychological stress and wound healing, which form the basis of this thesis (chapter 2). Following this, the data from the two studies conducted as part of the present thesis are presented (chapter 3). Finally, the thesis is concluded with a

general discussion of the obtained results and their implications for future studies (chapter 4).

2 Theoretical background

In this thesis, three large subject areas are integrated, namely macrophages (section 2.1), psychological stress (section 2.2), and wound healing (section 2.3). Since each of these subject areas is quite detailed and complex in its own right, they are first presented in separate sections before being integrated in sections 2.4 and 2.5.

2.1 Macrophages

Macrophages are tissue-based leukocytes that can be found throughout the body in almost all lymphoid and non-lymphoid tissues (Gordon, 2007). They play a crucial role in both innate and adaptive immunity. Among other things, these cells are able to engulf and kill microbes, infected or unwanted cells, and to activate T-cells by presenting antigens. Moreover, macrophages are highly secretory cells. They can secrete molecules that promote or inhibit inflammation, growth regulation and hematopoiesis, influence lymphocyte function, affect tissue repair and turnover, act as autoregulatory factors or enhance the cell's microbicidal potential (Adams & Hamilton, 1984). The main effector functions of macrophages are illustrated in Figure 1.

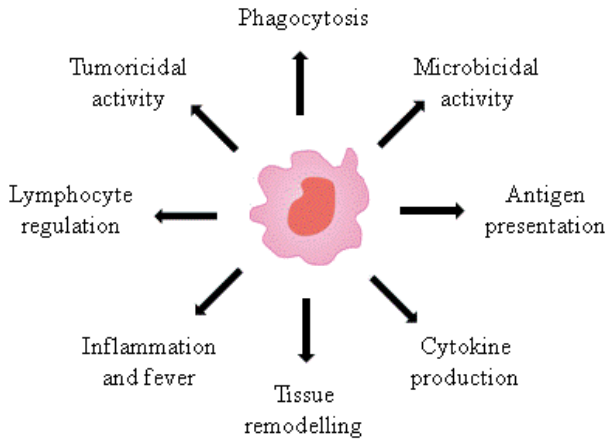

Figure 1. Major effector functions of macrophages. Adapted from Woods et al. (2000).

The following chapters will describe the characteristics and functions of these cells. Finally, practical aspects regarding the assessment of the microbicidal potential of human macrophages will be addressed.

2.1.1 The mononuclear phagocyte system

Macrophages are the terminally differentiated cells of the mononuclear phagocyte system (MPS). This system comprises bone marrow monoblasts and promonocytes, peripheral blood monocytes and tissue macrophages (van Furth et al., 1972). All cells of this lineage, together called mononuclear phagocytes, derive from a common pluripotent stem cell in the bone marrow, the so-called hematopoietic stem cell (HSC; see Figure 2).

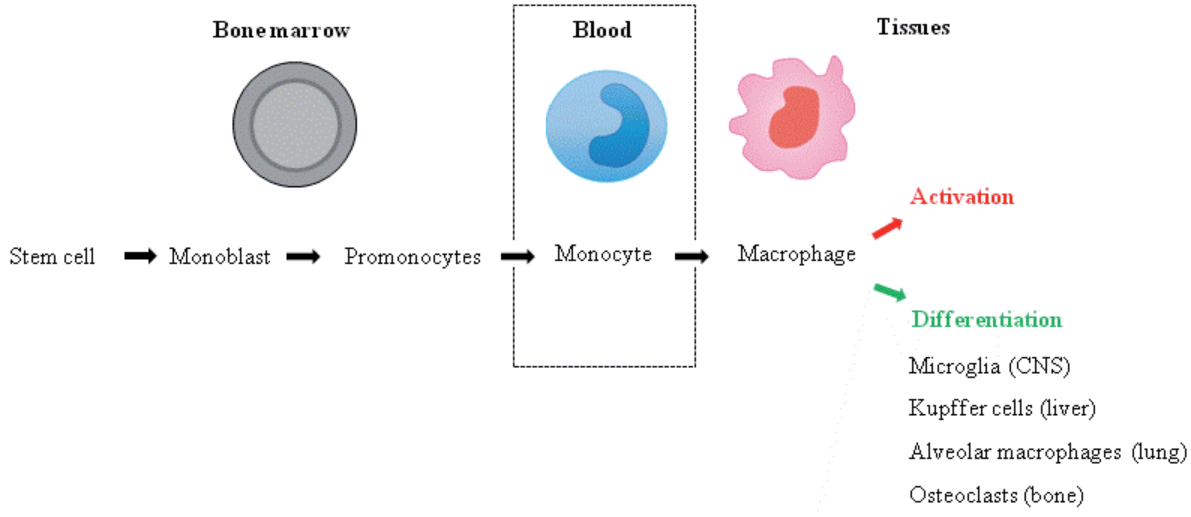

Figure 2. Differentiation of mononuclear phagocytes. Adapted from Abbas et al. (2007).

The primary function of the mononuclear phagocytes is phagocytosis. In addition, all cells of the MPS share further similarities in terms of functional, cytochemical, and morphological characteristics.

2.1.1.1 Monocytes and monocyte-derived macrophages

The precursor cells of tissue-based macrophages are monocytes. Monocytes, in turn, differentiate from precursor cells in the bone marrow (see Figure 2). This differentiation is orchestrated by sequential expression and action of different transcription factors, such as CCAAT/enhancer-binding protein β (C/EBPβ), acute myeloid leukemia, and IFN regulatory factor 8 (IRF8; Nagamura-Inoue, Tamura, & Ozato, 2001). The newly-formed monocytes remain in the bone marrow for about 24 hours before migrating into the blood (van Furth & Sluiter, 1986).

Monocytes circulate in the blood, bone marrow, and spleen (Auffray, Sieweke, & Geissmann, 2009). In humans, circulating blood monocytes represent 10% of the peripheral blood leukocytes (Auffray et al., 2009). Together with lymphocytes, they constitute the so-called peripheral blood monocytes (PBMC).

When monocytes migrate into tissues, they differentiate further into macrophages or dendritic cells (DC). This migration can occur either in response to an inflammatory stimulus or at a much lower level in the absence of any specific cues. Accumulating evidence demonstrates that there are at least two functionally distinct subpopulations of monocytes that can differentiate into tissue macrophages (Geissmann, Jung, & Littman, 2003; Gordon & Taylor, 2005).

In a murine model, it has been shown that under steady-state conditions, i.e. in the absence of inflammation, tissue-resident macrophages are generally differentiated from a monocyte subpopulation characterized as Gr-1_{low}CX3CR1_{high}CCR2^{-}CD62L^{-} monocytes (Zhang & Mosser, 2009) (Tacke & Randolph, 2006). These cells are functionally most similar to human CD14^{+}CD16^{+}CX3CR1_{high}CCR2^{-}CD62L^{-} monocytes (Tacke & Randolph, 2006), representing less than 10% of circulating blood monocytes (Serbina, Jia, Hohl, & Pamer, 2008). The migration of these monocytes into tissue under steady-state conditions contributes to the maintenance of constant numbers of macrophages in tissues. Notably, a further significant contribution to the maintenance of tissue-resident macrophage is made by proliferation of local progenitor cells (Daems & de Bakker, 1982; Landsman, Varol, & Jung, 2007; Mildner et al., 2007; Sawyer, Strausbauch, & Volkman, 1982; Tarling, Lin, & Hsu, 1987).

During inflammation, however, the dynamics of accumulating macrophages is considerably altered. Newly recruited blood monocytes, which differentiate rapidly into so-called inflammatory macrophages, contribute substantially to the increase of macrophages at inflamed tissue sites. Studying the origin and characteristics of skin macrophages during inflammation in a mouse model, van Furth and colleagues even found that 99% or more of the macrophages occurring in the inflammatory exudates derive from circulating blood monocytes (newly recruited inflammatory macrophages), while 1% or less originate through local proliferation of tissue-resident skin macrophages (van Furth, Nibbering, van Dissel, & Diesselhoff-den Dulk, 1985). The newly recruited monocytes typically correspond to GR-1_{high}CX3CR1_{low}CCR2^{+}CD62L monocytes in mice or to $CD14_{high}CD16^{-}$CXCR1_{low}CCR2^{+}CD62L monocytes in humans (Gordon, 2007; Tacke & Randolph, 2006). $CD14_{high}CD16^{-}$CXCR1_{low}CCR2^{+}CD62L monocytes, also known as inflammatory monocytes, represent about 80-90% of circulating human blood monocytes (Serbina et al., 2008).

The recruitment of inflammatory monocytes from the bloodstream into inflamed tissue is a multi-step process, which is significantly mediated by adhesion molecules and cytokines.

Step 1: After the occurrence of different stimuli, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α from activated tissue-resident macrophages and bacterial peptides, endothelial cells express the adhesion molecule P-selectin on their surface within a few minutes, and then the adhesion molecule E-selectin with a time lag of about 1-2 hours (Abbas et al. 2007). On the other hand, monocytes express carbohydrate ligands for P- and E-selectins, which allow them to interact with the selectins on the endothelium. Selectin-selectin ligand interactions reveal a low affinity and fast off-rate. A stable binding of

monocytes to the endothelium is therefore not possible. Instead, the monocytes "roll" along the endothelium as a consequence of this unstable bond (Abbas et al. 2007).

Step 2: A stable bond between monocytes and the endothelium takes place as a response to the interaction between the monocyte integrins very late antigen-4 (VLA-4; CD49dCD29), leukocyte function-associated antigen one (LFA-1; CD11aCD18), macrophage 1 antigen (Mac1; CD11bCD18) and corresponding adhesion molecules of the endothelium such as vascular cell adhesion protein (VCAM)-1, inter-cellular adhesion molecule (ICAM)-1-3, iC3b, fibronectin and Factor X (Imhof & Aurrand-Lions, 2004). This interaction is mainly promoted by the chemotactic cytokine monocyte chemoattractant protein (MCP)-1 (CCL2) bound on the luminal surface of endothelial cells. MCP-1 is secreted by tissue-resident macrophages and endothelial cells in response to microbial products, IL-1, and TNF-α. MCP-1 interacts with corresponding CCR2 (receptors for CCL2) on monocytes. This causes a conformational change of integrins on the rolling monocytes, thereby increasing the affinity of the monocyte integrins to adhesion molecule of the endothelium. A stable bond is formed between the endothelium and monocyte (Imhof & Aurrand-Lions, 2004).

Step 3: As a result of the stable bond, monocytes pass through the vessel wall and migrate into the connective tissue. This process is called diapedesis.

Step 4: The monocytes migrate along the chemokine concentration gradient to the site of infection and differentiate into inflammatory macrophages. Notably, the essential factor regulating the differentiation process from monocytes into macrophages is macrophage colony-stimulating factor (M-CSF; Hume, 2006; Hume et al., 2002). It has been shown that M-CSF is produced by both tissue cells and macrophages. Hence, macrophages can

control their differentiation by using M-CSF in an autocrine manner (Popova, Kzhyshkowska, Nurgazieva, Goerdt, & Gratchev, 2011).

2.1.1.2 Macrophage activation during inflammation

In order to act in an effective way at sites of inflammation, macrophages need to be activated. Macrophage activation is the result of stimulus-induced modification of gene expression such that the functional competence of macrophages is either increased or acquired where it did not previously exist (Adams & Hamilton, 1984; Barish et al., 2005).

Traditionally, activated macrophages have been described as antigen-presenting phagocytes with high microbicidal activity and enhanced secretion of pro-inflammatory cytokines (Mosser, 2003). However, a series of *in vitro* studies investigating the macrophages attracted to inflamed tissue sites – that is, inflammatory monocyte-derived macrophages – revealed phenotypically polarized macrophages, depending on the stimuli used to activate them (Gordon, 2007; Gordon & Taylor, 2005).

Activated macrophages can differ in terms of receptor expression, cytokine production, chemokine repertoires and effector function (Mantovani et al., 2004). They are generally referred to as pro-inflammatory (M1) or classically activated macrophages and anti-inflammatory (M2) or alternatively activated macrophages (Mantovani et al., 2004; Pelegrin & Surprenant, 2009).

2.1.1.2.1 Classically activated macrophages

Classically activated M1 macrophages are characterized by an up-regulation of the surface molecules major histocompatibility complex (MHC) class II and B-7 (CD86; a

costimulator for T-cell activation) accompanied by a simultaneous down-regulation of mannose receptors (this receptor binds mannose and fucose residues on microbial walls and mediates phagocytosis). Furthermore, their secretory profile is dramatically changed. They secrete high levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12, IL-15, IL-18, and TNF-α and therefore propagate the T helper cell (Th)1 response (Gordon, 2003; Martinez et al., 2008; Mosser, 2003). They also secrete high amounts of the chemokines CCL15/HCC-2 (for mixed leukocyte recruitment), CCL20/MIP-3α, CXCL9/Mig (for effector T-cell recruitment), CXCL10/IP10 (for effector T-cell recruitment), CXCL13/BCA-1 (for B-cell migration; Martinez, Gordon, Locati, & Mantovani, 2006).

Functionally, M1 macrophages display elevated endocytic functions and enhanced microbicidal activity, i.e. killing of microbes (Martinez et al., 2008). The latter is mainly mediated by increased secretion of microbe-killing, highly oxidizing agents, the so-called reactive oxygen species (ROS; e.g. superoxide anions [O_2^-]), and reactive nitrogen species (RNS; e.g. nitrogen oxide [NO]; Dale, Boxer, & Liles, 2008; Taylor et al., 2005).

Regarding their involvement in biological processes, it has been verified that M1 macrophages increase the magnitude of inflammatory processes and play a significant role during the early phases of wound healing primarily due to their pro-inflammatory and microbicidal activity (Mahdavian Delavary et al., 2011; for details see section 2.3). However, it is also known that uncontrolled activity of M1 macrophages is associated with chronic inflammation and tissue damage (Mosser, 2003). The latter is caused by synthesized radicals (e.g., ROS; see section 2.1.2.1) and by the proteolytic enzymes matrix metalloproteinase (MMP)-1, -2, -7, -9 and -12 secreted by M1 macrophages, which are known to degrade collagen, elastin, fibronectin, and other components of the extracellular

matrix (ECM; Chizzolini, Rezzonico, De Luca, Burger, & Dayer, 2000; Gibbs, Warner, Weiss, Johnson, & Varani, 1999).

M1 macrophages are formed in response to interferon (IFN)-γ, alone or in combination with microbial products such as lipopolysaccharides (LPS), or further cytokines (e.g. TNF-α; Martinez et al., 2008).

Under physiological conditions, IFN-γ is primarily secreted by natural killer (NK) cells or activated Th1 and CD8+ cytotoxic lymphocytes (Martinez et al., 2008). The binding of IFN-γ to IFN- γ receptors on the surface of macrophage cells induces a change in the conformation of the receptor, which in turn activates the Janus kinase /Signal transducers and activators of transcription protein (Jak-STAT) signaling pathway. The Jak-STAT signaling pathway transmits signals rapidly and directly to the cell nucleus and to gene promoters that are regulated by IFN-γ (Schroder, Hertzog, Ravasi, & Hume, 2004). In addition to features characteristic of M1, such as the subunit genes of the enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (i.e. gp91phox, p67phox, and p47phox), which is necessary for ROS production and thus microbicidal activity (see section 2.1.2), IFN-γ boosts transcription of the LPS receptor (toll-like receptor [TLR] 4) gene in macrophages, which primes the cells for a more rapid and increased LPS-induced response (Dalton et al., 1993; S. Huang et al., 1993; Sweet, Stacey, Kakuda, Markovich, & Hume, 1998).

LPS is the principal cell wall component of gram-negative bacteria. It is recognized by the soluble LPS-binding protein and delivered to a cell surface receptor complex that consists of the TLR 4 and the protein MD2 (Guha & Mackman, 2001). Several intracellular signaling pathways are activated in LPS-stimulated mononuclear phagocytes, such as

IkappaB kinase (IKK)- nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway as well as three mitogen-activated protein kinase (MAPK) pathways, which are extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK), and p38 (Guha & Mackman, 2001). These signaling pathways directly or indirectly phosphorylate and activate various transcription factors, including NF-κB and activator protein 1 (AP-1; c-Fos/c-Jun), which in turn coordinate the gene transcription of many inflammatory mediators, such as TNF-α, and subunits of the NADPH oxidase (i.e. p47phox, p67phox, and Rac; Guha & Mackman, 2001). LPS increases the transcription of features typical for M1 via activation of these signaling pathways.

TNF-α is synthesized primarily by macrophages themselves as a response to LPS or IFN-γ (Takashiba et al., 1999). Like LPS, TNF-α contributes to the M1 phenotype via activation of the IKK-NF-κB pathway and the MAPK pathway (Huang, Krein, Muruve, & Winston, 2002; Takashiba et al., 1999). TNF-α is also known to potentiate the expression of the NADPH oxidase subunits p47phox, p67phox, and gp91phox und thus microbicidal activity of M1 macrophages (Gauss et al., 2007).

IL-10 and transforming growth factor (TGF)-β are some of the cytokines that inhibit the activity of M1 macrophages. These cytokines may be derived from adjacent cells or secreted from macrophages themselves following endocytosis of apoptotic cells (Fadok et al., 1998; Huynh, Fadok, & Henson, 2002).

2.1.1.2.2 Alternatively activated macrophages

M2 macrophages have been described in different variants, depending on the stimuli used to activate them (Gordon & Taylor, 2005; Mantovani et al., 2004; Martinez et al., 2008).