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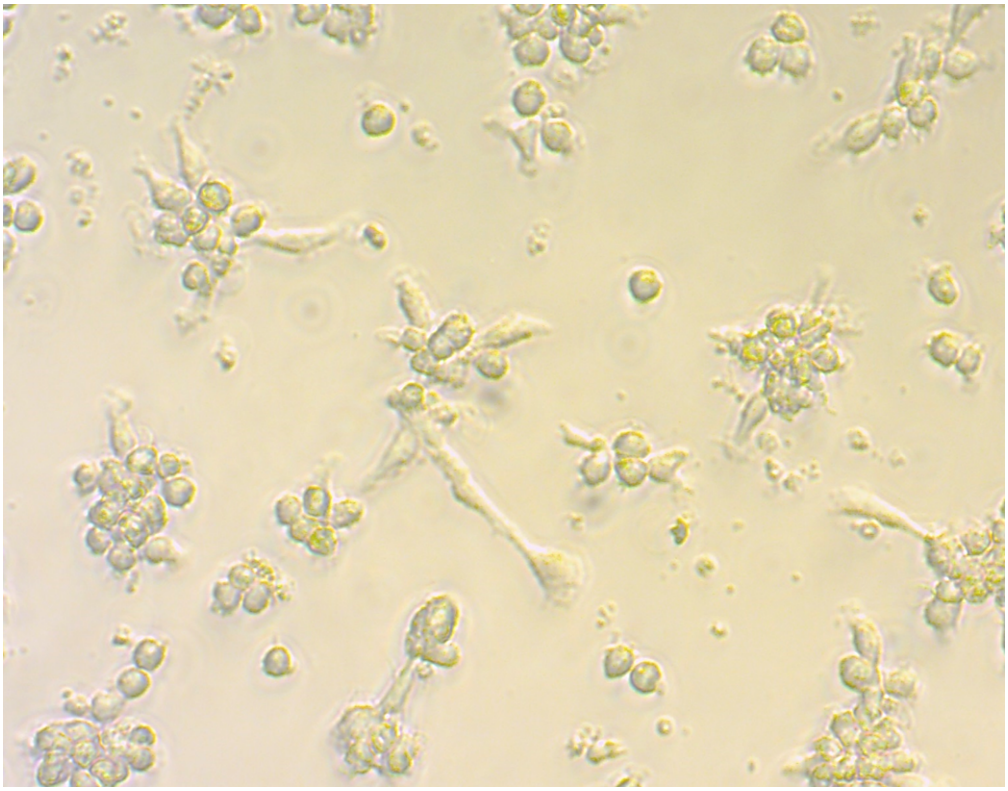

In vitro culture of human monocyte-derived macrophages with regards to M1/M2 polarization

Master's thesis in Biotechnology

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Department of Physics
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2016

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This thesis work was performed at Mölnlycke Health Care in Gothenburg, Sweden.

Cover: Image of polarized macrophages in clusters, taken in a bright-field microscope at 20x.

Typeset in LaTeX
Printed by [Chalmers Reproservice]
Gothenburg, Sweden 2016

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Abstract

Wound healing is a complex process where macrophages are highly involved, both by clearing the tissue from bacteria and debris, but also by regulating the inflammation and healing outcome by production of various cell mediators. A characteristic feature of macrophages is their ability to display a spectrum of different phenotypes. They can roughly be divided into two extreme phenotypes; M1 macrophages which produce pro-inflammatory cytokines and metalloproteinases and is strongly antimicrobial and M2 macrophages which produce anti-inflammatory cytokines, resolving inflammation and promoting tissue repair. Although both phenotypes are key in wound healing, an imbalance of M1 macrophage domination can result in nonhealing ulcers. At Mölnlycke Health Care effort is being paid at developing wound care products that could enhance the healing. In order to develop such concepts, an *in vitro* inflammatory model with M1/M2 macrophages could be utilized.

The aim of the thesis is to identify and study the relation of cell mediators and markers related to M1/M2 polarization in cultured human monocyte-derived macrophages and also to create a theoretical link of relevance of chosen markers to data in murine models and the human diabetic ulcer.

Monocytes were isolated from buffy coats, obtained from healthy blood donors. To induce M1 and M2 macrophages, cells were stimulated with LPS and IFN-γ (M1) or IL-4 (M2) for 6, 24, 48 or 72 hours prior to analysis. M1 cells where further reversibly polarized with IL-4 after 48 hours in order to switch phenotype from M1 to M2 cells. Secreted markers of M1/M2 macrophages were studied with ELISA. Production of TNF-α and IL-1β indicated a M1 phenotype and CCL18 production indicated a M2 phenotype. Production of IL-10 which is a M2 phenotype marker was produced by M1-stimulated macrophages. No production of IL-23 and IL-1ra was seen. Further, no significant CCL18 production was seen after reversible polarization, indicating that M1 macrophages did not switch phenotype to M2 macrophages. Different assay setups of lactate and reactive oxygen species production (M1 markers) did not display any significant results in this study.

Keywords: macrophages, polarization, M1, M2, LPS, IFN-γ, IL-4, ROS, lactate, cytokines, wound healing, inflammation, nonhealing ulcers, diabetes, ELISA.

Acknowledgements

I would like to sincerely thank my supervisors Sofia Almqvist and Fredrik Osla for all support and wise guidance throughout the project. Also a great thanks to the people in the Scientific Research group at Mölnlycke Health Care for making me feel so welcome. Last but not least I would like to thank my examiner Julie Gold for her enthusiastic and engaged way of teaching which made me wanting to learn more about the wound healing field.

Julia Gutman, Gothenburg, June 2016

Contents

Acronyms

AP-1	Activator protein 1
CCL18	Chemokine (C-C motif) ligand 18
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGR	Early growth response
FGF	Fibroblast growth factor
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GPR	G protein-coupled receptor
IFN-γ	Interferon-gamma
IFNGR	Interferon-gamma receptor
IL	Interleukin
IL4Rα	Interleukin-4 receptor alpha
IRF	Inteferon regulatory factor
JAK	Janus kinase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MMP	Metalloproteinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PDGF	Platelet derived growth factor
ROS	Reactive oxygen species
Slc25a1	Solute carrier family 25 member 1
STAT	Signal transducer and activator of transcription 1
TF	Tissue factor
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

1
Introduction

Mölnlycke Health Care is a provider of single-use surgical and wound care products. Their wound care products focus on preventing ulcer formation and enhancing wound healing. Some products are addressing chronic wounds including venous, pressure or diabetic ulcers where the latter will be particularly in focus in the thesis. Chronic ulcers is a clinical problem leading to morbidity and mortality throughout the world if not treated correctly. To prevent or treat these ulcers, knowledge of the complex process of wound healing is crucial. Macrophages are essential in wound healing in terms of clearing the tissue from invading microorganisms and enhancing tissue repair by releasing growth factors and cytokines which recruits new cells to the site of injury [1]. Diversity and plasticity are characteristic features of macrophages [2]. Macrophage phenotypes can vary in a spectrum between pro-inflammatory M1 cells and anti-inflammatory M2 cells, known as polarized macrophages which is a term used to describe these phenotypes. A disruption in the functioning of macrophages can result in prolonged inflammation and impaired healing, leading to non-healing ulcers where pro-inflammatory M1 macrophages dominate the wound (Fig. 1.1) [3][4]. The regulatory role of macrophages in wound repair make them a valuable target during development of new wound care solutions. Methods to locally control the polarization of macrophages are being evolved by for instance developing biomaterials with the ability to release polarization factors; a stimuli such as cytokines that can induce a M1 or M2 phenotype [5].

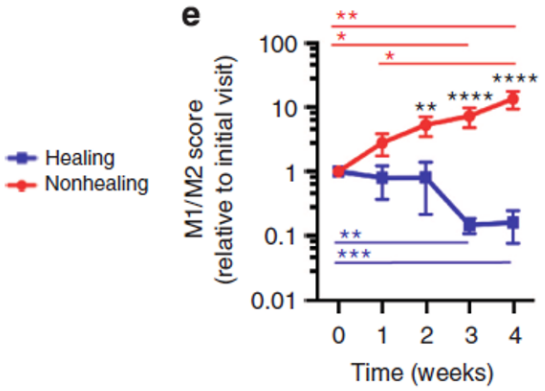


Figure 1.1: M1/M2 score of macrophages in human chronic diabetic foot ulcers [4].

At Mölnlycke Health Care effort is being paid at developing new wound care products that improves healing. In order to be able to study the relevance of a product which

is designed to promote healing, it is important to have an accurate *in vitro* model for studying the interaction of human macrophages with the substance or material being evaluated. In addition, such a model is also significant from a regulatory aspect. Due to the plasticity of macrophages, certain parameters including culturing conditions, cell source or handling can lead to varying phenotypes. Therefore, characterization of phenotype during the specific culturing conditions at Mölnlycke Health Care is required to set up an *in vitro* test model.

1.1 Aim of thesis

The aim of the thesis is to identify and study the relation of cell mediators and secreted markers related to M1/M2 polarization in cultured human monocyte-derived macrophages. Also, to create a theoretical link of relevance of the chosen markers to data in murine models and the human nonhealing wound.

Protocols and decision of cell mediators and markers are based on published research by Sofia Almqvist [6][7] in combination with literature studies. At Mölnlycke Health Care, previous work on isolation of monocytes and differentiation of monocytes to M1 respectively M2 macrophages have demonstrated promising results. It is of interest to evaluate how the previous work can be upscaled with further differentiation of M1 to M2, using several blood donors as well as additional characterizing methods.

1.2 Scientific questions

It is hypothesized that M1 is the dominating polarization of macrophages in nonhealing wounds like diabetic foot ulcers. In order to promote healing, it is hypothesized that a M2 polarized macrophage will be favorable.

- Is it possible for isolated human monocytes to be differentiated from nonstimulated M0 to M1 respectively M2 macrophages?
- Is it possible to switch phenotype from M1 to M2 macrophages?
- Is it possible to use other methods than ELISA to characterize the phenotype of macrophages?

1.3 Delimitations

Due to time and other limiting factors, following part will describe aspects not included in the thesis.

- The studied cells in the thesis will only be human monocytes, isolated from buffy coat obtained from healthy blood donors from Laboratoriemedicin at hospital of Kungälv. Primary cells are used since it is assumed that they provide a response more similar to the clinical conditions than using a cell line. However, donor-to-donor variability must be taken in concern.
- Six blood donors is desirable to use for each experiment. However, due to practical issues, no more than two or maximum three can be handled at the

same time during experimentation, leading to repeated experiments in order to obtain a significant result.

- Differentiation from monocytes to M1 and M2 respectively will be performed. Additionally, differentiation from M1 to M2 will also be evaluated.
- For differentiation of M1 macrophages, LPS and IFN-γ will be used. For differentiation of M2 macrophages, IL-4 will be used. In terms of time and results, further activators for polarization of M1 and M2 or M1 to M2 might be evaluated.
- ELISA kit for following markers will be used; TNF-α, IL-23 and IL-1β for M1 and CCL18, IL-1ra and IL-10 for M2.

2

Theory

This section aims at providing an overview of the physiology of skin and the four overlapping phases of wound healing and the difference between acute and chronic wounds. Furthermore, it describes the important role of macrophages in wound healing and features of M1 and M2 polarized macrophages. There are numerous cytokines and chemokines as well as surface markers describing macrophage polarization and a background of the most common markers in murine and human macrophages is provided. A brief description of main experimental methods is also included in this part.

2.1 Function and structure of skin

The largest organ of the human body is skin, contributing to 16 % of the total body weight with an average area of 1.5-2.0 m^2 [8]. It acts as a protective barrier against infections, chemical and mechanical insults, heat and excessive water loss [9][10]. Skin consists of three major layers: **epidermis**, **dermis** and **hypodermis** (Fig. 2.1).

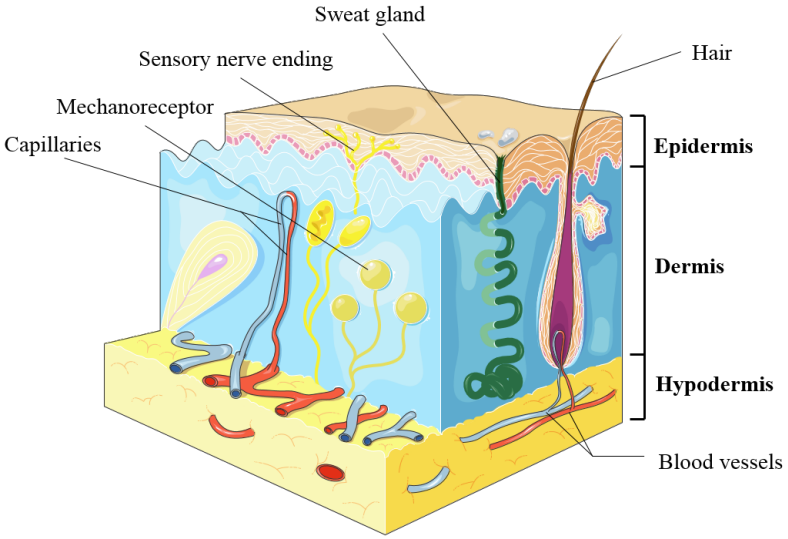


Figure 2.1: Skin consists of the three major layers epidermis, dermis and hypodermis [8].

The outermost layer of the skin is **epidermis**, containing bedded epithelium which constitute 95 % keratinocytes, important in inflammation and wound healing, and

which are generated and maturated within the epidermis [8]. Epidermis can be further subdivided into five layers: *stratum basale, stratum spinosum, stratum granulosum, stratum lucidum ad stratum corneum. Stratum basale* is the deepest layer of epidermis, comprising the basal keratinocytes which frequently divide, mature and replace the above layers. Other cells like melanocytes, Langerhans cells and Merkel cells are also found in this layer. Next layer is *stratum spinosum*, consisting of polyhedral keratinocytes with large pale-stained nuclei [8]. Adjacent cells in this layers are interconnected by desmosomes, a dense meshwork of intracelular keratin filaments which provides mechanical strength to the epidermis[11]. In third layer *stratum granulosum*, the uprising keratinocytes from lower layers lose their nuclei and organelles, thus becoming nonviable and flattened, forming the fourth layer, *stratum lucidum* and fifth layer *stratum corneum* [8]. The gradual loss of organelles is a consequence of the terminal differentiation (keratinization/cornificaiton) to corneocytes that keratinocytes undergo. This leads to cellular changes involving production of keratin, a fibrous protein which becomes incorporated into longer intermediate filaments. Keratin together with lipids and the tight junctions between cells in epidermis prevent the loss of water [11][8]. Moreover, cells form a cornified envelope, acting as a protection against chemical resistance [8].

Dermis is the second layer of skin, tightly connected to epidermis by a dynamic and stabilizing interphase, called basement membrane [11]. Two layers of connective tissue can be distinguished in dermis. Closest to epidermis is papillary dermis, a loose connective tissue, consisting of blood capillaries and different mechanoreceptors [8]. The other layer is reticular dermis, a deeper and thicker connective tissue which provides the tensile strength and elasticity of dermis due to a high concentration of type I and II collagen and elastin fibers [11][8]. Additionally, roots of hair, nails, sweat glands and blood vessels are distributed in the reticular dermis.

The innermost skin layer is **hypodermis**, also called subcutis [8]. It mainly contains adipocytes, being responsible for the homeostasis and thermoregulation. Hypodermis is also composed of various fibers, blood and lymphatic vessels, roots of hair, free nerve ends and mechanoreceptors for sensing vibration and pressure.

2.2 Introduction to wound healing

Despite the ability to act as a protective barrier, skin is nevertheless the most easily injured organ. There is two ways of healing after an injury; tissue repair and regeneration. Tissue repair or wound healing is the vital process which aim at repairing and restoring the damaged tissue in order to maintain the function of the skin [1]. The result is a scar tissue, which may have a different structure, biochemical compositions and/or mechanical properties compared to native tissue [12]. Regeneration is the secondary procedure which can form functional tissue, resulting in an identical tissue to that which was there before damage [12]. It takes place if the injury only occurs in the epidermal layer. However, deeper wounds extend to the dermal layer where the wound healing via tissue repair takes place.

The wound healing process is highly complex and involves numerous cells, cell mediators and extracellular matrix components. Immune cells including neutrophils, macrophages, lymphocytes and dendritic cells along with endothelial cells,

keratinocytes and fibroblasts undergo phenotypical changes, leading to cell proliferation, differentiation and migration [13]. The cellular events are coordinated by signals involving cytokines, chemokines and growth factors [14]. Cytokines are low molecular weight soluble proteins that are produced by cells to act as a chemical messenger for regulation of innate and adaptive immune system [15]. They bind to specific cytokine receptors on immune cells and affects their activity by enhancing or suppressing responses. Cytokines that enable cell migration are called chemokines [15]. Growth factors are also soluble secreted proteins which stimulate cell proliferation and differentiation by promoting synthesis of proteins and other macromolecules and by inhibiting their degradation [16].

The process of wound healing can be divided into four distinct but overlapping phases; hemostasis, inflammation, proliferation and remodeling (Fig. 2.2).

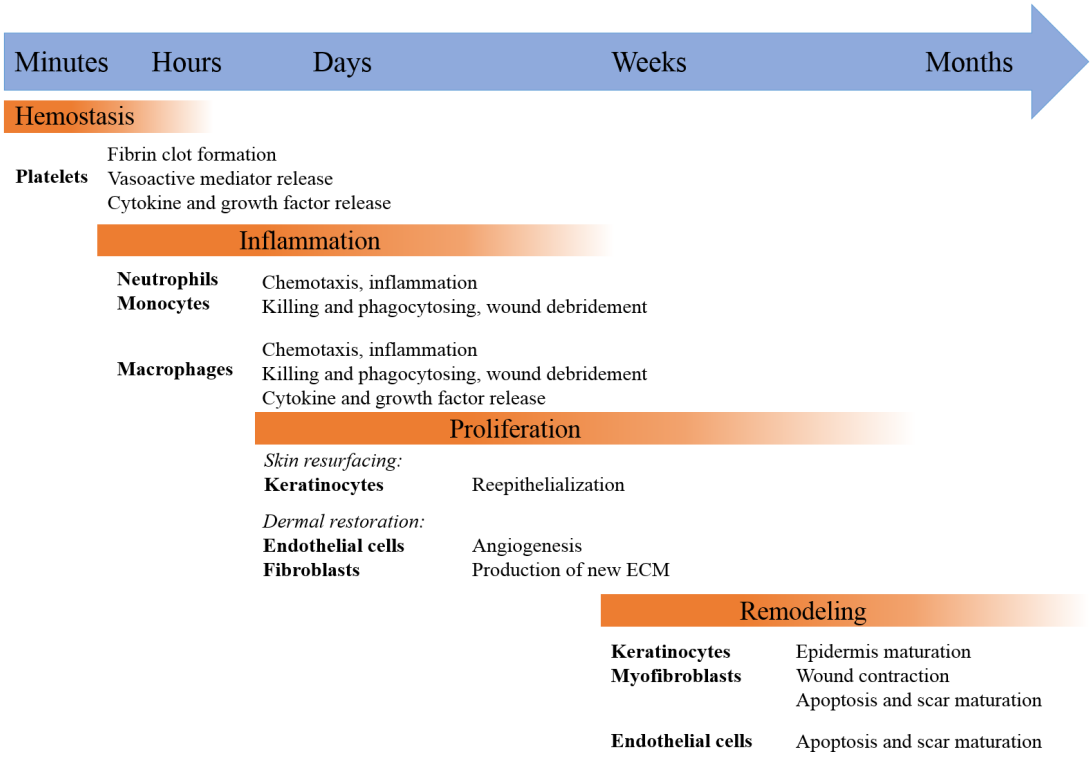


Figure 2.2: Wound healing consists of the four overlapping phases hemostasis, inlammation, proliferation and remodeling. Numerous cells with different tasks are involved. (Inspired by Li et al. [17])

2.2.1 Hemostasis

Hemostasis is achieved upon damage of blood vessels in order to prevent blood loss. The blood vessel is clogged within seconds after injury due to two main mechanisms; platelet aggregation and the induction of coagulation cascade (thrombosis) [9][12]. The platelets become activated upon various stimuli, including interaction with extracellular matrix (ECM), soluble factors and/or cells of injured vessel walls [12]. Common activators are collagen and von Willebrand factor (vWF) which interacts

with receptors on the cell membrane of the platelets. The activated platelets undergo several changes including taking on an irregular form and extending pseudopodia, leading to platelet adhesion.

The coagulation cascade consists of two main mechanisms; intrinsic and extrinsic pathways which end in a common pathway leading to conversion of fibrinogen to fibrin. Fibrin together with platelets form a clot. The intrinsic pathway is activated due to trauma to blood itself or exposure of ECM molecules in a damage vessel wall. In contrast, the extrinsic pathway is initiated by release of tissue factor (TF), synthesized by endothelial cells and macrophages which have been stimulated by interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α). IL-1 is the first signal that alerts the surrounding cells that a damage of tissue has occurred [14]. It is stored in keratinocytes and released upon damage of epidermis.

The hemostasis results in formation of a provisional and dynamic matrix, constructed of dense cross-linked fibrin, entrapped platelets and adhesive proteins [18]. Activated platelets release their stored granule contents, consisting of further activators of platelets and coagulation cascade, adhesive proteins and growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β) and vascular endothelial growth factor (VEGF) [14][9]. This early release of growth factors and chemokines stimulate inflammatory cells and induce cell migration to the wound site where the matrix acts as a scaffold for the arriving cells.

2.2.2 Inflammation

Inflammation is the second wound healing phase where inflammatory cells have been recruited by chemotaxis; a gradient of chemokines [9]. Released histamine causes the blood vessels to vasodilate which facilitates cell migration from the blood stream into the tissue. The initial function of inflammatory cells is to provide a specific and non-specific defense against pathogens. Among the first cells to arrive within hours after injury are neutrophils, attracted by cell mediators as PDGF and IL-1 [14]. Neutrophils and monocytes undergo transendothelial migration into the wound site. Transendothelial migration starts with initial contact of glycoprotein ligands on the neutrophils and monocytes and selectins on endothelial cells [19]. The weak binding to the endothelium and flowing blood results in rolling cells along the vessel wall [12]. The neutrophils and monocytes are during this process further activated by cytokines and chemokines, leading to activation of integrins. Subsequently, the integrins interact with intracellular adhesion molecules (ICAM) or vascular cell-adhesion molecule (VCAM) on endothelial cells, leading to interruption of rolling and a firm adhesion [19]. The transendothelial migration into the wound area is mediated via diapedesis [12]. Further chemokines allows the cells to migrate towards the exact location of where they are most needed.

Neutrophils become predominant within 24 hours, cleaning the wound from bacteria, foreign material and tissue debris and releasing cytokines [20]. Subsequently, about five to six hours past inflammatory phase initiation, monocytes arrive to the wound site in response to chemotaxis. Monocytes mature into macrophages, a process that can take up to eight hours [12]. The maturation into macrophages is

induced by several cytokines including IL-4, IL-10, IFN-γ, IL-13 or bacterial products like LPS and ECM-components [9]. For the next days to weeks, macrophages become the dominant inflammatory cell type. Similar to neutrophils but with a greater capacity, they continue the phagocytic activity, cleaning the wound as well as presenting antigens to T-cells. Furthermore, the macrophages release various pro-inflammatory cytokines including IL-1α, IL-1β, IL-6 and TNF-α [21]. When the inflammation phase is coming to an end, macrophages involved in cleaning the wound from pathogens or dead tissue undergo apoptosis [9]. Surviving macrophages remains in the wound bed and start stimulating collagen production, angiogenesis and reepithelialization.

2.2.3 Proliferation

The proliferative phase is characterized by degradation of provisional matrix, angiogenesis and formation of granulation tissue [9]. This phase starts approximately four days after injury [21]. Both tissue resident and recruited fibroblasts produce new extracellular matrix which is required for supporting cells and blood vessels [9]. A stimulator of ECM production is TGF-β which is primarily produced by macrophages. TGF-β1 is in wound healing important for inflammation, angiogenesis and reepithelialization and also acts as an inhibitor of metalloproteinases [14]. Further stimulators for ECM production are platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF2) and insulin like growth factor 1 (IGF-1). Additionally, persistent production of pro-inflammatory interleukins as IL-1α, IL-1β, IL-6 and TNF-α by macrophages controls the inflammatory cell adhesion and migration and also stimulates fibroblasts and keratinocytes to proliferate [22]. The fibrin clot formed during hemostasis is gradually transformed into connective tissue which is rich in blood vessels [9]. A balance between matrix degradation and production is required.

Angiogenesis which refers to formation of new capillaries from pre-existing blood vessels is necessary for providing nutrients and oxygen required for the increased cell growth and proliferation [9]. Macrophages are important for this process in terms of releasing metalloproteinases (MMPs) and serine proteases which mediate the ECM degradation in order to facilitate migration of endothelial cells which also release MMPs. The migration is stimulated by fibroblast and macrophage produced VEGF, FGF and TGF-β which stimulates integrin receptors on the endothelil cells.

The otherwise highly ordered epidermis become disordered or damaged during disruption of skin in an injury. Reepithelialization aims at re-establish the intact epidermis over the newly formed granulation tissue [9]. It is characterized by migration and proliferation of keratinocytes which are stimulated by TGF-β1, epidermal growth factor (EGF), TGF-α and keratinocyte growth factor (KGF) [23]. (Myo)fibroblasts, stimulated by PDGF and TGF-β1, facilitate the reepithelialization by contracting the underlying connective tissue in order to bring the wound edges towards each other. After the proliferation phase, the wound is filled with granulation tissue with an overlaying epidermal layer [9].

2.2.4 Remodeling

The remodeling phase is the last and longest wound healing phase, proceeding from weeks to years [9]. This phase is characterized by slower cell proliferation, decreased protein synthesis and specifically the remodeling of collagen (type III) into larger, more organized fibrils (type I). (Myo)fibroblasts which are the main producers of ECM proteins decrease their subsequent collagen production after being stimulated with for instance interferon-gamma (IFN-γ) and TNF-α.

Remodeling is a balance between extracellular matrix production, degradation and remodeling. Important regulators of the proteolytic activity are the MMPs and tissue inhibitor of metalloproteinases (TIMP). The MMPs have the ability to degrade most of the ECM components and many of them, including MMP-2, MMP-12 and MMP-19, are produced by macrophages. Furthermore, macrophages are also producers of TIMP for metallo- and serine proteases.

The newly formed capillaries are degenerated due to decreased nutrient and oxygen demand. Finally, endothelial cells, macrophages and fibroblasts undergo apoptosis or leave the wound [13]. Although the wound healing is completed, the scar tissue will never recover to the same properties as uninjured tissue.

2.3 Macrophages

Macrophages play a substantial role throughout the wound healing, being involved in numerous processes and most of the healing phases. As the role of macrophage phenotype in wound healing and various diseases has been highlighted, the interest in these cells is rising. Macrophages are hematopoietic cells from the myeloid lineage, specialized in phagocytosis and with a great ability to respond to numerous environmental signals [24]. They are found in two different populations where one population is resident in the tissue while the other circulates in the bloodstream in form of haematopoetic precursor cells, known as monocytes [9]. Approximately 10 % of human nucleated cells in blood are monocytes [2]. Monocytes in humans can be further divided into two subpopulations; $CD14^+$, precursors of macrophages, and $CD14^{low}CD16^+$, involved in patrolling and inspecting the endothelium in search for damaged cells. Although tissue macrophages and monocytes originates from the same cell lineage, they have crucial but distinct roles in the tissue homeostasis. Monocytes are highly involved during inflammation and pathogen elimination while tissue-resident macrophages are responsible in development, tissue homeostasis and resolution of inflammation. Furthermore, tissue-resident macrophages have different roles, depending on the tissue in which they reside [24]. For instance, spleen red pulp macrophages phagocyte erythrocytes and recycle heme in order to maintain the iron homeostasis while peritoneal cavity macrophages interact with B1 cells to regulate the production of gut immunoglobulin (Ig) A. Until recently, it was believed that tissue-resident macrophages relies on the constant recruitment of blood monocytes [2]. The explanation is rather that each organ has its own composition of embryonically and adult-derived macrophage subsets although monocyte derived macrophages replace tissue resident macrophages to some extent [25]. Especially during inflammation, monocytes are triggered to differentiate to macrophages. Lu-

cas et al. showed the importance of monocyte derived macrophages when showing in a mouse model that depletion of macrophages in the initial phase of tissue repair results in reduced or impaired healing in granulation tissue deposition, epithelialization and scar formation [26].

The diverse functions of macrophages are connected to their ability to display a spectrum of different phenotypes in response to temporally and dynamic signals. Macrophages in wound healing can roughly be divided into two extreme types; M1 (classically activated) and M2 (alternatively activated) [9]. Both macrophage phenotypes are important for the different phases in wound healing. M1 is dominating the early inflammatory phase while M2 is mainly anti-inflammatory, reducing inflammation and inducing formation of new tissue in the later inflammatory phase. What separates these so called polarized M1 and M2 macrophages is the receptor expression, cytokine and chemokine production as well as effector function. A schematic overview, relevant for this project, is seen in Figure 2.3.

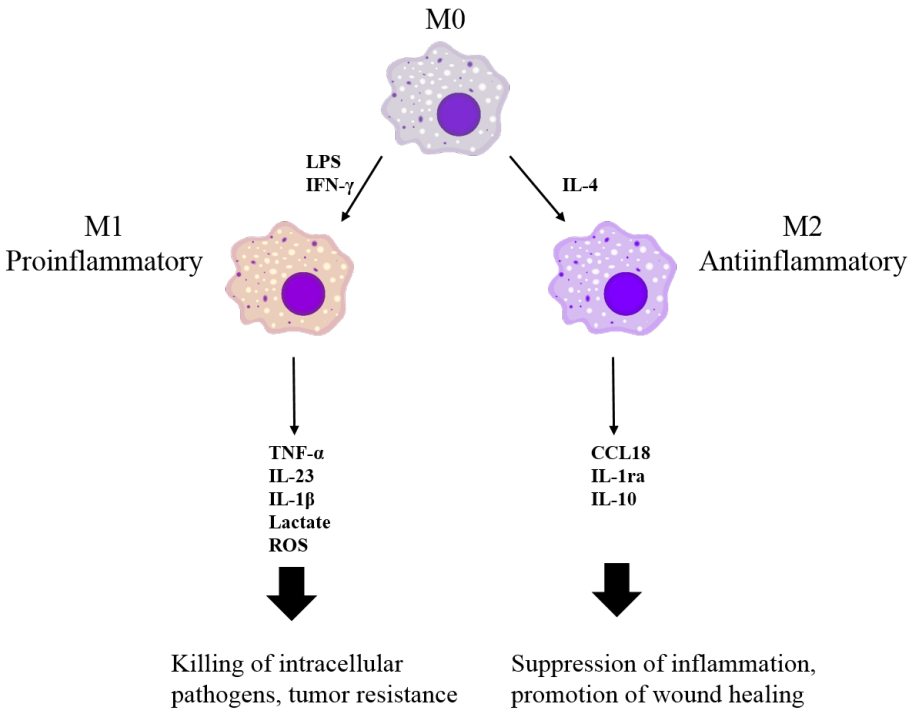


Figure 2.3: Schematic overview of M1 and M2 polarization in terms of activators, secretion of cytokines and function.

The terms M1 and M2 are often linked with the T helper cell 1 (Th1) or Th2 responses respectively [27]. This originally refers to a hypothesis stating that there are two subsets of helper T cells which after their activation can be distinguished by their cytokine secretion resulting in different regulatory and effector functions including macrophage activation. The *in vitro* M1 and M2 model of macrophages has been helpful in terms of describing the immune response during acute infections, asthma, allergies and obesity [28]. However, such a classification cannot represent the complex *in vivo* environment involving numerous cytokines and chemokines, constantly interacting with macrophages [3]. Further classification of M2 have been

made (M2a, M2b, etc.) but these classifications supports the idea that macrophage activation exists on a spectrum rather than being defined in groups [29]. As the diversity and plasticity of phenotype are the characteristic features of macrophage, they also most probably lead to various experimental results due to varying experiments. Subsequently, this can cause some confusion in the scientific literature, leading to widespread definitions of macrophage activation, terminology and characterization. For further guidance, Murray et al., have suggested nomenclature and experimental guidance for research within this subject [29].

2.3.1 M1 and M2 polarization

Inflammatory stimuli including lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) induces the classically activated macrophage M1, characterized by its antimicrobial and tumoricidal properties [2][9]. Release of inflammatory mediators including TNF-α, IL-1 and nitric oxide activates the anti-microbial defense which includes the oxidative processes by production of reactive oxygen species (ROS) that contributes to the killing of invading pathogens.

The main cytokine associated with macrophage M1 activation is IFN-γ which is produced by Th1 cells [27]. Natural killer cells and macrophages themselves have also been shown to produce this cytokine. The IFN-γ receptor is formed by two chains; IFNGR-1 and IFNGR-2 (Figure 2.4). The receptor further recruits the Janus kinase (JAK)1 and JAK2 adaptors which in their turn activate signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factors (IRF) including IRF-1 and IRF-8. IFN-γ activation leads to specific gene expression of cytokine receptors as IL12RA and IL6R, cell activation markers as CD38 and CD69 and adhesion molecules as ICAM1.

Toll-like receptors (TLR) are pattern recognition receptors which recognizes major parts of pathogens including lipopolysaccharide (LPS) and lipoteichoic acid [27]. LPS is the best studied macrophage M1 signal and after its binding to TLR4, the receptor induces MyD88 and MaL/Tirap (toll-interlukin 1 receptor domain containing adaptor protein)-dependent pathway (Figure 2.4. This leads to expression of pro-inflammatory cytokines (e.g. IL-12, TNF, IL-6, IL-1β, IFN-β), chemokines and antigen-presenting complexes as major histocompatibility complex (MHC). The expression is controlled by the gene enhancer nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), IRFs, STAT1, and the early growth response (EGR) family.

Gene expression profiles includes the combination of IFN-γ and LPS as their combined profiles are different from LPS and IFN-γ profiles alone [27]. There is some overlapping between gene profiles of IFN-γ and LPS but the similarities are not enough to consider the stimuli to be homologous.

Alternative activation of macrophages, M2, is stimulated by interleukin-4 (IL-4) alone or IL-4/IL-13, leading to anti-inflammatory features including suppression of inflammatory responses and induction of wound healing [9]. IL-4 is produced by Th2 cells, eosinophiles, basophils or macrophages. It is recognized by three different receptor pairs where IL-4Rα1 pairs up with its corresponding gamma chain (γc), allowing IL-4 or IL-13 binding (Fig. 2.4). Subsequently, the receptor ac-

tivates JAK1 and JAK3, leading to STAT6 activation and translocation. Other transcription factors involved are c-Myc and IRF4. Subsequently, transcription of anti-inflammatory cytokines such as IL-1ra and IL-10 is induced. These cytokines signal to the macrophages to decrease the production of pro-inflammatory cytokines as TNF-α and IL-1 and also the overall macrophage activity.

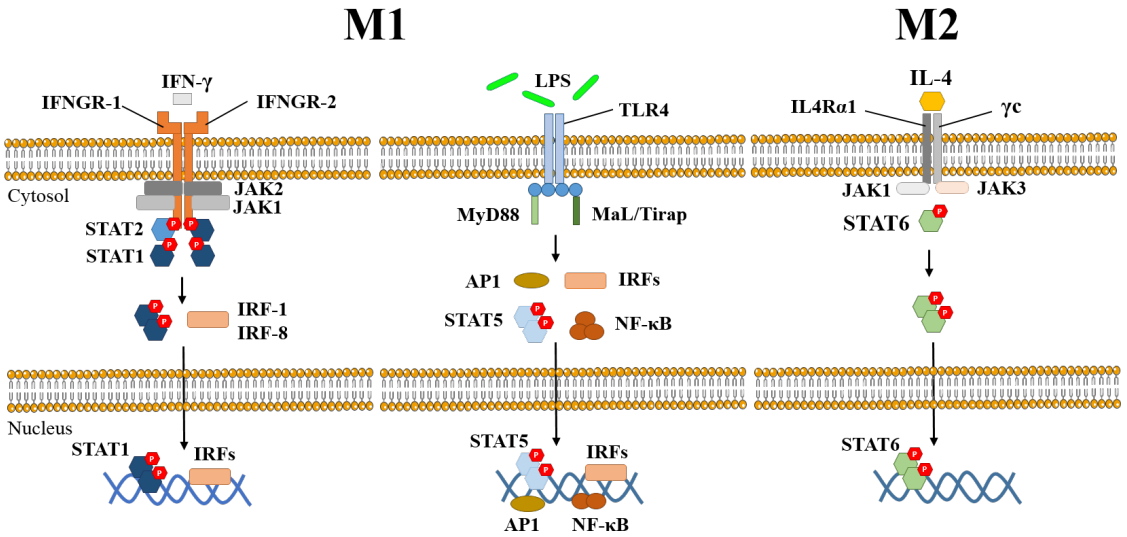


Figure 2.4: Schematic overview of M1 and M2 activators and their corresponding receptors. Activation results in different signaling pathways, activating various transcription factors which travels to nucleus and activate genes. (Inspired by Martinez et al. [27])

The latest among the *in vitro* stimuli for M2 polarization is macrophage colony-stimulating factor (M-CSF/CSF-1) or similarly granulocyte/macrophage colony-stimulating factor (GM-CSF) for M1 polarization. A difference in the transcriptome of macrophages have been noticed during growth in GM-CSF or M-CSF as well as cytokine secretion but there is not further evidence that these alone can generate M1 or M2 macrophages [30][31]. Other have reported that expression levels of some surface markers were independent of GM-CSF and M-CSF induction although they were dependent on the usual polarization stimulation including LPS, IFN-γ, IL-4/IL-13 [32].

2.3.2 Metabolism in M1 and M2 macrophages

Macrophage phenotype subsets were discovered as a consequence of two opposing metabolic pathways of one amino acid; arginine, which was metabolized via nitric oxide synthase (NOS) to nitric oxide (NO) and citrulline or via arginase to ornithine and urea, resulting in definitions of M1 (NOS) and M2 (arginase) [33]. Mills with colleagues were among the first to discover this after studying wounds and observing a high production of ornithine and depletion of the substrate arginine by macrophages *in vivo* [34]. Further experiments were conducted with murine macrophages, stimulated with IFN-γ or LPS which confirmed the metabolic difference resulting in either

NO or ornithine production. Subsequently, Mills with colleagues hypothesized that these metabolites seemed to act as a "Stop" or "Go" signal, where NO and citrulline resulted in inhibition of cell proliferation while ornithine and urea resulted in cell proliferation and repair.

M1 cells have a different metabolism from M2 cells as they require rapid energy in form of ATP for the bactericidal activity. The metabolism is characterized by induction of glycolysis and pentose phosphate pathway (PPP) [35]–[37]. This leads to increased glucose consumption and lactate release, an effect which is also found in tumor cells and known as the Warburg effect [38][37]. Enhanced PPP results in increased purine and pyrimidine production, used for biosynthesis. In addition, it also provide NADPH and NADPH oxidase enzyme which produces ROS. The reduction of oxidative phosphorylation (OXPHOS) and increased glycolysis can be induced by different stimuli including LPS [37].

M1 cells have an enhanced fatty acid synthesis and an elevation in certain intermediates as succinate, citrate and itaconic acid due to a broken Krebs cycle (TCA cycle) [35]. In LPS-activated macrophages, succinate facilitates the signal leading to stabilization of hypoxia inducible factor-1α (HIF-1α) which positively regulates IL-1β, thereby driving the inflammation further [39].

Extracellular succinate can signal via the G protein-coupled receptor (GPR) 91 and synergize with TLR signaling, leading to further production of pro-inflammatory TNF-α [37]. Citrate, another TCA cycle intermediate, is formed by oxaloacetate and acetyl-CoA in the mitochondria. LPS increase the expression of the mitochondrial citrate carrier; solute carrier family 25 member 1 (SlC25a1), via NF-κB pathway, leading to accumulation of citrate in the cytosol [37]. Citrate is required for the fatty acid synthesis and is in the cytosol cleaved back into acetyl-CoA and oxaloacetate, precursor for nitric oxide and ROS [35]. Oxaloacetate is further metabolized to pyruvate which generates NADPH from $NADP^+$. NADPH oxidase uses NADPH and oxygen to generate ROS. NADPH is further required for the conversion of arginine to NO and citrulline. NO is continuing inhibiting oxidative phosphorylation and in conformity with ROS, also stabilizes HIF-1α.

Complex I of the electron transport chain is another major producer of ROS since the superoxide is here produced from O_2 with help from a cofactor. The cofactor is dependent on electrons from NADH, leading to ROS being dependent on the NADH/NAD^+ ratio. It has been shown that LPS increases the NADH/NAD^+ ratio by possibly attenuating mitochondrial function [40][41]. ROS production is important for more reasons than only being bactericidal. For instance, it enhances production of pro-inflammatory cytokines as IL-1β, IL-6 and TNF-α [35].

While M1 macrophage metabolism is well studied, less is known about the M2 macrophage metabolism [41]. However, it is understood that metabolism of M2 macrophages is characterized by increased fatty acid oxidation and increased oxidative phosphorylation [35]. Further, there is difference in the iron metabolism [41]. It is believed that the fatty acid oxidation (FAO) allows the M2 macrophages to sustain longer as it generates more energy in terms of ATP [36]. In contrast to M1 macrophages, M2 macrophages have an intact Krebs cycle. A simplified summary of M1 and M2 metabolism is seen in Figure 2.5.

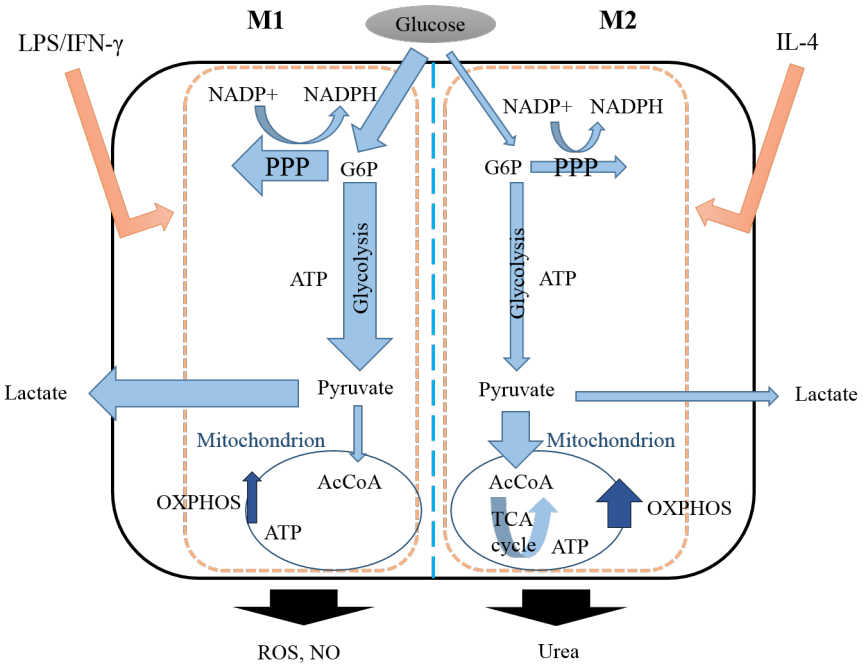


Figure 2.5: A graphic overview of M1 and M2 metabolism. Glucose is metabolized to glucose 6-phosphate (G6P) before going into glycolysis or pentose phosphate pathway (PPP). M1 metabolism is characterized by an increased glycolysis, PPP, NADP+/NADPH rate and a broken TCA cycle. M2 metabolism has an unbroken TCA cycle and increased fatty acid oxidation.

The difference in metabolism between phenotypes can be used for characterization of M1 and M2 cells. For example, quantification of ROS *in vitro* has been used when comparing how human and murine M1 macrophages respond to various stimuli [42]–[44]. Furthermore, increased glucose consumption is seen during LPS and/or IFN-γ stimulation [40][38] and increased lactate production when comparing to IL-4/IL-13 stimulated macrophages [38].

Since most experiments include murine cells, not all metabolic pathways can be as clearly identified or detected in human macrophages. For instance, a study with human macrophages showed only moderate changes of oxidative phosphorylation and fatty acid oxidation (FAO) during IL-4 induction [45]. Another example of possible differences in metabolism between human and murine cells is the expression of NOS (also called iNOS) and NO production in M1 cells. Expression of this enzyme and NO production has been established in murine cells [30]. Similar *in vitro* experiments with human cells have not yielded any iNOS nor NO [30]. However, other publications have reported abundant expression of iNOS in human tissue samples [30]. One explanation might be that commercially available media as RPMI lacks something needed to generate iNOS expression and NO production in monocyte-derived macrophages [30]. Another explanation is that human macrophages lack the obligatory cofactor tetrahydrobiopterin (BH4), necessary for stabilizing iNOS [46]. Similarly to iNOS expression, arginase expression (Arg1/Arg2) has been widely discussed. Arg1 is one of the most well-known markers of murine M2 cells [30]. However, in human macrophages it appears to only be expressed in neutrophils [30].

Despite some differences in metabolism between murine and human cells, there is a core set of pathways identical between macrophages in these species, leading to similar markers. Following section will provide an overview of similarities and differences in human and murine M1 and M2 macrophages.

2.3.3 Comparison of M1/M2 macrophage markers

Polarized macrophages have been evaluated in terms of gene expression, surface markers, secreted molecules and difference between human and murine species as well as cell lines [47][48]. However, the exact role of macrophages, especially in non-healing wounds is still incompletely understood. Studies of macrophages can be complicated because of their plasticity leading to different gene expression. Therefore, gene expression across multiple studies are difficult to compare due to different experimental conditions including cell culturing, sampling conditions, probe set design and donor-to-donor variability [47]. Characterization based on murine macrophages or cell lines is favorable due to a large number of cells with high reproducibility, but are harder to compare with human macrophages [30]. Highlighting differences in human versus mouse models is key in order to link *in vivo* murine models with human clinical data in order to develop new therapies for chronic wounds.

The current distinct classification of M1 and M2 markers is to some extent limiting for several reasons [27]. Firstly, it ignores the source and context of the stimuli. Secondly, the stimuli do not exist alone in a tissue and finally, macrophages may not form clear-cut activation subsets. However, the *in vitro* polarization can still be viewed as an useful and simplified tool to learn the continuum *in vivo*.

A summary of commonly used markers is made in Table 2.1, containing references which aim at characterizing murine and human macrophage phenotype and function.

Marker	M1		M2	
	Human	*Mouse*	*Human*	*Mouse*
Cytokine secretion	IFN-γ^{a}, TNF-$\alpha^{a,b,c,l}$, IL-1$\beta^{a,b,l}$, IL-6^{b,c,d}, IL-8^{a,d}, IL-12A^{b}, IL-15^{d}, IL-12B^{b,c}, IL-23^{b,k}, CCL2^{d}, CCL8^{e}, CCL15^{e}, CCL19^{e}, CCL20^{e}, CXCL9^{b,d}, CXCL10^{a,e,b,d}, CXCL11^{b}, CXCL13^{e}, RANTES^{a}	TNF-α^{b}, IL-6^{b}, IL-27^{b}, IL-23a^{b}, IL-12a^{b}	IL-13^{a}, CCL4^{b}, CCL13^{e,b}, CCL14^{e}, CCL17^{a,e,b}, CCL18^{a,e,b,d,h}, CCL23^{e}, IL-1ra^{l}, IL-10^{l}	CCL17^{b}, CCL22^{b}, CCL24^{b}
Surface markers	CD64^{a}, CD80^{a,l}, CD40^{c}	MHC class II^{i}, CD86^{i}	CD1a^{a}, CD1b^{a}, CD200R^{a}, CD209^{a}, MR^{c,d}, MRC1/CD206^{h,l},	MRC1/CD206^{j}
Gene expression	TNF-$\alpha^{f,a}$, CXCL9^{e}, CXCL10/IP10^{f,e}, CXCL11^{f,a,e}, CXCL13^{e}, CCL19^{e}, IL-1β^{d}, IL6^{e,d}, IL-8^{f}, IL-15^{e}, Cox-2^{a} , APOL3^{a}, IRF5^{a}, CCL1^{f}, CCL2^{d}, CCL5^{e}, CCL15^{e}, CCL20^{e}	NOS2^{f,g,e}, TNF-$\alpha^{f,g}$, CCL1^{f}, CXCL10^{f}, CXCL11^{f}, IL-1β^{g}, IL-6^{g}, IL-12β^{g}	CCL13^{e}, CCL17^{f}, CCL18^{a,d}, CCL22^{f}, FN1^{a}, IRF4^{a}	Arg1^{g,e}, Chi3l3/Ym1^{g,i}, Retnla/Fizz1^{g,i}, Egr2^{g}, Fn1^{g}, Mrc1/CD206^{g}, CCL17^{f}, CCL22^{f}

Table 2.1: Human cells are monocyte-derived macrophages. Murine cells can vary between monocyte-derived or bone-marrow derived macrophages. Important to note that concentrations of polarization activators LPS, IFN-γ, IL-4 and IL-13 may vary between studies. In most cases, cells are grown in medium supplied with M-CSF or GM-CSF. Genes presented in this table are commonly employed phenotype markers, see references for more detailed M1/M2 gene profiles. [32]^{a} [29]^{b} [31]^{c} [49]^{d} [50]^{e} [47]^{f} [48]^{g} [51]^{h} [52]^{i} [53]^{j} [54]^{k} [55]^{l}.

2.4 Impaired wound healing

Nonhealing ulcers are evolved due to failure of the normal wound healing process. The failure is a consequence of postponed, incompleted or uncoordinated healing, leading to a state of pathological inflammation [56]. Subsequently, the tissue becomes poorly anatomical and functional [57]. What characterizes these wounds is the chronicity and frequent occurrence, often caused by infection or underlying diseases as ischemia, diabetes mellitus, venous stasis or external factors as shear [57][56].

There is a great difference between the inflammatory reaction of an acute wound versus a chronic wound. In the normal wound healing, there is a preparation of healing by removal of necrotic tissue, debris and pathogens in combination with recruiting and activating fibroblasts and endothelial cells. In a chronic wound on the other hand, the inflammation is sustained and stalled, leading to further injury

[57]. As previously mentioned, macrophages are initiators of inflammation and their clearance of invading organisms and tissue debris may trigger indirect tissue damage due to for instance the toxic activity of reactive oxygen and nitrogen species [3]. Additionally, macrophages continue to release pro-inflammatory cytokines and metalloproteases (MMPs). Levels of the cytokines IL-1β and TNF-α are elevated in chronic venous and pressure ulcers [58][59]. The increased levels of MMPs including collagenases and elastinases and reduction of inhibitors (TIMPs) are believed to be induced by IL-1β and TNF-α. Due to MMP activity, the extracellular matrix is degraded which hinder cell migration and collagen deposition. Furthermore, MMPs also target growth factors and their corresponding cell surface receptors [60]. In nonhealing ulcers, elevated levels of specifically MMP-1, MMP-2, MMP-9 and MMP-13 have been identified [61].

A hypothesis of nonhealing wounds is that they contain cells that are phenotypically altered and are less responsive to activation signals which may affect the healing process [17]. As an example, fibroblasts of diabetic ulcers have shown a decreased response to growth factors [62].

Another critical affected component of nonhealing wounds is oxygen. Oxygen is critical for the cellular metabolism and energy maintenance in terms of ATP [56]. It induces angiogenesis, increases keratinocyte and fibroblast proliferation, migration and re-epithelialization as well as enhances collagen synthesis. Furthermore, ROS produced by macrophages kills pathogens. The wound environment becomes hypoxic due to the high oxygen demand, a condition especially found in chronic wounds. During hypoxia, pyruvate in macrophages is via anaerobic metabolism metabolized to lactate instead of being fed to the TCA cycle [37]. A significant elevation of lactate has been seen in infected compared to non-infected diabetic foot ulcers [63].

To sum up, observations in terms of secreted cytokines and metabolites in nonhealing wounds shows involvement of M1-like macrophages. Therefore, if an elevation of pro-inflammatory macrophage response is not controlled in time, it can become pathogenic, leading to disease progression as seen in several chronic inflammatory and autoimmune diseases including diabetes [3].

2.4.1 Diabetic foot ulcers

Diabetes mellitus is characterized as a group of metabolic diseases caused by hyperglycemia as a result of defects in insulin secretion, insulin action or both [64]. The wound healing ability of diabetic patients is reduced or impaired, often causing nonhealing foot ulcers which can maintain for weeks to months [65]. If not healed, they may cause lower limb amputation, leading to severe morbidity and mortality. The reduced healing ability is associated with delayed immune cell migration and altered macrophage activity. Macrophages are regulated by the cytokine milieu in the wound and are characterized by upregulation of pro-inflammatory cytokines and downregulation of anti-inflammatory cytokines. A review about the cytokine mileu in diabetic wounds suggests that by blocking the activity of pro-inflammatory cytokines as IL-1β, TNF-α and C-reactive protein, the diabetic wound healing in both animal models and humans have been improved [65]. Additionally, an improvement was seen during increased expression of anti-inflammatory cytokines IL-10 and

TGF-β [65].

Studies of gene expression in human ulcers are usually complicated due to inherently heterogeneous tissue and sampling variability. In a study of human diabetic ulcers, it was revealed that there was a major difference in the score of M1/M2 cells [4]. The M1/M2 score was increased during 4 weeks in a non-healing wound while the M1/M2 score was decreased during the same period of time in a healing wound, indicating that the M1 macrophages was dominating the nonhealing wounds.

2.5 Enzyme-linked immunosorbent assay

There are various techniques to characterize macrophage polarization. One commonly utilized assay is enzyme-linked immunosorbent assay (ELISA). This method identifies specific proteins in a solution by utilizing enzyme-detection in combination with antibodies [12]. The antibodies have an unique design in order to recognize specific proteins and bind to them. A schematic view of ELISA is seen in Figure 2.6. The primary antibody is immobilized to the surface of the well and binds to the protein of interest. A secondary antibody which is conjugated to an enzyme is added and also binds to the protein of interest. Enzymes including alkaline phosphatase, horseradish peroxidase and p-nitrophenol phosphatase are frequently used. A substrate is added, leading to a color change when enzyme interacts with it. The color change is quantified on a plate reader/spectrophotometer and is proportional to the amount of protein present.

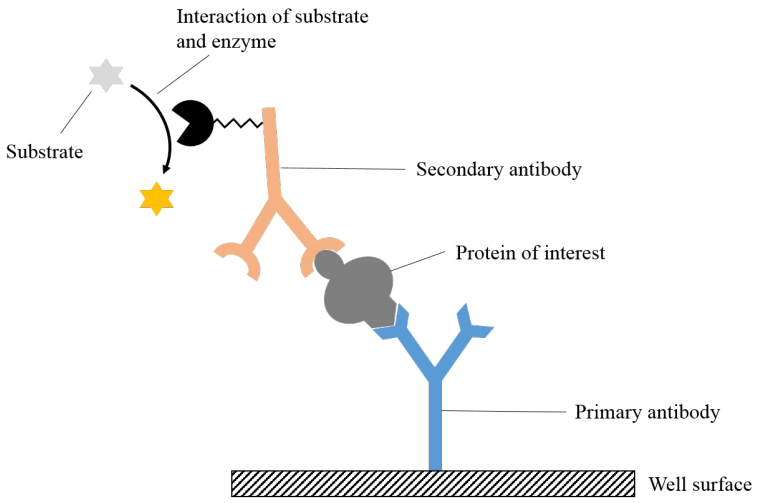


Figure 2.6: Schematic view of ELISA.

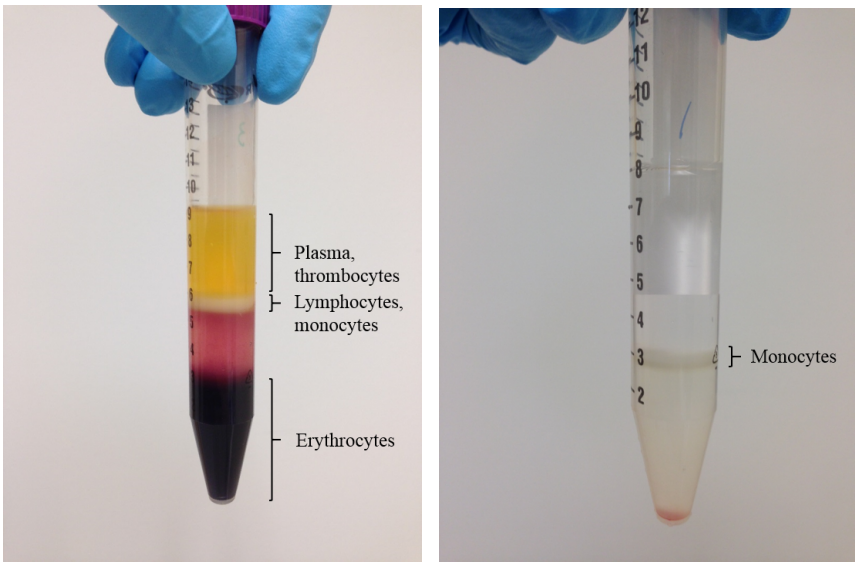
3
Methods

3.1 Materials

Monocyte isolation and cell culturing: Monocytes in a buffy coat were obtained from healthy blood donors at Laboratoriemedicin, hospital of Kungälv. Percoll, Roswell Park Memorial Institute 1640 medium (RPMI), Hanks balanced salt solution (HBSS) without calcium and magnesium, Percoll, fetal bovine serum (FBS), Penicillin-streptomycin (PEST), phosphate-buffered saline (PBS), trypsin, Phorbol myristate acetate (PMA).
Equipment: 15 and 45 ml conical centrifugation tubes, glass Pasteur pipettes, finnpipette, centrifuge with temperature adjustment, ice, 96 well tissue culture plate, 96 well black tissue culture plates, 96-well black assay tissue culture plate, 96 well non-tissue culture (polypropylene) plate, Vivaspin 500 (10 000 MWCO, GE Healthcare).
Reagents: Lipopolysaccharide from *Escherichia Coli* (L3880, Sigma-Aldrich), interleukin 4 (204-IL, R&D Systems), recombinant human interferon gamma (PHC4031, Thermo Fisher).
Assay kit: Human TNF-alpha ELISA kit (RAB0476, Sigma-Aldrich), human PARC/CCL18 ELISA kit (RAB0051, Sigma-Aldrich), human IL23A ELISA kit (RAB0697, Sigma-Aldrich), human IL-1 beta ELISA kit (RAB0273, Sigma-Aldrich), human IL-1RA ELISA kit (RAB0283, Sigma-Aldrich), human IL-10 ELISA kit (RAB0244, Sigma-Aldrich), fluorometric intracellular ROS kit (MAK144, Sigma-Aldrich), Lactate Colorimetric/Fluorometric Assay Kit Catalog L(+)-Lactate (K607-100, Biovision).

3.2 Isolation and polarization of monocytes-derived macrophages

Isolation of monocytes was conducted according to a protocol by Pertoft et al [66]. The isolation of monocytes was performed in a two-step procedure using the separation agent Percoll during centrifugation, see figure 3.1. Following description of procedure is based on isolation of monocytes from one donor ($\approx$ 45 ml buffy coat).



(a) **(b)**

Figure 3.1: Buffy coat is obtained from gradient 1 separation (a) and monocytes is obtained from gradient 2 separation (b).

Firstly, 3 ml of gradient 1 (1,076 kg/l Percoll) was added to 8 15 ml tubes and 6 ml buffy coat was carefully layered on top. Tubes were centrifuged at 800g for 30 min at room temperature. After centrifugation the cells were kept on ice and the white band with mononuclear cells (see figure 3.1 (a)) was removed with a glass Pasteur pipette and transferred to two new 45 ml centrifugation tubes. To wash the cells, cold HBSS(-) was added to the new tubes and then cells were centrifuged at 150g with a light brake (deceleration set at 2) for 5 min and 4^{o}C. Pellet was resolved with a pipette and washing procedure repeated twice. After last wash, the pellet was re-suspended in 5 ml cold HBSS(-) and carefully layered on top of 3 ml gradient 2 (1,064kg/l Percoll) in two new 15 ml tubes. Subsequently, cells were centrifuged at 800g for 60 min at 4^{o}C, separating monocytes from lymphocytes. The white layer consisting of monocytes (see figure 3.1 (b)) was harvested with a glass Pasteur pipette and transferred to a new 50 ml tube. Monocytes were washed three times with cold HBSS(-) and centrifuged at 150g without brake. Pellet was resolved with a pipette. Thereafter, monocytes were re-suspended in 5 ml culture medium (RPMI, 5% FBS, 1% Penicillin-streptomycin). Cell number was estimated with a MOXI Z^{TM}, an automatic cell counter. Cells were diluted to a concentration of 5x10^5 cells/ml and seeded in a 96 well tissue culture plate (150 000cells/well/300μl). Cells used for reactive oxygen species assay where added in an amount of 50 000cells/well/100μl and in a black tissue culture plate for improved fluorescent measurement. Cells were cultured at 37^{o}C and 5% CO_2 and 95% humidity for 24 hours. Subsequently, supernatant and non-adherent cells were removed in order to increase the purity of monocyte-derived macrophages. New medium with polarization activators according to M1 (LPS [100ng/ml] and IFN-γ [20ng/ml]) or M2 (IL-4 [40ng/ml]) in medium were added to the cells. M0 macrophages were negative controls, cultured in only medium without simulation. For reversible po-

larization (M1 to M2), medium was changed after 48 hours M1 polarization and IL-4 (40ng/ml) were added to M1 cells. This procedure was repeated for three separate blood donors.

3.3 Characterization of polarized macrophages

Cells were observed after 6, 24 and 48 hours after polarization where number of cells, morphology and secreted cytokines were studied. Generation of ROS and lactate production was also analyzed. Cells after reversible polarization (M1 to M2) were observed after 24 and 72 hours.

3.3.1 Cell number

Macrophages do not proliferate and it is therefore assumed that their number do not change throughout the experiments. However, a cell number study was still performed to evaluate if a significant difference between M0, M1 and M2 in terms of viability could be seen. Prior to cell quantification, three different cell detachment procedures were evaluated (Supp. data Fig. A.1). In the first procedure, medium was removed, then 100 μl trypsin added before cells were incubated 2-3 min at 37^{o}C. Trypsin was thereafter neutralized with 100 μl medium and number of cells was quantified. The second procedure was conducted in the same way as the first except that the cells were washed with 200 μl PBS before addition of trypsin. Last procedure involved removal of medium, addition of 200 μl cold PBS before cells was freezed for 2-3 min at -20^{o}C. When cells were quantified in later experiments, the first procedure with trypsin was used (Supp. data Fig. A.2).

The cells were automatically counted by adding 75 μl sample to a MOXI Z. Cells within the diameter of 7-20 μm were counted. An example of MOXI results is presented in Supplementary data Figure A.3.

3.3.2 Cytokine analysis

Supernatants for cytokine analysis and lactate analysis were stored in a non-proteinbinding (polypropylene) 96 well plate at -80°C. ELISA sandwich assay were used for studying following secreted cytokines; TNF-α, IL-23 and IL-1β (M1) and AMAC-1/CCL-18, IL-1ra and IL-10 (M2). ELISA was conducted according to manufacturer's description. Briefly, the supernatant was diluted 1:2 with dilution buffer and sample added to an ELISA plate with immobilized specific cytokine-detecting antibodies. The plate was incubated at room temperature for 2,5 h with gentle shaking. The solution was discarded and the wells washed 4 times with wash solution. After removal of the last wash, secondary biotin labeled detection antibodies were added to wells and the plate was incubated as previously for 1 h. Solution was discarded and wash procedure repeated as previously mentioned. Horseradish peroxidase (HRP) conjugated with streptavidin were added to wells in order to conjugate the enzyme with detection antibody and plate were incubated as previously for 45 min. Solution was discarded and wash procedure repeated as mentioned. Tetramethylbenzidine (TMB) reagent, a HRP substrate, was added to

wells and plate were incubated at room temperature in the dark at gentle shaking for 30 min. Then, stop solution was added to the wells and absorbance was read immediately at 450 nm in a PowerWave HT. A 5 parameter logistic fit standard curve was used in order to obtain concentrations of cytokines.

3.3.3 Reactive oxygen species analysis

The procedure was carried out according to manufacturer's instructions and with some further modifications that will be described. ROS was detected using a cell-permeable sensor. Reaction with ROS resulted in a fluorometric product, proportional to present ROS. Briefly, wells with 50 000 or 300 000 cells/well were cultured in a 96-well black assay plate overnight at 37 ^{o}C and 5% CO_2 and 95 % humidity. Subsequently, supernatant and non-adherent cells were removed and new medium with polarization activators according to M1 (LPS [100ng/ml] and IFN-γ [20ng/ml]) or M2 (IL-4 [40ng/ml]) was added to the cells. M0 macrophages were grown in medium without stimuli and was a negative control. The cells were incubated for 24 hours at 37^{o}C before 100 μL ROS green reaction mix were added to wells and cells were further incubated for 1 hours at 37^{o}C. Medium was thereafter removed and cells were washed twice with 200 μL PBS. 200 μL PBS or Phorbol myristate acetate (PMA) (500 ng/mL) were added to wells before cells were incubated for 15 min at 37^{o}C. The generated fluorometric product was measured at the intensity λ_{ex} = 490/λ_{em} = 520 nm in PowerWave HT. Cells were further incubated for 1 hour at 37^{o}C before absorbance was measured again. Control samples for background were wells with only PBS, empty wells, medium + ROS green mix, medium + PBS and PBS + ROS green mix.

3.3.4 Lactate analysis

Lactate was determined by an enzymatic assay which resulted in a calorimetric product, proportional to present lactate. Procedure was carried out according to manufacturer's instructions. Prior to analysis, 300 μL supernatant were spin filtered in vivaspin 500 tubes at 14 000 g for 10 min in order to concentrate the sample and deproteinize it from lactate dehydrogenase which degrades lactate. Thereafter 50 μL sample was mixed with 50 μL reaction mix and incubated for 30 minutes at room temperature. Thereafter, a calorimetric measurement was performed at OD 570 nm in PowerWave HT.

3.3.5 Statistics

Experiments were carried out in triplicates and results are presented as mean + SEM of donors. A two-way analysis of variance (ANOVA) was performed on data from each time period using the software Minitab, version 16. Detailed ANOVA results and confidence intervals for donors and macrophages are presented in the appendix.

4
Results

Monocytes were isolated from buffy coats obtained from healthy blood donors and cultured for 24 hours prior to polarization. 3-9 donors were used for main experiments. M0 cells were negative controls, grown in medium without polarization activators. M1 macrophages were obtained by induction with LPS and IFN-γ and M2 macrophages with IL-4. Cells were induced for 6, 24, 48 or 72 hours depending on experiment. Macrophage polarization was evaluated with ELISA analysis where following cytokines were analyzed; TNF-α, IL-23a and IL-1β as markers for a M1 phenotype and CCL18, IL-1ra and IL-10 as markers for a M2 phenotype. Results of IL-1ra and IL-23a secretion are not presented as the analysis did not yield in any detectable levels. Furthermore, analysis of produced ROS and lactate did not either yield in any significant results.

As macrophages do not proliferate, it was assumed that the same amount would be present throughout the experiment and no normalization to cell number is therefore performed in the graphs of the ELISA results. However, cells were still quantified after 0, 6 and 24 hours of polarization to study the viability between phenotypes (Supp. data A.2). There was difficulties with detaching all cells for quantification, leading to less cells being quantified than were present in wells. Therefore, it is difficult to conclude if the small decrease after polarization time (0 h) to 6 or 24 hours is due to less viable cells or less detached cells because of a stronger adherence. However, no significant difference in cell number could be seen between M0, M1 and M2 after 24 hours.

A two-way ANOVA was performed for analyzing the variance of mean between donors and macrophages. $p<0.05$ for donors and macrophages, indicating there was a significant variability between different donors as well as M0, M1 and M2. Detailed results on ANOVA analysis on data for each time point is found in Supplementary data.

4.1 Evaluation of M2 polarization

In Figure 4.1, M2 cells were stimulated with IL-4 from R&D Systems. No CCL18 production is seen at 6 hours. At 24 hours, there are more CCL18 produced in M2 macrophages than M1 macrophages (individual confidence intervals of Fig. A.9 in Supp. data) while no further difference is seen after 48 hours (individual confidence intervals of Fig. A.10 in Supp. data). Although the result from six donors were used, only one donor showed any response to IL-4, see Supplementary data Figure A.8-A.10. Finally, large error bars is seen in Figure 4.1.

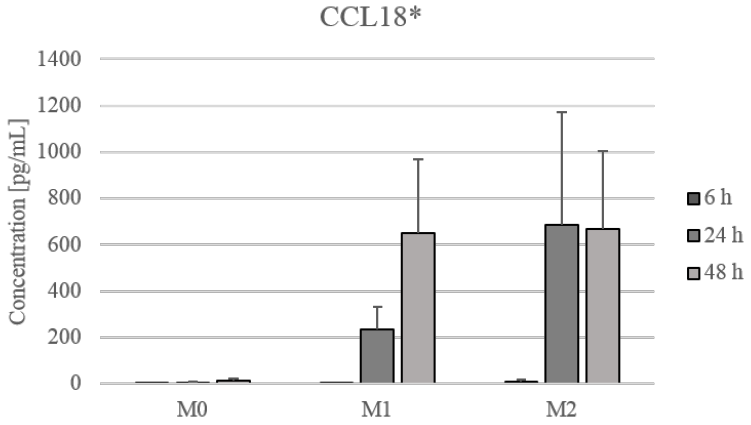
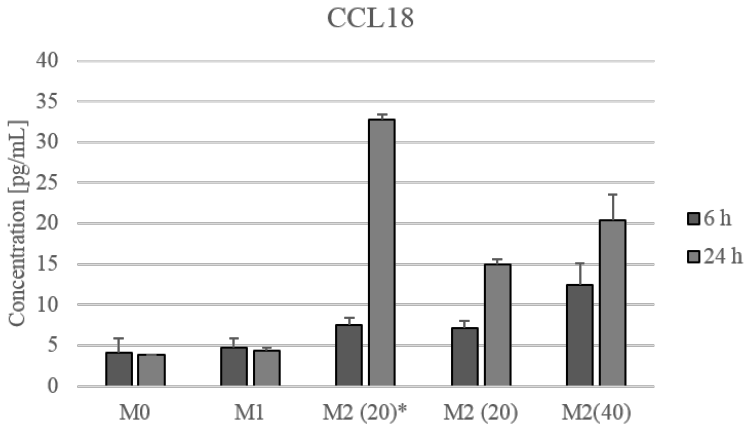


Figure 4.1: Graph illustrates ELISA analysis of CCL18 production in unstimulated macrophages M0, and stimulated macrophages M1(LPS, IFN-γ) and M2(IL-4) during 6, 24 and 72 hours of stimulation. Graphs represent M2 cells obtained with IL-4 from R&D Systems*. Bars represent results presented as mean value + SEM. n=6 blood donors.

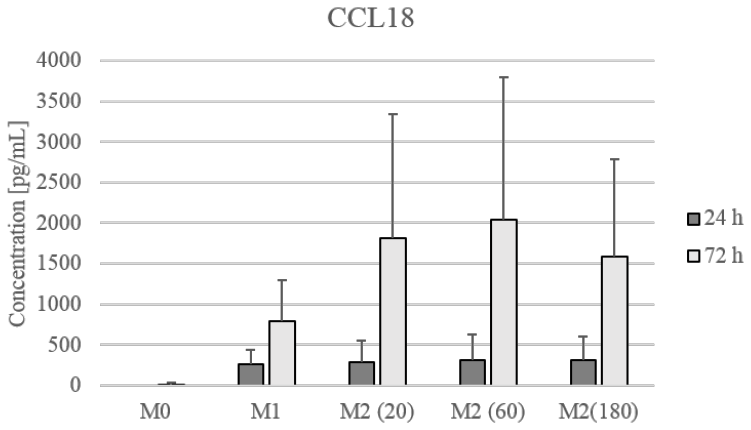
As M2 polarization by IL-4 induction did not result in a significant increase of CCL18 production in M2 macrophages compared to M1 macrophages (Fig. 4.1), an evaluation of IL-4 as M2 inducer was performed. Therefore, different distributors of IL-4, different concentrations, different polarization times as well as different ELISA kits were tested. As the CCL18 response in both ELISA kits was similar (results not presented), CCL18 production was continued to be analyzed with the ELISA kit from Sigma-Aldrich.

M2 macrophages stimulated with IL-4 from Sigma-Aldrich and R&D Systems* produced a very low amount of CCL18 after 24 hours (Fig. 4.2 (a)) compared to previous performed experiments using the same protocol where CCL18 production is around 2000 pg/mL. Additionally, no significant difference is seen between cells stimulated with different IL-4 distributors or with different concentrations (also see Supplementary data, Figure A.13 and Figure A.14). Therefore, the next experiment was performed with M2 macrophages stimulated with IL-4 from Sigma-Aldrich and with concentration of 20, 60 and 180 ng/mL IL-4 over a longer time period, see Figure 4.2 (b). After 72 hours, it is seen that induction of M2 macrophages leads to a higher production of CCL18 (Fig. 4.2 (b)) than previously (Fig. 4.1), despite the concentration of IL-4 cells were induced with. M0 cells did not produce any CCL18

and M1 cells produced approximately one 1/3 of what M2 cells produced. There was a large difference in response between the two donors used for the experiment, clearly seen in Supplementary data, Figure A.15-A.16.



(a)



(b)

Figure 4.2: Graphs illustrate ELISA analysis of CCL18 production in unstimulated macrophages M0, and stimulated macrophages M1 (LPS, IFN-γ) and M2 (IL-4) during 6, 24 or 72 hours of stimulation. Graphs represent M2 cells obtained with IL-4 from Sigma-Aldrich or R&D Systems* (a) and M2 cells obtained with different concentrations in ng/mL of IL-4 from R&D Systems (b). Bars represent results presented as mean value + SEM. In (a) n=7 blood donors for 6 h and n=3 for 24 h, (b) n=2 for all time points.

4.2 Characterization of M1 and M2 phenotypes

The production of IL-1β in response to LPS and IFN-γ stimulated M1 cells is displayed in Figure 4.3 (a). IL-1β production is seen in M1 cells while no production is seen in M0 or M2 macrophages. Similarly, in Figure 4.3 (b), IL-10 production is seen after 24 and 48 hours for M1 macrophages but not for M0 and M macrophages.

However, the maximum response is unknown since it was higher than the maximum standard concentration the ELISA analysis was calibrated with.

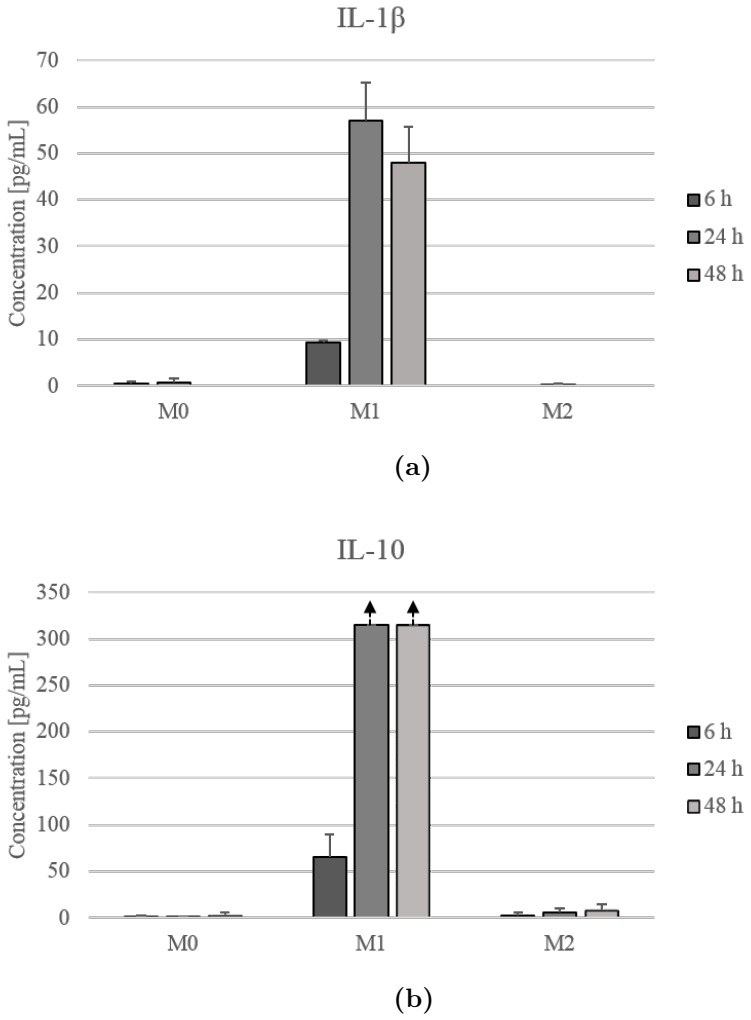
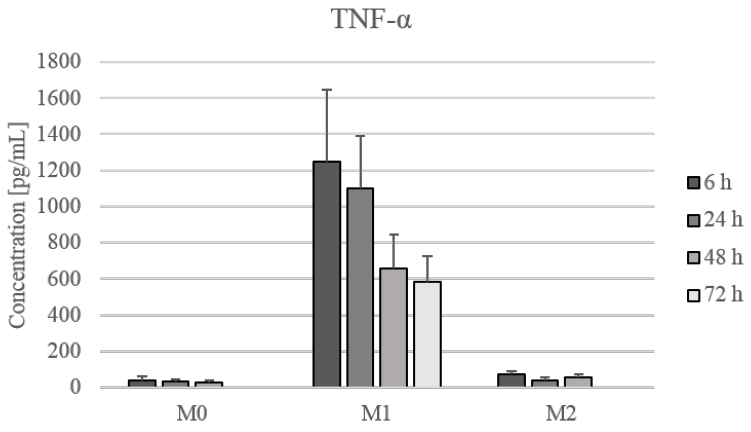
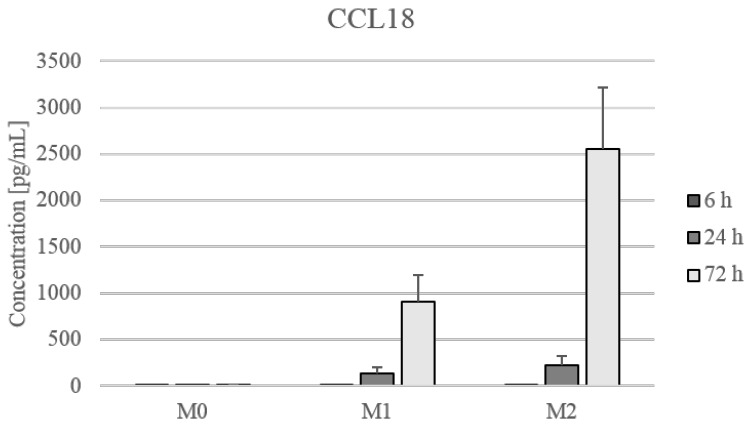


Figure 4.3: Graphs illustrate ELISA analysis of IL-1β production (a) and IL-10 production (b) in unstimulated macrophages M0, and stimulated macrophages M1 (LPS, IFN-γ) and M2 (IL-4) during 6, 24 or 48 hours of stimulation. Bars represent results presented as mean value + SEM. n=3 blood donors.

In Figure 4.4 (a) it is seen that M1 cells produced TNF-α in response to LPS and IFN-γ stimulation. A response in M1 cells is seen at all time points compared to no significant production of TNF-α in M0 and M2 macrophages. That M1 cells are different from M0 and M2 cells is statistically verified in confidence intervals of macrophages, Supplementary data Figure A.4-A.7. In Figure 4.4 (b), the CCL18 production is stronger for M2 cells than M1 cells after 72 hours of IL-4 induction, verified by confidence intervals for macrophages found in Supplementary data, Figure A.12. Although M1 cells did not receive any IL-4 stimuli, some CCL18 production is seen in those cell. However, the response is approximately 1/3 of the M2 response.



(a)



(b)

Figure 4.4: Graphs illustrate ELISA analysis of TNF-α production (a) and CCL18 production (b) in unstimulated macrophages M0, and stimulated macrophages M1 (LPS, IFN-γ) and M2 (IL-4) during 6, 24, 48 or 72 hours of stimulation. Bars represent results presented as mean value + SEM. For TNF-α: n=6 blood donors for 6 and 48 h, n=9 for 24 h, n=3 for 72 h. For CCL18: n=2 for 6 h, n=7 for 24 h, n=3 for 72 h.

Morphology of macrophages is seen in Figure 4.5. M0 and M2 macrophages have similar morphology; a rounded/oval with some protrusions, marked with arrows. However, no further difference is seen over time. M1 cells form clusters with rounded cells and the shape of some cells becomes elongated after 24 hours. After 72 hours some cells become spindle-like but clusters of rounded cells are still seen. Additionally, clusters of cells tend to grow over time, seen in Supplementary data, Figure A.2.

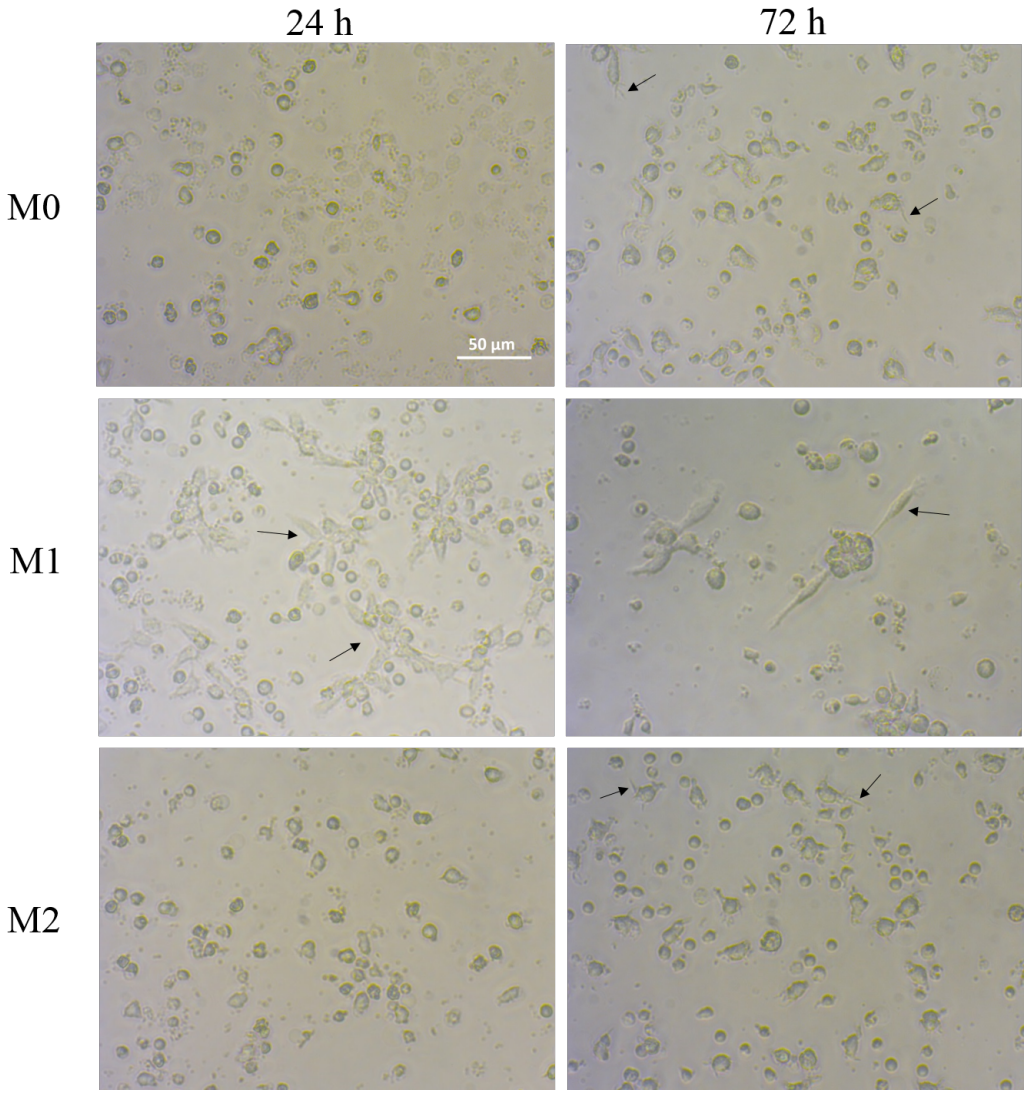
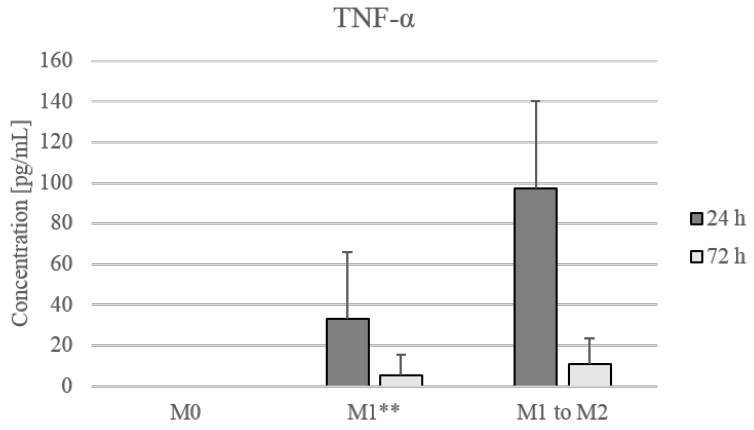


Figure 4.5: Morphology of unstimulated macrophages M0, and stimulated macrophages M1 (LPS, IFN-γ) and M2 (IL-4) during 24 and 72 hours of stimulation.

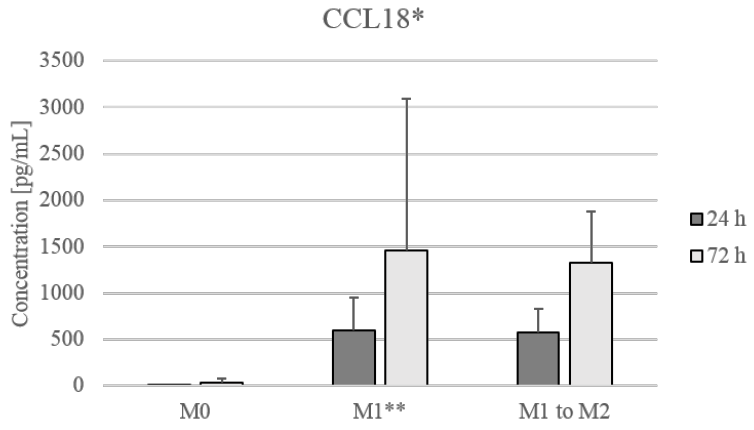
4.3 Evaluation of reversible polarization of M1 to M2 phenotype

A reversible polarization of M1 macrophages to M2 macrophages was also evaluated. M1 macrophages were induced with LPS and IFN-γ for 48 hours prior to reversible polarization. During reversible polarization, IL-4 were added to M1 cells.

After reversible polarization, it is seen in Figure 4.6 (a) that the TNF-α production in M1** cells grown in medium, and M1 to M2 cells grown in medium supplemented with IL-4, is decreased from 24 to 72 hours. The CCL18 production in Figure 4.6 (b), is increased from 24 to 72 hours. Large error bars are seen in the figure due to donors variation, see detailed donor data in Supplementary data, Figure A.23-A.26.



(a)



(b)

Figure 4.6: Graphs illustrate ELISA analysis of TNF-α production (a) and CCL18 production (b) in unstimulated macrophages M0 and M1**, and M1 macrophages stimulated with IL-4 (M1 to M2) during 24 or 72 hours of reversible polarization. *Cells are induced with IL-4 from R&D Systems. **Cells induced with LPS and IFN-γ for 48 h to become M1 cells and then culture in only medium without stimulation for further 24 or 72 hours. Bars represent results presented as mean value + SEM. n=3 blood donors. Data for each time point were analyzed in a two-way ANOVA test, $p<0.05$ for all time points except for TNF-α 72 h where $p<0.4$.

No difference in morphology or clustering of M0 macrophages is seen overtime in Figure 4.7. M1** macrophages have a few or no clusters after 24 hours. After 72 hours, more and larger cell clusters are seen and cells have a rounded morphology. Stimulated M1 to M2 cells form cluster after 24 hours. At 72 hours, cells are still in clusters but morphology of cells in the outer layer of the clusters are extended.

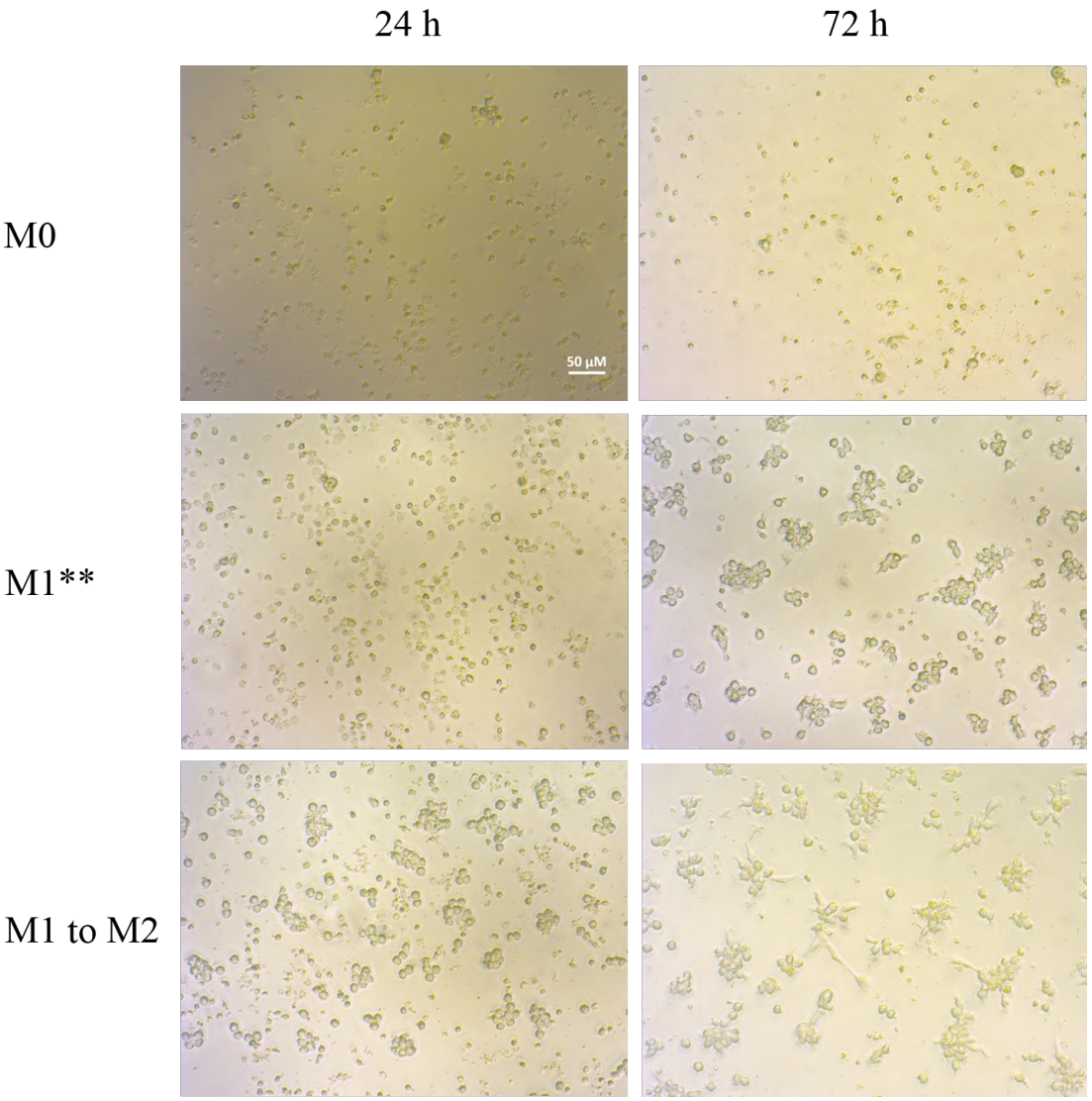


Figure 4.7: Morphology of unstimulated macrophages M0 and M1**, and M1 macrophages stimulated with IL-4 (M1 to M2) during 24 or 72 hours of reversible polarization.

5
Discussion

The aim of this study was to characterize polarized human macrophages for an *in vitro* inflammatory model for development of ulcer treatment products. Human monocyte-derived macrophages were polarized into M1 macrophage with LPS and IFN-γ and M2 macrophages with IL-4 during 6, 24, 48 or 72 hours. Nonstimulated macrophages M0 were a negative control. Characterization of M1 and M2 macrophages were performed by analyzing produced cytokines with ELISA. Analyzed M1 markers were TNF-α, IL-23 and IL-1β and analyzed M2 markers were CCL18, IL-1ra and IL-10. Furthermore, production of lactate and reactive oxygen species (ROS) was also studied as an increased production indicates a M1 phenotype. However, the analysis of IL-23, IL-1ra, lactate and ROS did not yield in any significant results (results not presented). When choosing markers it was desirably to use markers present in nonhealing ulcers, specifically diabetic ulcers, in order to be able to correlate the *in vitro* test model with clinical data or *in vivo* mouse models.

5.1 Evaluation of M2 polarization

As first experiments, an evaluation of M2 polarization was conducted. It was necessary as six different donors did not yield in any significant production of CCL18 due to inconsistent response among donors and no clear difference between M1 and M2 macrophages (Fig. 4.1). In Supplementary data, Figure A.8 (b)-A.10 (b), the individual value plots show that only a high response in donor 6 was seen and additionally, no clear difference is seen between M1 and M2 macrophages. Therefore, a decision was taken to induce macrophages with IL-4 from another distributor; Sigma-Aldrich and test different concentrations of IL-4 as well as induce the cells for a longer time period (Fig. 4.2). A reason for why 72 hours were not tested earlier was that other studies, using the same experimental setup and IL-4, showed a high CCL18 production already after 24 hours [6] [7]. Furthermore, an evaluation of IL-4 was necessary as there was two main differences between the two IL-4 used. Firstly, the amino acid sequence was longer in IL-4 from R&D Systems. Secondly, in contrast to IL-4 from Sigma-Aldrich, IL-4 from R&D Systems was also delivered with a carrier protein which should stabilize IL-4 during storage. Whether the longer amino acid sequence lead to a slightly different folding of the IL-4 protein or whether the carrier protein interfered when IL-4 bound to the IL-4 receptor on the cell is not known but only a speculation.

An important factor after IL-4 evaluation seemed to be the longer time of

polarization since one of the two donors showed a high CCL18 production in M2 cells, significantly different from M1 cells (Fig. 4.2 (b)). Furthermore, the different concentrations of IL-4 did not either affect the response significantly. For the first time during the project, relevant concentrations of CCL18 was detected and in consistency with previous CCL18 responses regarding produced amounts of CCL18 [6][7]. Therefore, a decision was taken to continue further experiments with IL-4 from Sigma-Aldrich and prolong the induction time to 72 hours (Fig. 4.2 (b)). As time seemed to be an important factor, if a longer induction with IL-4 from R&D Systems (Fig. 4.1) would have been performed earlier, it might have resulted in a high CCL18 response also. These speculations are based on the fact that M2 cells, although the response was very low, still were significantly different from M0 cells which did not receive any stimuli.

5.2 Donor variability

Not only time of polarization is important in this study. The responsiveness between donors is very different, resulting in large error bars when results are presented as mean of donors. Donor variability is a consequence of genetic and epigenetic variations together with age. Macrophages in young people generally have a higher responsiveness to stimuli [67]. In case of stimuli of LPS or IFN-γ, the reduced sensitivity in elderly people can be explained by reduced tyrosine phosphorylation of signal transducer and activator of STAT1 along with depletion of STAT1 due to inhibition of STAT1 gene transcription [67]. Finally, in elderly people, expression of cytokines is decreased together with a reduced ability of phagocytosis and clearance of pathogens [68].

Donor variability is consistent throughout these experiments and will have a great impact on the results when working with primary cells. It was also expected as this was a small scale study with limited amount of donors. What is important to see in the results is a clear difference between M0, M1 and M2 cells within the same donors and desirably that confidence interval of donors (presented in Supplementary data) lays within the same range. Furthermore, after performing an ANOVA, it would be desirable to obtain a result showing that donor variability is not significant. However, such a result was never obtained when analyzing six or more donors due to donor variability. Additionally, it is still important to mention that despite a variability, the advantage of utilizing primary cells is that they provide a result closer to *in vivo* responses than for instance a cell line does. To reduce the donor variability, the number of donors can be increased. However, time and cost must also be taken in consideration and only three donors are possible to work with at a time in terms of sample handling and available equipment.

5.3 Verification of M1 and M2 macrophages

It is possible to differentiate nonstimulated M0 macrophages to M1 and M2 phenotypes which is indicated by TNF-α production in M1 macrophages and CCL18 production in M2 macrophages (Fig. 4.4). Similar results were obtained when cells

in the same concentration were grown in a 24-well plate instead of 96-well plate, indicating well size (cells/area) is not important in this study (Supp. data Fig. A.1). The highest TNF-α response is after 6 hours while the highest CCL18 response is after 72 hours (Fig. 4.4). It is not known if the macrophages continuously produce the cytokines over a time period or if there is a burst of them after a specific time point.

M1 cells also produced CCL18 after 72 hours although the response is three times lower than that of M2 cells (Fig. 4.4 (b)). However, CCL18 production in M1 macrophages should be inhibited by IFN-γ [69][70]. This is because IFN-γ and IL-4 have an antagonistic effect on CCL18 expression as they induce transcription factors that binds competitively; IFN-γ induces STAT1 and IL-4 induces STAT6 [69].

A reason for why M1 cells produce CCL18 after 72 hours can be that the added IFN-γ is consumed after 72 hours which allows CCL18 production. On the other hand, M1-activated macrophages might produce other cytokines that induce the CCL18 production. As an example, IL-10 is also an inducer of CCL18 [69][51]. A high production of IL-10 was seen in M1 cells (Fig. 4.3 (b)) already after 24 hours. Further, IL-10 is a downregulator of TNF-α which might explain the decreased concentration of TNF-α over time (Fig. 4.4). On the other hand, the decreased TNF-α concentration can also be because macrophages consume the cytokines. Another speculation is that cytokines spontaneously degrade due to a short half-time in medium. Another argument for TNF-α depletion over time might be decreased cell viability. Cell viability was not enough assessed during the experiment due to difficulties detaching the cells and counting them. However, as the CCL18 response is increasing and therefore showing the opposite, cell viability most probably do not affect the results.

Important to mention about the data seen in Figure 4.3 is that during that specific experiment where IL-1β, IL-10 and CCL18 were measured, no clear M2 macrophage phenotype was seen due to not significant CCL18 production as seen in Figure 4.1. Therefore, production of IL-1β and IL-10 in M2 macrophages cannot be correctly evaluated and compared to M0 or M1 macrophages. On the other hand, what can be concluded is that a significant production of IL-1β and IL-10 can be seen in M1 cells compared to M0 cells. As IL-1β is an important inflammatory cytokine produced by M1 cells, the result indicates that also this cytokine can be used as a marker for M1 macrophages. That TNF-α and IL-1β can be used as markers for this model is relevant as these are typically elevated in diabetic ulcers [65][61]. Further, TNF-α elevation has been seen when culturing murine cells in a diabetic milieu with high glucose concentrations and also in diabetic mouse models [71][72].

IL-10 has been considered to be a M2 macrophage marker [27]. Although this experiment cannot say if M2 cells do produce IL-10 after 48 hours, other studies have shown a higher production of IL-10 by LPS and IFN-γ stimulated macrophages than IL-4 stimulated macrophages, both in murine [73] and human cells [32][74].

The results of IL-23a (subunit of IL-23) and IL-1ra are not presented as the analysis did not yield in a detectable response. What can be discussed is whether other time points would have resulted in a response. Otherwise, a cause could be repression of other produced cytokines. For example, a reason for why IL-23 was

not produced could be due to stimulation with IFN-γ, as the IL-23 response seems to be induced by LPS, but reduced when also IFN-γ is present [54]. IL-1ra on the other hand might not have been produced as no clear M2 response was seen due to insignificant CCL18 production (Fig. 4.1) and IL-1ra induced by IL-4 is associated with M2 activation [75][76]. However, similarly to IL-10 the results for this cytokine in literature vary; Isidro et al. showed a higher production by M1 cells than M2 cells while Rey-Giraud et al. showed the opposite [77][55].

5.4 Reversible polarization of M1 to M2 macrophages

A reversible polarization was conducted in order to see whether M1 cells could switch phenotype to M2 cells. M1 cells grown in only medium (M1**) were used as a control to see if IL-4 had any effects or whether a decreasing M1 response could be due to removed stimuli of LPS and IFN-γ. No significant difference in the CCL18 production was seen between M1** and M1 cells stimulated with IL-4, concluding that markers used (TNF-α and CCL18) were not able to prove that a M2 phenotype were obtained 4.6. However, a plasticity of cells is still seen as the TNF-α and CCL18 response is decreasing and increasing respectively (Fig. 4.6). On the other hand, in that specific experiment IL-4 was from R&D Systems was used, which previously caused an unclear M2 response due to low CCL18 production (Fig. 4.1). Another point of discussion is whether the M2 phenotype in M0 to M2 polarization differ from M0 to M1 to M2 polarization. There might be a difference in markers or function when polarizing already activated macrophages compared to non-activated macrophages.

The morphological changes of M1 cells seen in this study (Fig. 4.5, Fig. A.2) is similar to results of Isidro et al. where M1 cells formed aggregates of round/oval cells with a few fibroblast-like cells [77]. The formation of aggregates or clusters of cells could indicate that multinucleated giant cells will be formed as IFN-γ promotes macrophage fusion [78]. Maturation and activation of macrophages likely affects cytoskeletal rearrangements, generally resulting in a rounded M2 morphology and spindle-like M1 morphology [74][31]. Morphological comparisons of cells within this study and other studies is difficult as other comparable studies let their M0 cells grow for days, often supplemented with GM-CSF or M-CSF before induction which might lead to different morphologies.

5.5 Evaluation of ROS and lactate assays

The analysis of ROS production did not generate any results due to high background fluorescence and no difference between M0, M1 or M2 macrophages. Troubleshooting involved addition of PMA to cells in order to test whether ROS production could be primed in LPS-stimulated cells according to Pabst et al. [79]. From analysis of different backgrounds a conclusion was that probably phenol red in medium is generating a high background fluorescence. However, although cells were incubated with

PBS instead of medium, no ROS production was obtained. For future experiments, medium without phenol red can be tested. Experiments in this study indicate that M1-cell are present around 24 hours as a result of high TNF-α production, thus should the ROS production be high at 24 hours since ROS enhances TNF-α production. Nevertheless, other time point for ROS production other than 24 hours could also be evaluated in future studies. Further, the ROS kit measured intracellular ROS as superoxide and hydroxyl radicals while it would be interesting to test other kits which can measure extracellular ROS and also other radicals. ROS production is interesting to study in this model since ROS as a marker can be connected to clinical studies of oxidative stress in chronic diabetic foot ulcers [80][81]. In addition, lactate production is also interesting to study in macrophages as it also has a clinical connection; lactate concentration is increased in diabetic chronic foot ulcers [63][82]. However, the analysis performed in this study did not yield in any results due to high background absorbance, which after further evaluation came from fetal bovine serum. For future studies, an alternative would be to shortly incubate cells without serum or try to purify lactate from collected supernatant. Additionally, glucose consumption could also be measured instead of lactate consumption. In this study there were thoughts of evaluating the function of macrophages by for instance their phagocytic capacity which can be assessed by using beads or bacteria/yeast particles [32][75][55][83]. However, this kind of *in vitro* test would be more difficult to correlate with *in vivo* studies of nonhealing ulcers.

5.6 Suggestions for future studies

Further experiments and characterization of macrophages is required in order to develop an *in vitro* inflammatory model for Mölnlycke Health Care. Although it seems like M1 and M2 phenotypes are obtained due to TNF-α and CCL18 production, further characterizations need to be performed to strengthen the results. Additionally, some parts of the experimental procedures can be optimized or more evaluated.

Firstly, in order to evaluate if IFN-γ has an effect on CCL18 production in M1 macrophages, a future study could involve measurement of CCL18 production in M1 cells polarized with LPS and different concentrations of IFN-γ versus polarization with LPS alone.

Secondly, that macrophages are plastic and can be reversibly polarized from M1 to M2 and vice verse have previously been shown [84] [51]. From an ulcer treatment development view, it is an advantage to test substances or material that can drive the M1 cells towards a M2 anti-inflammatory phenotype. Further evaluation of switching phenotype could include different time points of polarization and reversible polarization as the M1 cells were polarized for 48 hours before being switched. Furthermore, a new reversible study would also involve a control with M1 cells continuously cultured in LPS and IFN-γ to see whether also that stimuli after a certain time drives cells towards a M2 cell phenotype.

As monocytes adhere to the plate bottom, it is assumed that they have spontaneously differentiated to macrophages. In this study, it is assumed that after 24 hours monocytes are differentiated to macrophages. However, other studies let their monocytes differentiate to macrophages for days to one week with supplements as

GM-CSF (for M1 cells) or M-CSF (for M2 cells), before stimulating them to M1 and M2 cells. It has been shown that macrophages are obtained after 6 days with spontaneous monocyte differentiation without GM-CSF or M-CSF [74]. A future experiment could involve longer incubation than 24 hours prior to polarization which might be necessary for obtaining a more differentiated macrophage which express more markers. GM-CSF and M-CSF have shown to increase but also decrease cytokine production compared to cells grown in medium without these supplements prior to polarization [31]. However, as it is shown that M1 and M2 macrophages can be obtained anyway, it is probably unnecessary to add further stimulation. Additionally, it is more difficult to draw a conclusion what really stimulates the cells after polarization if they have been cultured with different supplements before polarization stimulation.

Finally, a suggestion for other characterization methods of M1/M2 macrophages is analysis of surface markers by immunocytochemistry or fluorescence-activated cell sorting (FACS). Markers of macrophages are well studied and FACS is frequently used in these kind of experiments. Analysis of surface markers can for example identify what kind of macrophage phenotype is present during a specific time point while this is more difficult with cytokines as these are secreted during different time points.

6
Conclusion

Production of TNF-α and IL-1β indicated that a M1 phenotype was obtained after LPS and IFN-γ stimulation. Moreover, CCL18 production indicated that a M2 phenotype was obtained after IL-4 stimulation. On the other hand, production of IL-10 which is a M2 phenotype marker was produced by M1-stimulated macrophages, a phenomenon seen in other studies as well. M1 cells might produce IL-10 as a feedback mechanism to lower the pro-inflammatory response by inhibiting of TNF-α production. No detectable production of IL-23 and IL-1ra was seen which could be because of inhibition by other cytokines as IFN-γ in case of IL-23, or that time points of analysis did not correspond to when these markers were produced. Moreover, no significant production of CCL18 was seen after reversible polarization, indicating that M1 macrophages did not switch phenotype to M2 macrophages. Whether this was not seen because of the chosen CCL18 marker, IL-4 from R&D systems or time points of polarization that affected the reversible polarization, is not known but could be further evaluated. Different assay setups of lactate and ROS did not display any significant results in this study despite different experimental setups. This could be explained by high background fluorescence/absorbance during the spectrophotometric reading due to certain medium compounds as phenol red.

The concentration of cytokines vary during different time points which makes time point of analysis important. ANOVA analysis indicated that donor variability is consistent throughout the experiments. For a future *in vitro* inflammatory model TNF-α, IL-1β and CCL18 are suitable markers of M1 and M2 macrophage phenotypes as these were produced in substantial levels. Since IL-1ra, IL-23, ROS and lactate did not yield in detectable levels, these consider further evaluation.

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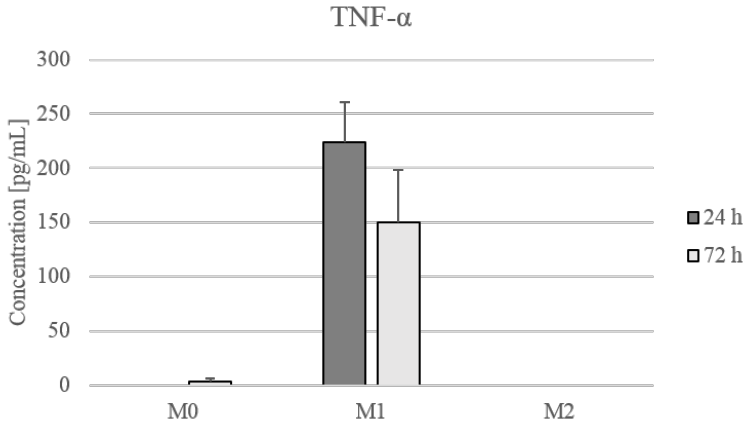
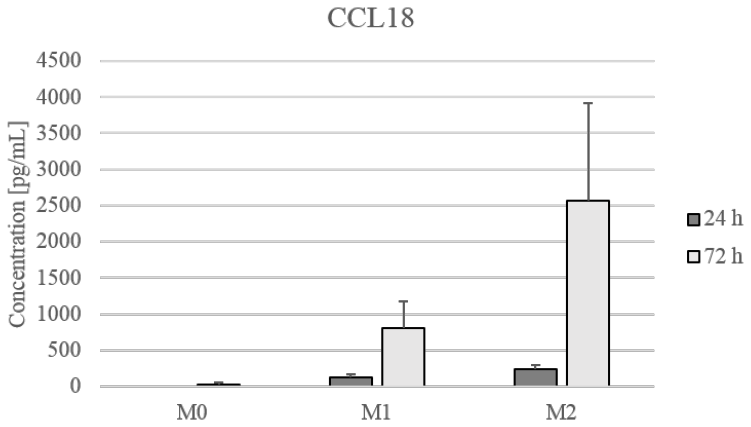
A

Supplementary data

A.1 Polarization of M1/M2 in a 24-well plate



(a)



(b)

Figure A.1: Graphs illustrate ELISA analysis of TNF-α production (a) and CCL18 production (b) in unstimulated macrophages M0, and stimulated macrophages M1(LPS, IFN-γ) and M2(IL-4) during 24 or 72 hours of stimulation. Bars represent results presented as mean value + SEM. n=3 for all time points. Data for each time point were analyzed in a two-way ANOVA test.

To the 96-well plate and 24-well plate, 300 μL cells and 1 mL cells respectively were added with a concentration of $5*10^5$ cells/mL. Area of 96-well plate and 24-well plate is 0.36 cm^2 and 2 cm^2 respectively. This leads to $4.17*10^5$ cells/cm^2 in a 96-well plate and $2{,}5*10^5$ cells/cm^2 in a 24-well plate. Cells per area needs to be taken in consideration when comparing results of 24-well plate with results of a 96-well plate.

A.2 Morphology of macrophages

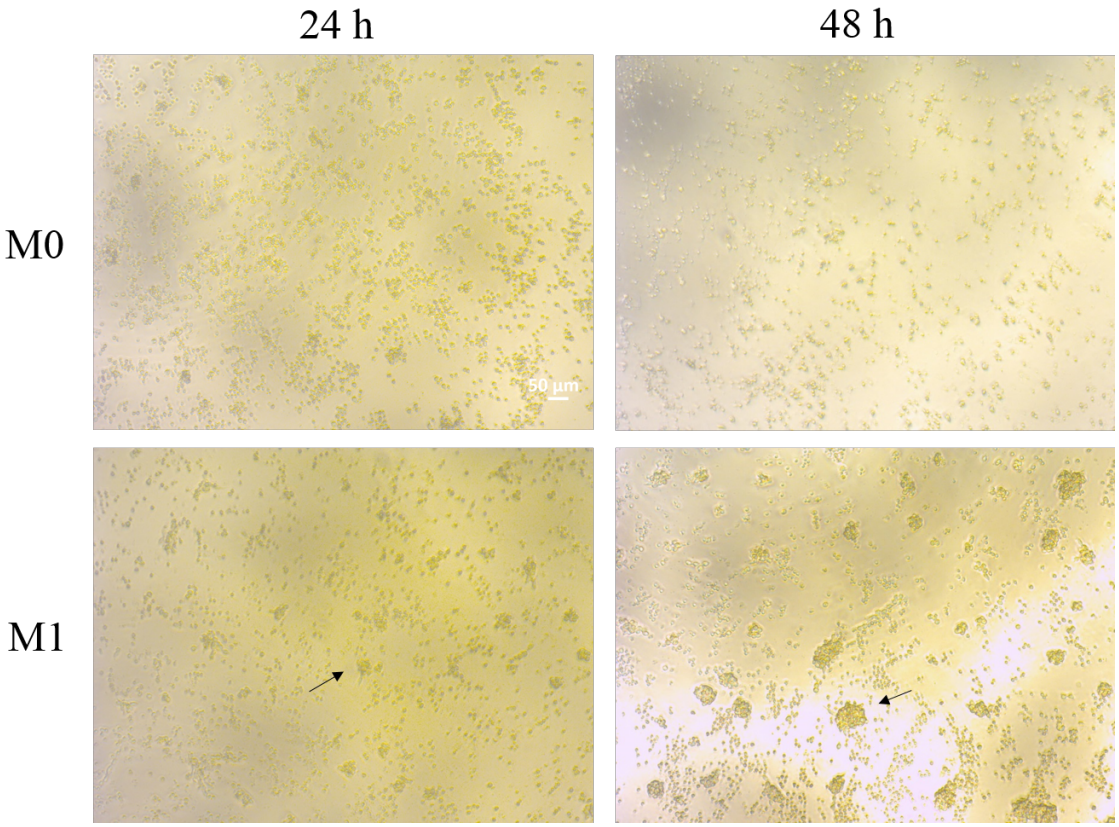


Figure A.2: Morphology of unstimulated macrophages M0 and stimulated macrophages M1(LPS, IFN-γ) during 24 and 48 hours of polarization.

A.3 Detachment of cells

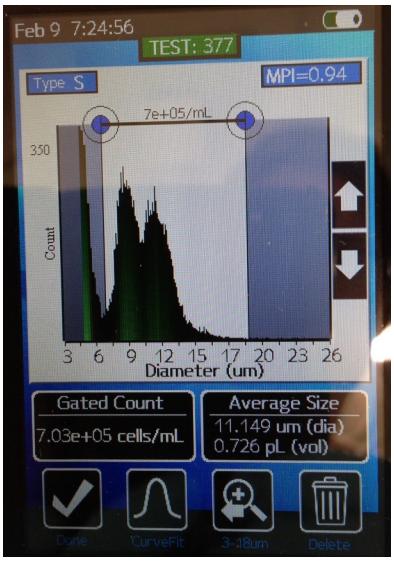


Figure A.3: Image of MOXI results showing size distribution of cells, average cell diameter and cell concentration.

Detachment method/ Time	Trypsin	PBS + freezing
0 h	81 %	83 %
6 h	35 %	46 %
24 h	66 %	62 %
48 h	21 %	33 %

Table A.1: Table showing how many percent cells are detached after 0, 6, 24 and 48 hours of culturing in only medium. Cells were detached with three different methods; trypsin, PBS + freezing and PBS + trypsin. As the PBS + trypsin only yielded in 41 % cells at 0 h, no further detachment with that method was performed. n=6.

A.4 Quantification of cells

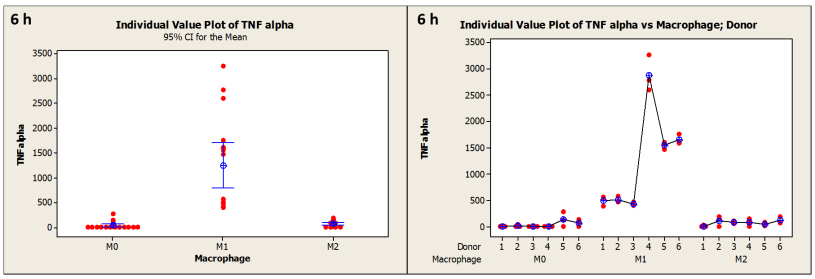
Macrophage/ Time	M0	M1	M2
0 h	68 %	68 %	68 %
6 h	56 %	48 %	72 %
24 h	58 %	50 %	56 %

Table A.2: Table showing how many percent cells are detached in wells after 0, 6, and 24 hours of culturing nonsimulated M0 macrophages and simulated M1 (LPS, IFN-γ) and M2 (IL-4) macrophages. Cells were detached with trypsin. n=6.

A.5 ANOVA analysis

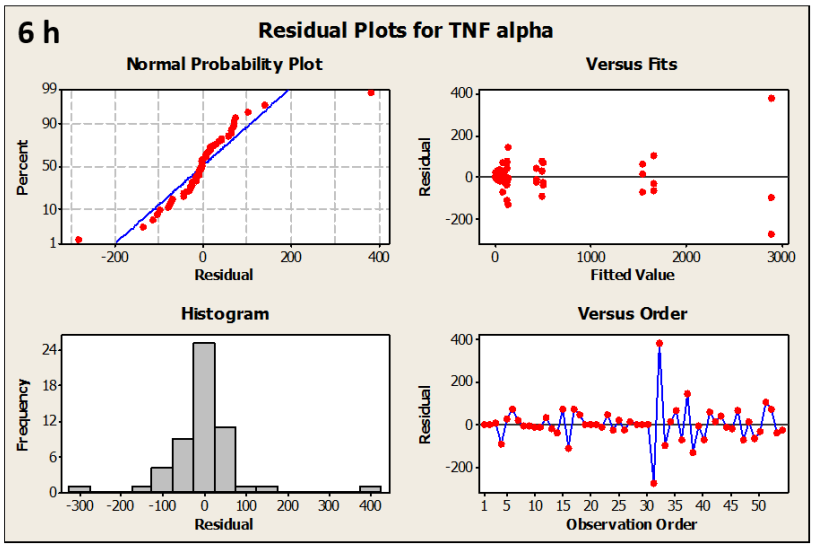
Different graphs were obtained from ANOVA results. The different individual value plots visualize the variations among macrophages and for each donor. The residual plots is used for examining the goodness of model fit in ANOVA. Data in normal probability plot should generally follow a straight line. Residuals versus fitted values show a random pattern of residuals on both sides of 0. No recognizable pattern should be distinguished in this plot. Histogram of residuals shows general characteristics of residuals including the values, spread and shape. A long tale might indicate a skewed distribution. If there are bars far from each other these might indicate outliers. Last plot of residual plots are residuals versus order of data. The graph is a plot of all residuals and is used to check whether residuals are correlated or not. Below each graph the result of ANOVA is presented together with confidence intervals for macrophages and donors.

A.5.1 ANOVA analysis of TNF-alpha ELISA results presented in Figure 4.4 (a)



(a)

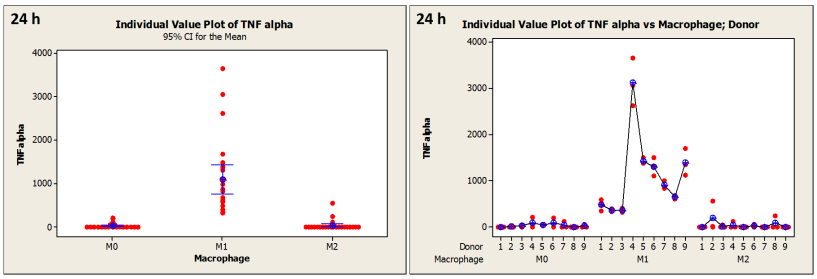
(b)



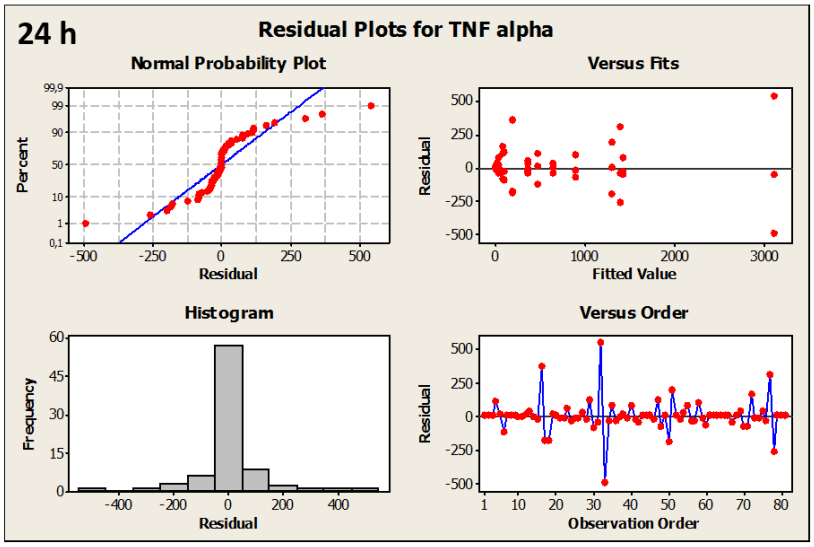
(c)

Figure A.4: ANOVA analysis of TNF-α ELISA after 6 hours.

```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF        SS        MS        F       P
Macrophage     2  17094553   8547277   814,04   0,000
Donor          5   4881653    976331    92,99   0,000
Interaction   10   9281601    928160    88,40   0,000
Error         36    377995     10500
Total         53  31635802
S = 102,5   R-Sq = 98,81%   R-Sq(adj) = 98,24%

Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  +---------+---------+---------+---------
M0             37,76  (*)
M1           1249,63                                    (-*)
M2             75,30   (*-)
                     +---------+---------+---------+---------
                     0        350       700       1050


Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ------+---------+---------+---------+---
1       168,160  (--*-)
2       211,410    (-*--)
3       171,881  (--*--)
4       985,006                                  (-*--)
5       574,129                    (--*--)
6       614,819                      (--*-)
               ------+---------+---------+---------+---
                   250       500       750      1000
```



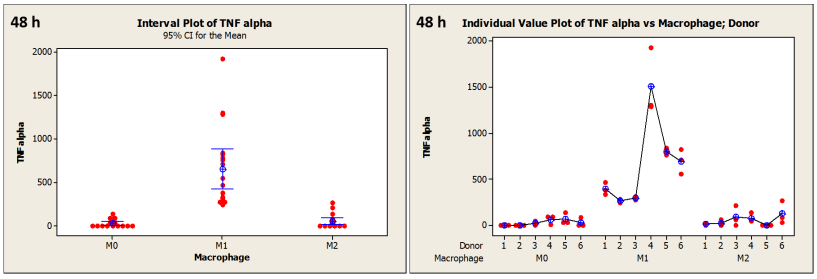
(a) (b)



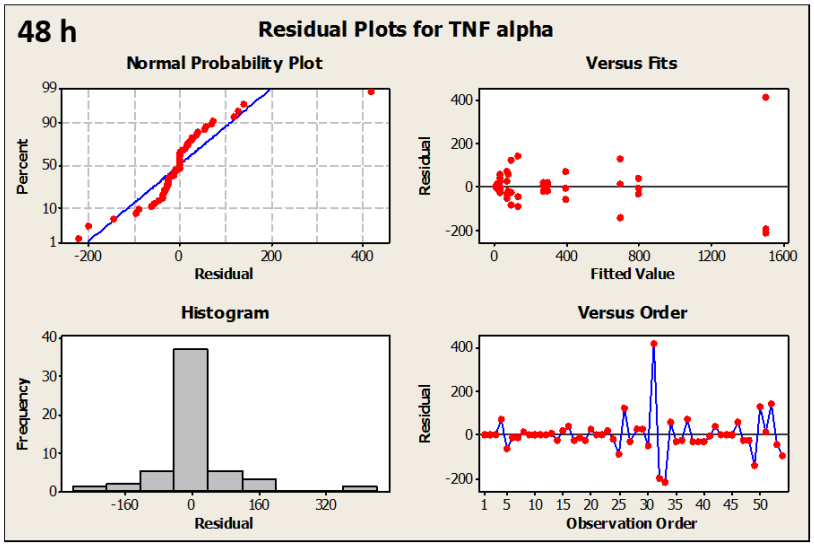
(c)

Figure A.5: ANOVA analysis of TNF-α secretion after 24 hours.

```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF         SS         MS        F       P
Macrophage     2   20581416   10290708   485,76   0,000
Donor          8    6205536     775692    36,62   0,000
Interaction   16   11960867     747554    35,29   0,000
Error         54    1143988      21185
Total         80   39891806
S = 145,6   R-Sq = 97,13%   R-Sq(adj) = 95,75%

Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  -+---------+---------+---------+--------
M0             31,47  (-*-)
M1           1104,27                                      (-*-)
M2             38,50  (-*-)
                     -+---------+---------+---------+--------
                      0        300       600       900

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ---------+---------+---------+---------+
1        157,01    (--*--)
2        184,23     (--*--)
3        128,26  (--*---)
4       1079,25                                  (--*--)
5        484,72             (--*--)
6        469,33            (---*--)
7        311,35         (--*---)
8        241,76       (--*--)
9        466,82            (---*--)
               ---------+---------+---------+---------+
                       300       600       900      1200
```



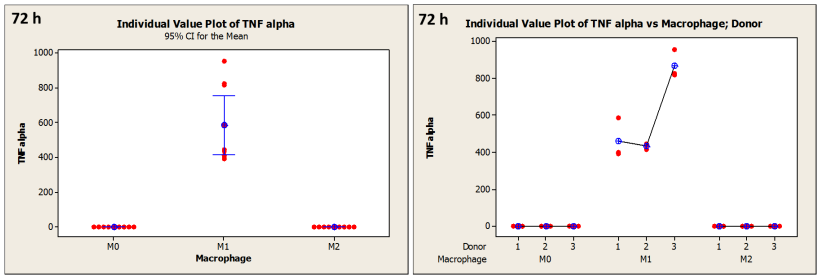
(a) (b)



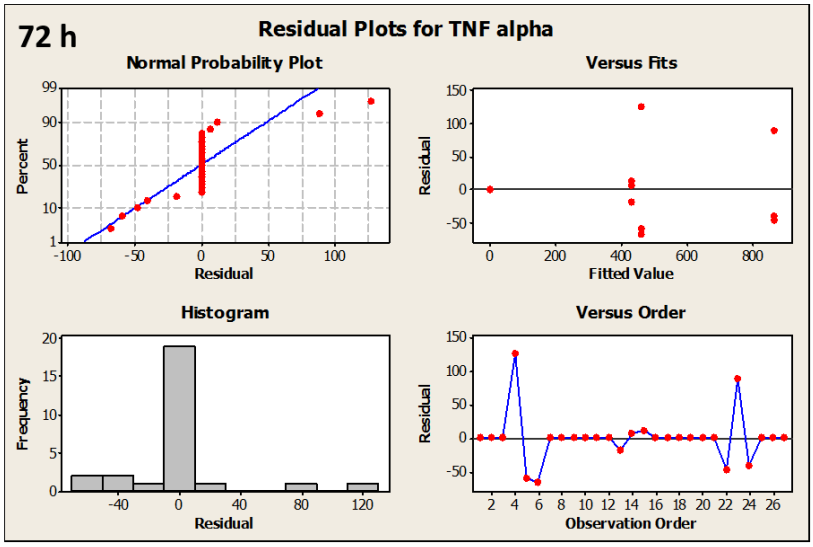
(c)

Figure A.6: ANOVA analysis of TNF-α secretion after 48 hours.

```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF       SS        MS        F       P
Macrophage     2  4537423   2268711   207,78   0,000
Donor          5  1265165    253033    23,17   0,000
Interaction   10  2061872    206187    18,88   0,000
Error         36   393071     10919
Total         53  8257531
S = 104,5   R-Sq = 95,24%   R-Sq(adj) = 92,99%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  -+---------+---------+---------+--------
M0            30,226  (--*-)
M1           657,125                                   (--*-)
M2            54,942    (--*-)
                      -+---------+---------+---------+--------
                       0        200       400       600

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --------+---------+---------+---------+-
1       134,475    (----*----)
2        96,754  (---*----)
3       135,408    (----*----)
4       546,838                                (---*----)
5       287,868              (----*----)
6       283,242              (----*----)
                --------+---------+---------+---------+-
                        150       300       450       600
```



(a) (b)



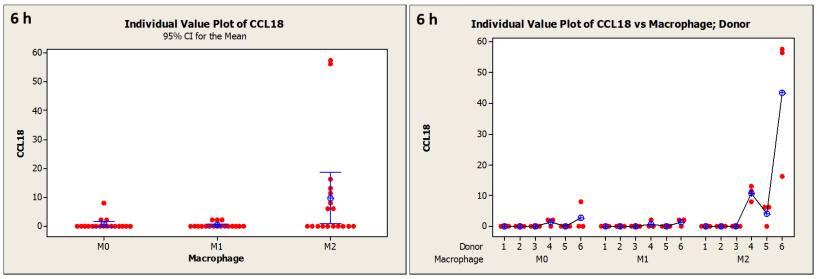
(c)

Figure A.7: ANOVA analysis of TNF-α secretion after 72 hours.

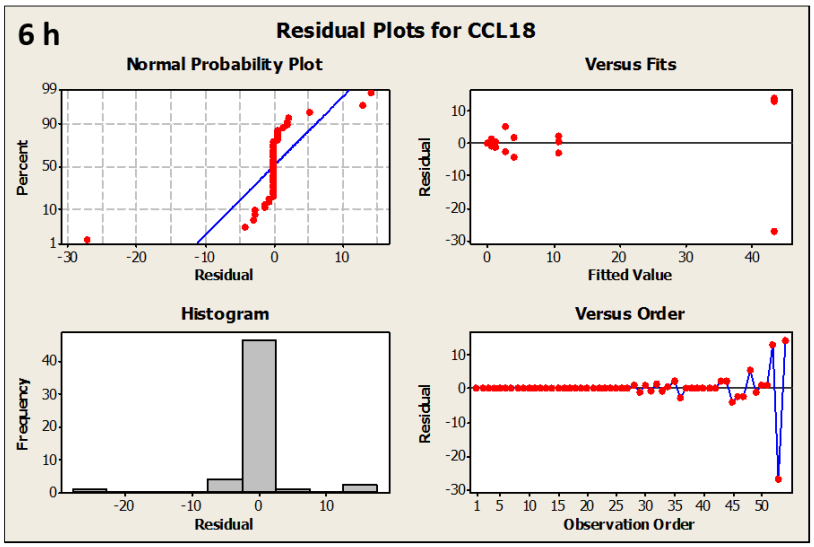
```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source       DF       SS        MS       F       P
Macrophage    2  2060084  1030042  506,35  0,000
Donor         2   118368    59184   29,09  0,000
Interaction   4   236736    59184   29,09  0,000
Error        18    36616     2034
Total        26  2451804
S = 45,10   R-Sq = 98,51%   R-Sq(adj) = 97,84%

Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  --+---------+---------+---------+-------
M0            0,000  (-*-)
M1          585,958                                  (*-)
M2            0,000  (-*-)
                     --+---------+---------+---------+-------
                       0        200       400       600

Individual 95% CIs For Mean Based on
Pooled StDev
Donor     Mean   -+---------+---------+---------+--------
1      153,178    (-----*----)
2      143,975   (----*----)
3      288,806                           (----*----)
                 -+---------+---------+---------+--------
                 120       180       240       300
```

A.5.2 ANOVA analysis of CCL18 ELISA results presented in Figure 4.1

A.5.2.1 Cells activated with IL-4 from R&D Systems



(a) (b)

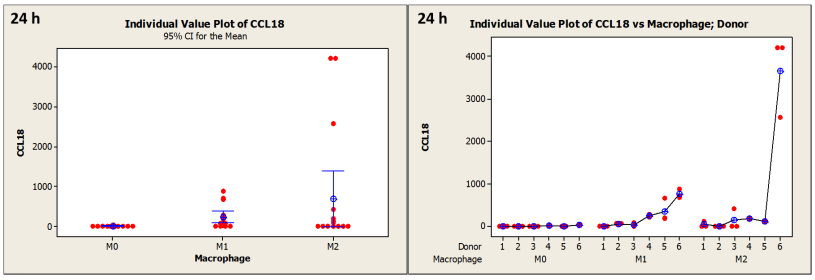


(c)

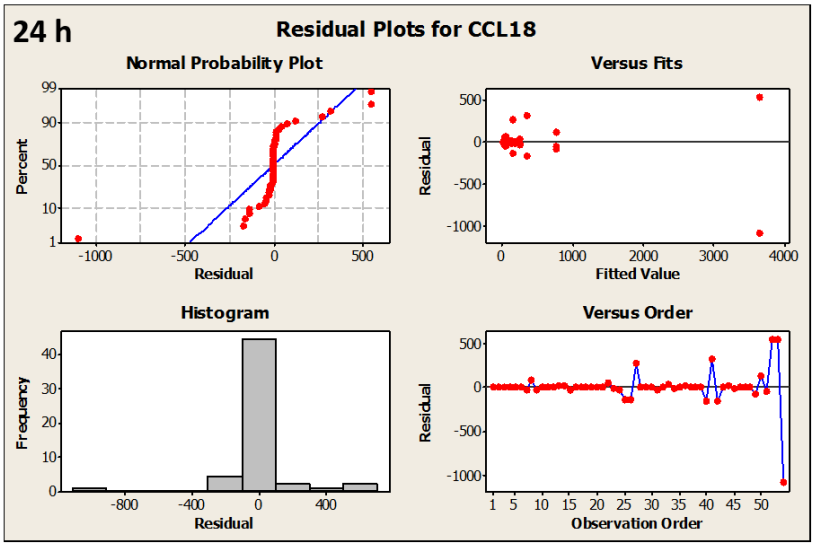
Figure A.8: ANOVA analysis of CCL18 secretion after 6 hours.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF       SS        MS       F       P
Macrophage     2  1017,87   508,937   15,35   0,000
Donor          5  1731,22   346,244   10,44   0,000
Interaction   10  2631,28   263,128    7,93   0,000
Error         36  1193,92    33,164
Total         53  6574,29
S = 5,759    R-Sq = 81,84%    R-Sq(adj) = 73,26%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  ------+---------+---------+---------+---
M0          0,66067   (------*------)
M1          0,32867  (------*------)
M2          9,70011                          (------*------)
                     ------+---------+---------+---------+---
                         0,0       4,0       8,0      12,0
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ------+---------+---------+---------+---
1        0,0000  (-----*-----)
2        0,0000  (-----*-----)
3        0,0000  (-----*-----)
4        4,2538         (-----*------)
5        1,3582    (-----*------)
6       15,7669                          (-----*------)
               ------+---------+---------+---------+---
                   0,0       6,0      12,0      18,0
```



(a) (b)

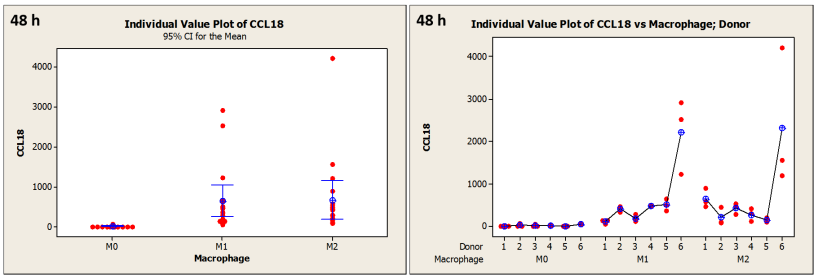


(c)

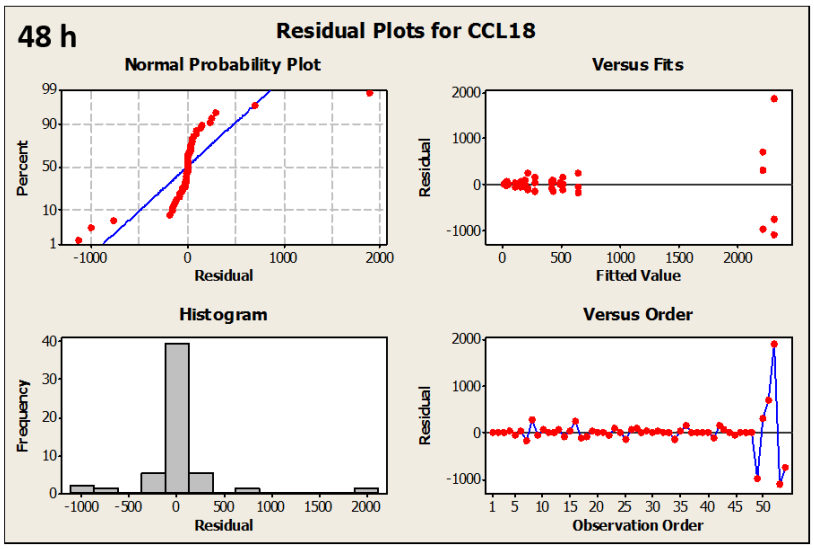
Figure A.9: ANOVA analysis of CCL18 secretion after 24 hours.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF        SS        MS       F       P
Macrophage     2   4329353   2164677   37,14   0,000
Donor          5  14896661   2979332   51,11   0,000
Interaction   10  18084899   1808490   31,02   0,000
Error         36   2098492     58291
Total         53  39409405
S = 241,4   R-Sq = 94,68%   R-Sq(adj) = 92,16%

Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  ----+---------+---------+---------+-----
M0             5,714  (---*----)
M1           236,056           (---*----)
M2           687,441                          (---*----)
                     ----+---------+---------+---------+-----
                         0       250       500       750
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ---+---------+---------+---------+------
1         12,91  (--*---)
2         16,49  (--*---)
3         55,64   (--*--)
4        142,26     (--*--)
5        153,46     (--*--)
6       1477,66                              (---*--)
                ---+---------+---------+---------+------
                   0        500       1000      1500
```



(a) (b)



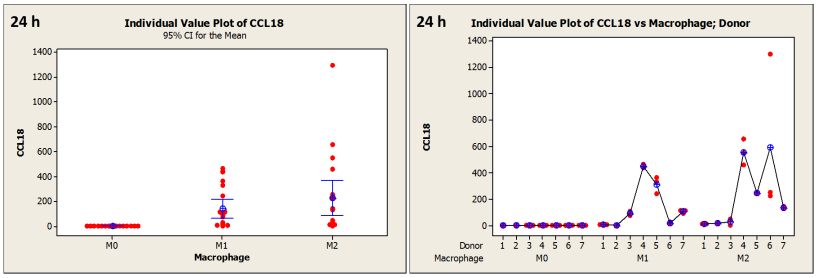
(c)

Figure A.10: ANOVA analysis of CCL18 secretion after 48 hours.

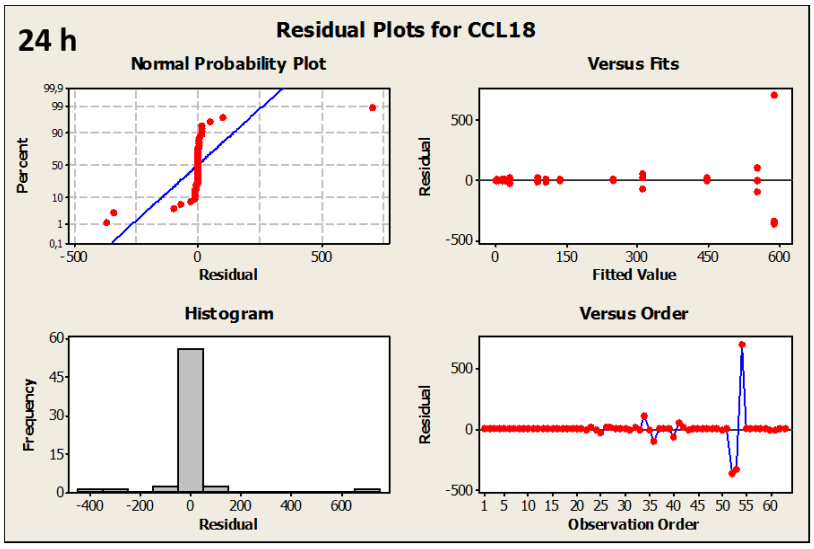
```
Source        DF        SS        MS      F      P
Macrophage     2   4968065   2484033  12,25  0,000
Donor          5  12616650   2523330  12,45  0,000
Interaction   10   6869721    686972   3,39  0,003
Error         36   7298022    202723
Total         53  31752458
S = 450,2   R-Sq = 77,02%   R-Sq(adj) = 66,16%

Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  -------+---------+---------+---------+--
M0            15,522  (-------*------)
M1           650,131                   (-------*------)
M2           667,430                    (------*------)
                     -------+---------+---------+---------+--
                            0       300       600       900
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --+---------+---------+---------+-------
1        249,00    (-----*-----)
2        213,35  (-----*-----)
3        209,07  (-----*-----)
4        253,38    (-----*-----)
5        216,86  (-----*-----)
6       1524,51                              (-----*------)
               --+---------+---------+---------+-------
                 0        500       1000      1500
```

A.5.2.2 Cells activated with IL-4 from Sigma-Aldrich



(a) (b)

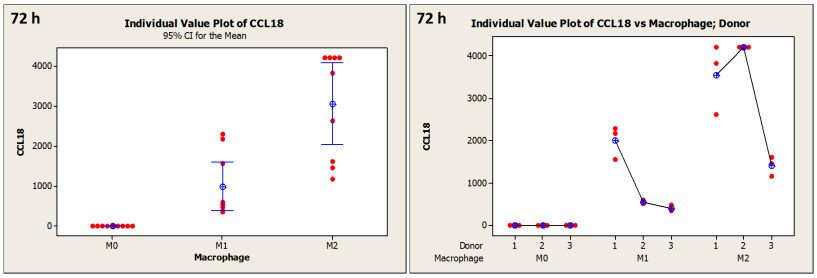


(c)

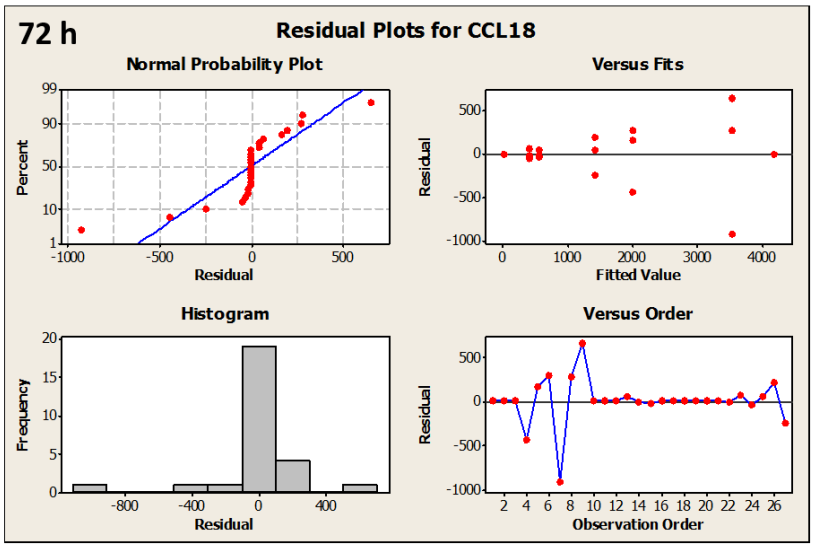
Figure A.11: ANOVA analysis of CCL18 secretion after 24 hours.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF       SS       MS       F       P
Macrophage     2   544797   272398   14,65   0,000
Donor          6   812951   135492    7,29   0,000
Interaction   12   853665    71139    3,83   0,001
Error         42   780920    18593
Total         62  2992332
S = 136,4   R-Sq = 73,90%   R-Sq(adj) = 61,48%
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  ------+---------+---------+---------+---
M0            1,113  (-----*-----)
M1          140,648               (-----*-----)
M2          226,802                          (-----*-----)
                    ------+---------+---------+---------+---
                          0        100       200       300

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ------+---------+---------+---------+---
1         7,638  (------*-----)
2         7,790  (------*-----)
3        39,543     (-----*-----)
4       334,000                      (-----*-----)
5       186,658               (-----*------)
6       203,299                (------*-----)
7        81,051        (-----*------)
                 ------+---------+---------+---------+---
                       0        150       300       450
```



(a) (b)



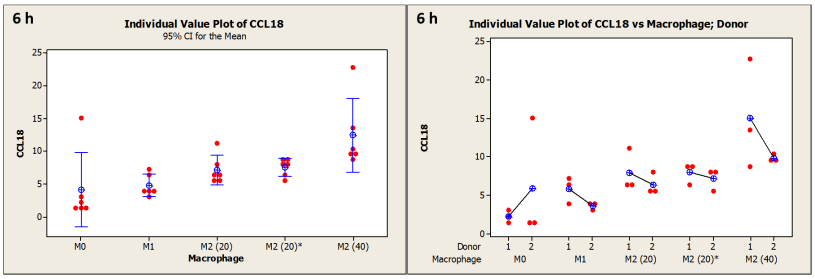
(c)

Figure A.12: ANOVA analysis of CCL18 secretion after 72 hours.

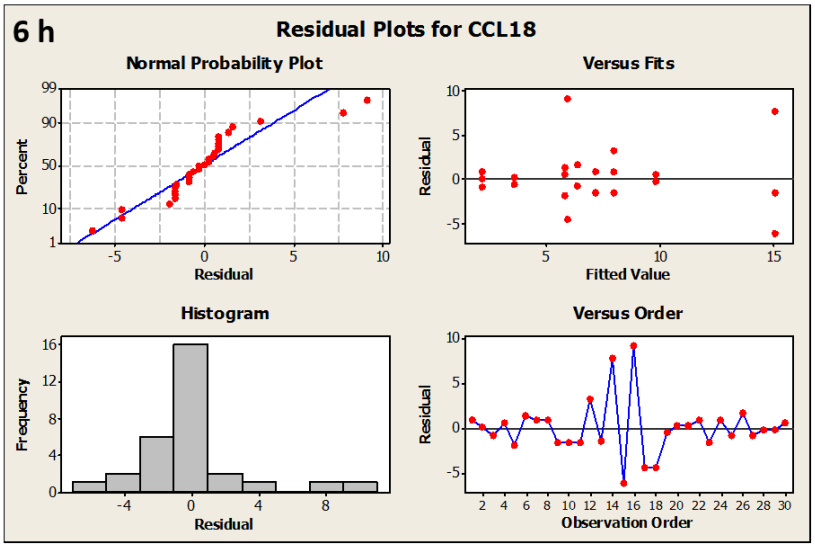
```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF         SS         MS       F      P
Macrophage     2   43695613   21847806  220,82  0,000
Donor          2    7748635    3874317   39,16  0,000
Interaction    4    9747392    2436848   24,63  0,000
Error         18    1780946      98941
Total         26   62972586
S = 314,5   R-Sq = 97,17%   R-Sq(adj) = 95,91%
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  --+---------+---------+---------+-------
M0             0,00  (-*-)
M1          984,45           (-*-)
M2         3052,64                                (--*-)
                     --+---------+---------+---------+-------
                       0       1000      2000      3000

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --+---------+---------+---------+-------
1       1851,17                        (---*---)
2       1581,69                  (----*---)
3        604,24  (---*---)
                 --+---------+---------+---------+-------
                  500       1000      1500      2000
```

A.5.2.3 Cells activated with IL-4 from Sigma-Aldrich or R&D Systems



(a) (b)

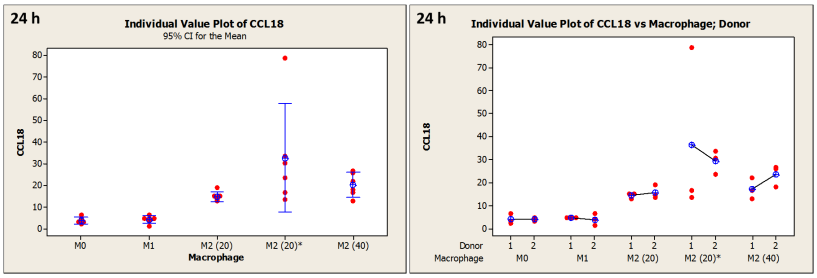


(c)

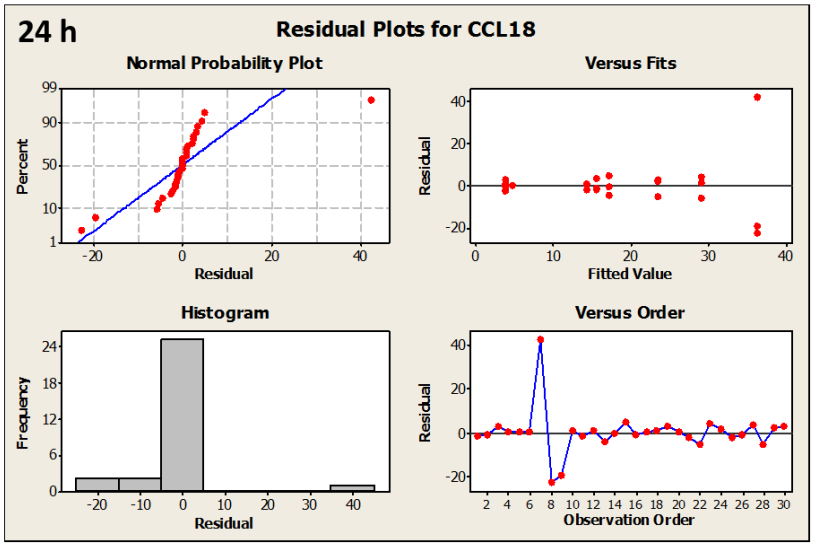
Figure A.13: ANOVA analysis of CCL18 secretion after 6 hours.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF       SS       MS     F       P
Macrophage     4  260,672  65,1679  4,96  0,006
Donor          1   11,066  11,0656  0,84  0,370
Interaction    4   62,129  15,5323  1,18  0,348
Error         20  262,605  13,1302
Total         29  596,472
S = 3,624   R-Sq = 55,97%   R-Sq(adj) = 36,16%
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  --------+---------+---------+---------+-
M0            4,0773  (-------*-------)
M1            4,7203    (-------*-------)
M2 (20)       7,1590            (-------*-------)
M2 (20)*      7,5680              (-------*-------)
M2 (40)      12,4353                        (-------*-------)
                     --------+---------+---------+---------+-
                           4,0       8,0      12,0      16,0

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ---------+---------+---------+---------+
1       7,79933           (------------*------------)
2       6,58467  (------------*------------)
                 ---------+---------+---------+---------+
                         6,0       7,5       9,0      10,5
```



(a) (b)



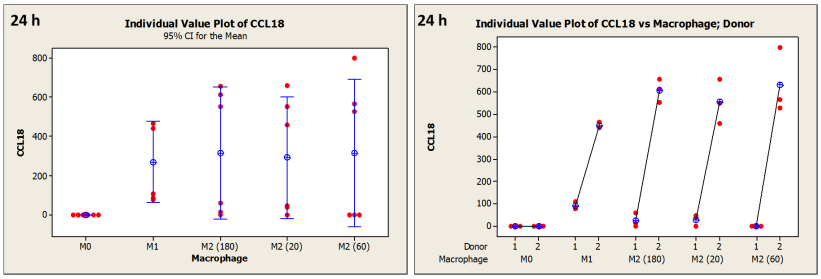
(c)

Figure A.14: ANOVA analysis of CCL18 secretion after 24 hours.

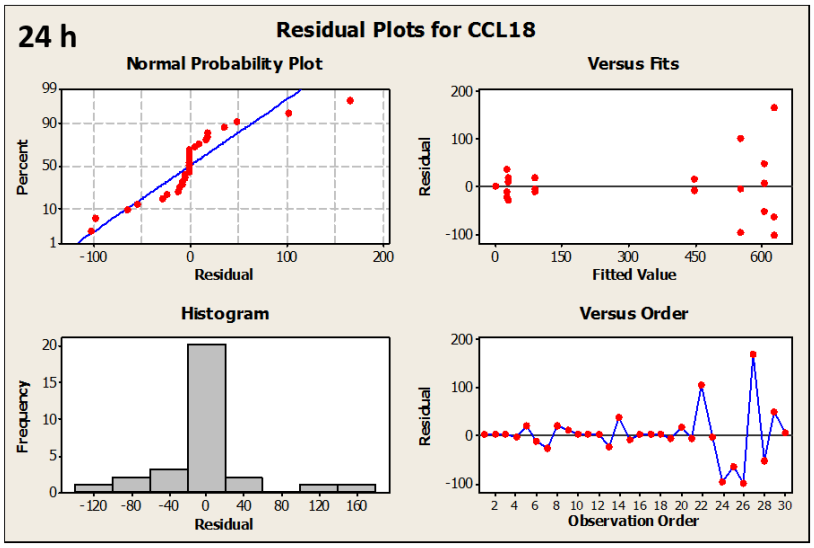
```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF       SS        MS     F      P
Macrophage     4  3478,18   869,545  6,04  0,002
Donor          1     0,01     0,011  0,00  0,993
Interaction    4   140,71    35,179  0,24  0,910
Error         20  2877,28   143,864
Total         29  6496,19
S = 11,99   R-Sq = 55,71%   R-Sq(adj) = 35,78%
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  ----+---------+---------+---------+-----
M0            3,8947  (------*-----)
M1            4,3033  (------*------)
M2 (20)      14,9443          (------*------)
M2 (20)*     32,7083                      (------*------)
M2 (40)      20,3527             (------*-----)
                     ----+---------+---------+---------+-----
                         0         15        30        45


Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  -----+---------+---------+---------+----
1       15,2595  (-------------------*-------------------)
2       15,2219  (-----------------*------------------)
                 -----+---------+---------+---------+----
                    10,5      14,0      17,5      21,0
```

A.5.2.4 Cells activated with different concentrations of IL-4 from Sigma-Aldrich



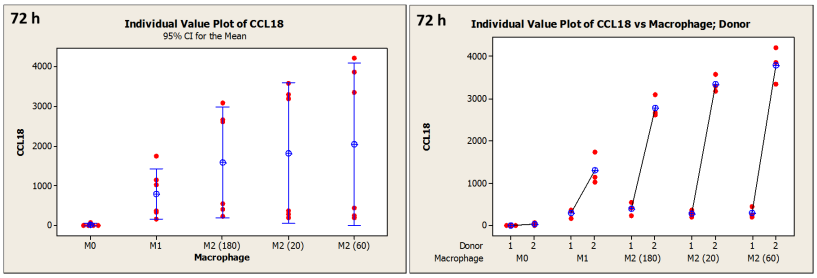
(a) (b)



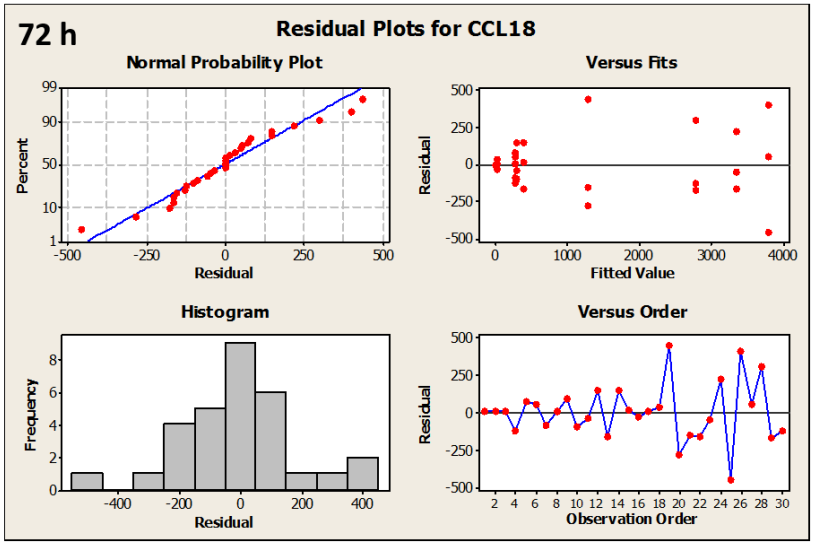
(c)

Figure A.15: ANOVA analysis of CCL18 secretion after 6 hours.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF       SS        MS        F      P
Macrophage     4   433471    108368    30,08  0,000
Donor          1  1315313   1315313   365,08  0,000
Interaction    4   391907     97977    27,19  0,000
Error         20    72056      3603
Total         29  2212747
S = 60,02   R-Sq = 96,74%   R-Sq(adj) = 95,28%

Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  ----+---------+---------+---------+-----
M0             0,000  (---*---)
M1           268,585                      (---*----)
M2 (180)     314,866                         (---*---)
M2 (20)      291,730                       (---*----)
M2 (60)      314,619                         (---*---)
                     ----+---------+---------+---------+-----
                         0        120       240       360

Individual 95% CIs For Mean Based on
Pooled StDev
Donor       Mean  +---------+---------+---------+---------
1         28,571  (-*-)
2        447,349                           (-*-)
                 +---------+---------+---------+---------
                 0        150       300       450
```



(a) (b)

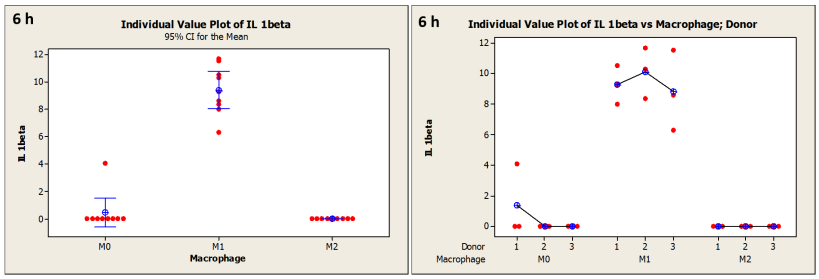


(c)

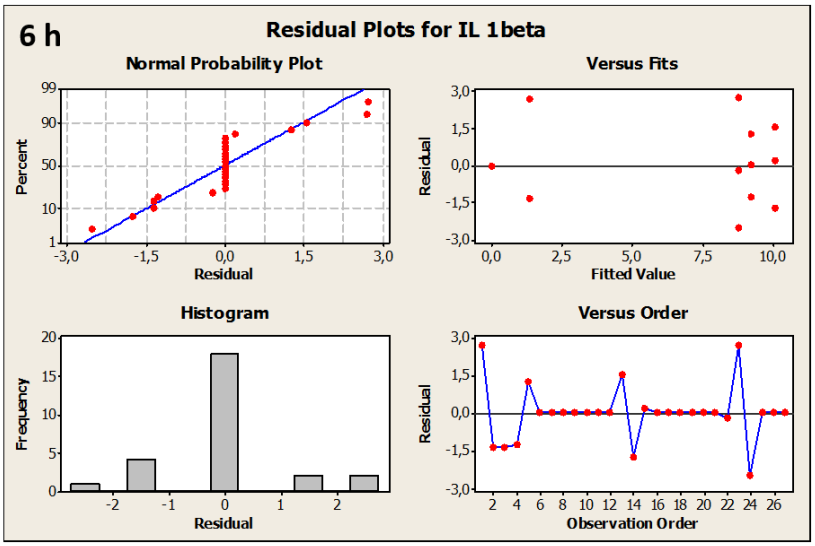
Figure A.16: ANOVA analysis of CCL18 secretion after 24 hours.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF         SS         MS        F       P
Macrophage     4   16743051    4185763    83,00   0,000
Donor          1   30083174   30083174   596,50   0,000
Interaction    4   12609852    3152463    62,51   0,000
Error         20    1008659      50433
Total         29   60444735
S = 224,6   R-Sq = 98,33%   R-Sq(adj) = 97,58%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  --+---------+---------+---------+-------
M0             16,98  (-*--)
M1            793,75            (-*--)
M2 (180)     1589,69                      (--*-)
M2 (20)      1812,84                         (--*--)
M2 (60)      2043,62                            (--*--)
                    --+---------+---------+---------+-------
                      0        700       1400       2100

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --------+---------+---------+---------+-
1        249,99  (-*-)
2       2252,76                                  (-*-)
                --------+---------+---------+---------+-
                        600       1200      1800      2400
```

A.5.3 ANOVA analysis of IL-1β ELISA results presented in Figure 4.3 (a)



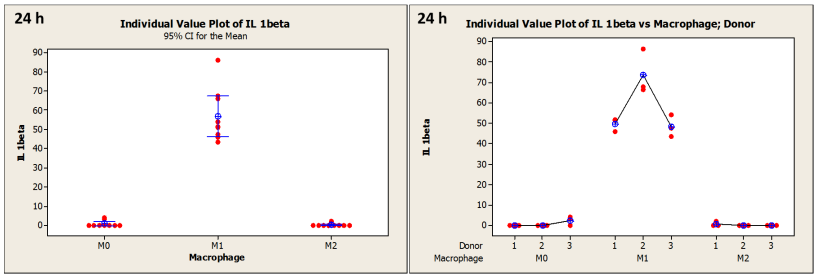
(a) (b)



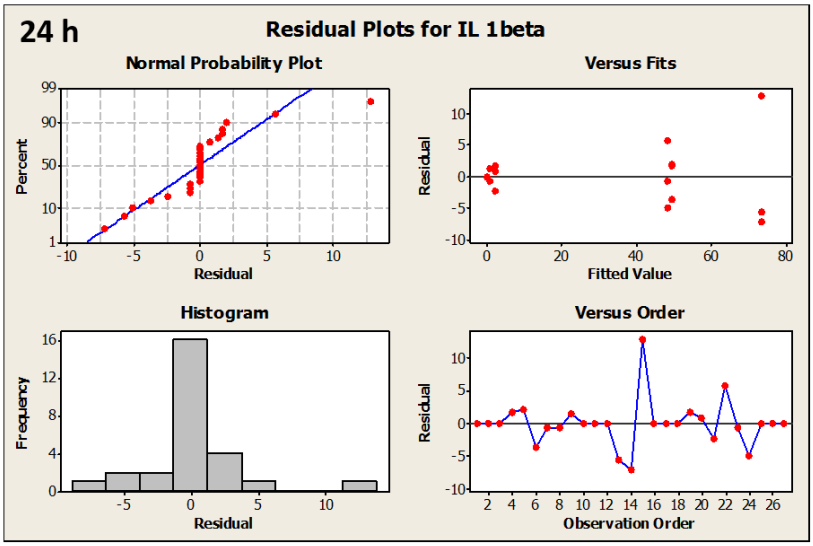
(c)

Figure A.17: ANOVA analysis of IL-1β secretion after 6 hours.

```
Two-way ANOVA: IL 1beta versus Macrophage; Donor

Source        DF       SS        MS        F       P
Macrophage     2  503,728   251,864   135,16   0,000
Donor          2    1,751     0,876     0,47   0,633
Interaction    4    4,509     1,127     0,60   0,664
Error         18   33,543     1,863
Total         26  543,531
S = 1,365   R-Sq = 93,83%   R-Sq(adj) = 91,09%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  ---+---------+---------+---------+------
M0           0,45133   (---*--)
M1           9,38000                                   (--*--)
M2           0,00000  (--*--)
                     ---+---------+---------+---------+------
                        0,0       3,0       6,0       9,0

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --+---------+---------+---------+-------
1       3,53667            (-------------*-------------)
2       3,36356         (-------------*-------------)
3       2,93111  (-------------*-------------)
                 --+---------+---------+---------+-------
                2,10      2,80      3,50      4,20
```



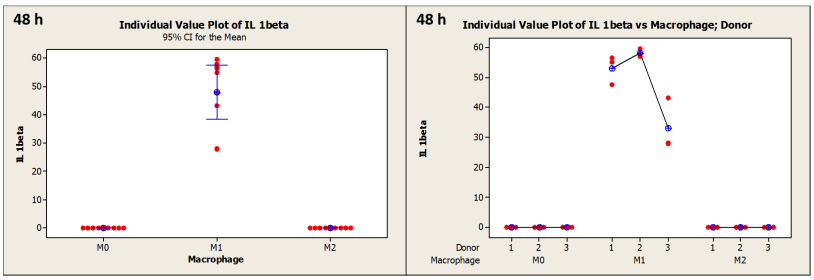
(a) (b)



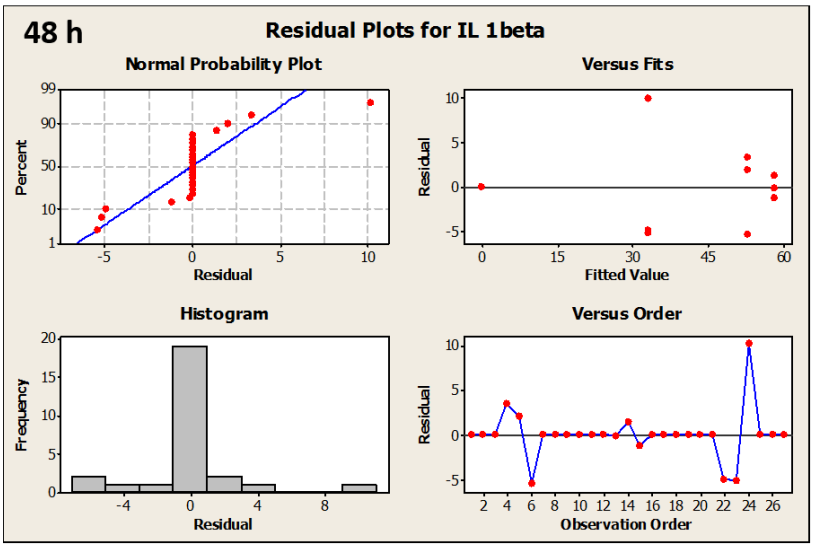
(c)

Figure A.18: ANOVA analysis of IL-1β secretion after 24 hours.

```
Two-way ANOVA: IL 1beta versus Macrophage; Donor

Source        DF        SS        MS        F       P
Macrophage     2  19221,2   9610,62   509,01   0,000
Donor          2     352,0    176,02     9,32   0,002
Interaction    4     864,2    216,04    11,44   0,000
Error         18     339,9     18,88
Total         26  20777,3
S = 4,345   R-Sq = 98,36%   R-Sq(adj) = 97,64%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  --+---------+---------+---------+-------
M0            0,7973   (*-)
M1           57,1089                                     (-*-)
M2            0,2251  (-*-)
                      --+---------+---------+---------+-------
                        0         16        32        48

Individual 95% CIs For Mean Based on Pooled StDev
Donor      Mean     -+---------+---------+---------+--------
1       16,7487     (--------*--------)
2       24,4829                        (--------*--------)
3       16,8998      (--------*--------)
                    -+---------+---------+---------+--------
                     14,0      17,5      21,0      24,5
```



(a) (b)



(c)

Figure A.19: ANOVA analysis of IL-1β secretion after 48 hours.

```
Two-way ANOVA: IL 1beta versus Macrophage; Donor

Source        DF        SS        MS        F       P
Macrophage     2  13797,0   6898,50   613,84   0,000
Donor          2     351,7    175,84    15,65   0,000
Interaction    4     703,4    175,84    15,65   0,000
Error         18     202,3     11,24
Total         26  15054,3
S = 3,352   R-Sq = 98,66%   R-Sq(adj) = 98,06%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  --+---------+---------+---------+-------
M0           0,0000  (-*-)
M1          47,9531                                   (-*-)
M2           0,0000  (-*-)
                     --+---------+---------+---------+-------
                       0         15        30        45

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  -----+---------+---------+---------+----
1       17,6329                    (-----*------)
2       19,3433                         (-----*------)
3       10,9769  (-----*------)
                 -----+---------+---------+---------+----
                     10,5      14,0      17,5      21,0
```

A.5.4 ANOVA analysis of IL-10 ELISA results presented in Figure 4.3 (b)

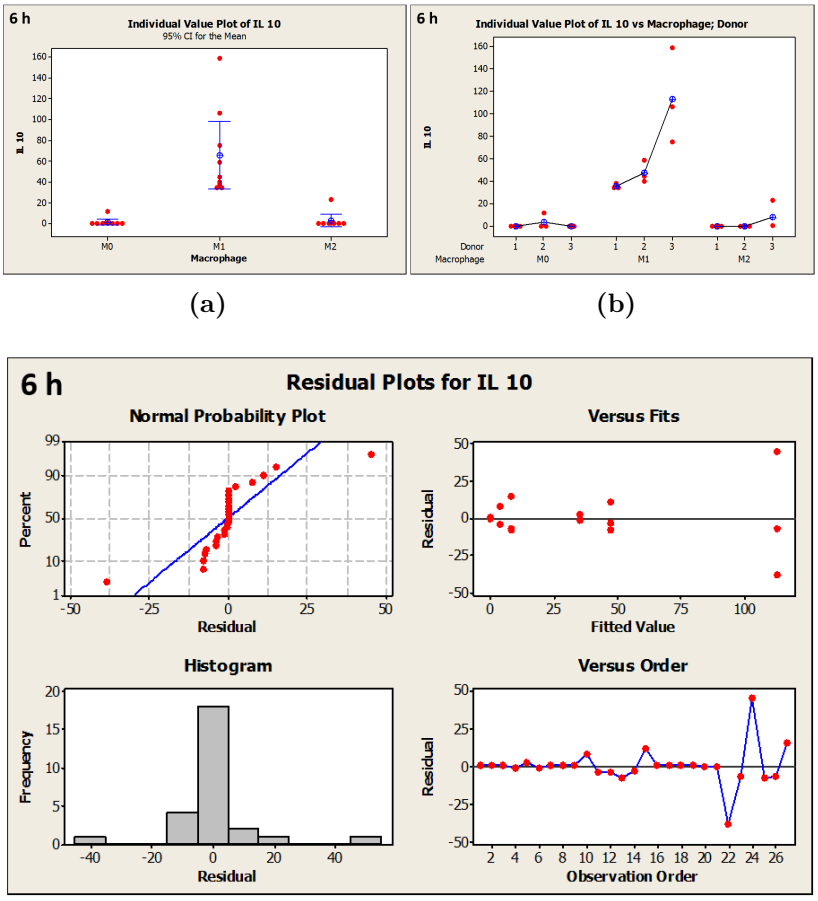
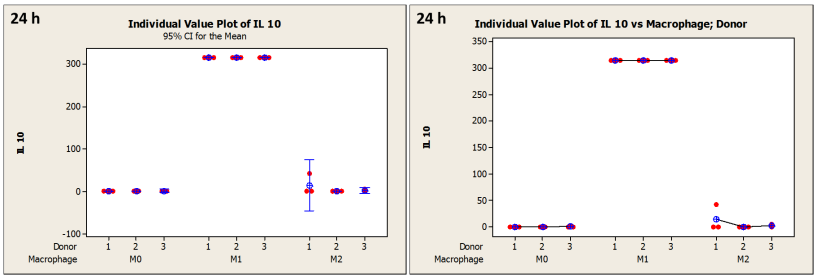
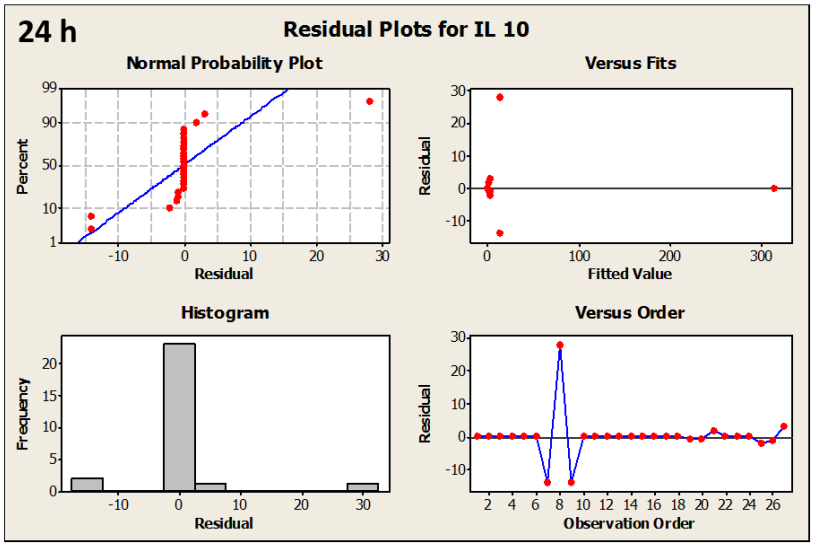


Figure A.20: ANOVA analysis of IL-10 secretion after 6 hours.

```
Two-way ANOVA: IL 10 versus Macrophage; Donor

Source        DF       SS        MS       F       P
Macrophage     2  24072,6  12036,3  51,51  0,000
Donor          2   4166,3   2083,1   8,91  0,002
Interaction    4   6464,8   1616,2   6,92  0,001
Error         18   4206,4    233,7
Total         26  38910,0
S = 15,29   R-Sq = 89,19%   R-Sq(adj) = 84,38%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  ----+---------+---------+---------+-----
M0           1,3033  (----*---)
M1          65,3238                           (---*---)
M2           2,6847    (---*---)
                     ----+---------+---------+---------+-----
                         0         25        50        75


Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ---------+---------+---------+---------+
1       11,7876  (------*------)
2       17,1253     (------*-------)
3       40,3989                    (------*------)
               ---------+---------+---------+---------+
                        15        30        45        60
```



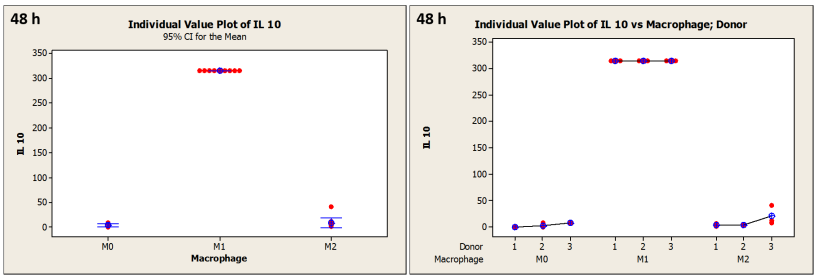
(a) (b)



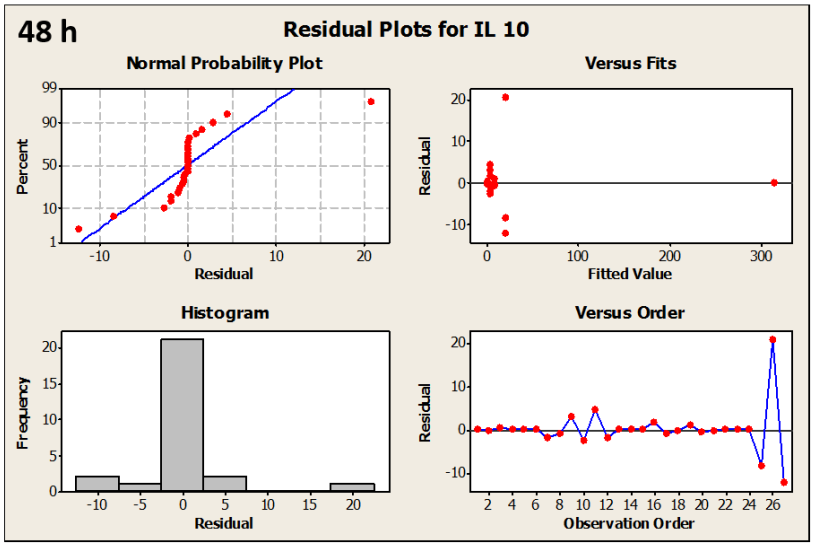
(c)

Figure A.21: ANOVA analysis of IL-10 secretion after 24 hours.

```
Two-way ANOVA: IL 10 versus Macrophage; Donor

Source        DF       SS       MS         F       P
Macrophage     2   583886   291943   4377,07   0,000
Donor          2      109       54      0,82   0,458
Interaction    4      237       59      0,89   0,491
Error         18     1201       67
Total         26   585432
S = 8,167   R-Sq = 99,79%   R-Sq(adj) = 99,70%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  -+---------+---------+---------+--------
M0             0,322  (*)
M1           314,766                                    *)
M2             5,366   (*
                       -+---------+---------+---------+--------
                        0        100       200       300

Individual 95% CIs For Mean Based on Pooled StDev
Donor      Mean     --+---------+---------+---------+-------
1       109,598               (----------*----------)
2       104,926     (----------*----------)
3       105,930       (----------*----------)
                    --+---------+---------+---------+-------
                    100,0     105,0     110,0     115,0
```



(a) (b)



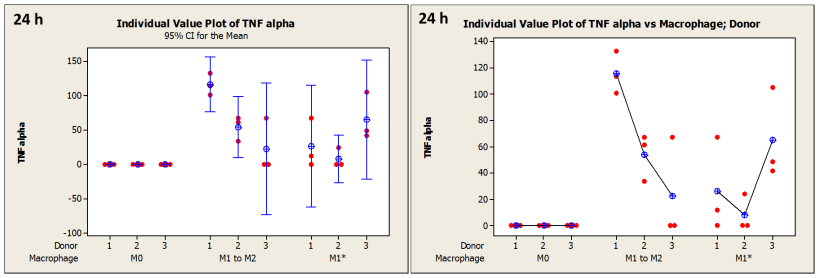
(c)

Figure A.22: ANOVA analysis of IL-10 secretion after 48 hours.

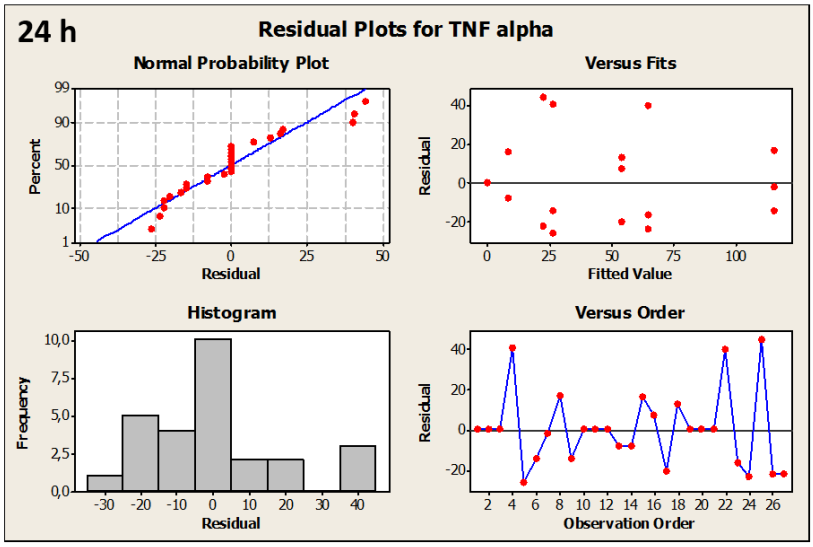
```
Two-way ANOVA: IL 10 versus Macrophage; Donor

Source       DF       SS       MS         F       P
Macrophage    2  571106  285553  7295,22  0,000
Donor         2      384     192     4,91  0,020
Interaction   4      313      78     2,00  0,138
Error        18      705      39
Total        26  572508
S = 6,256   R-Sq = 99,88%   R-Sq(adj) = 99,82%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  +---------+---------+---------+---------
M0            3,630  *)
M1          314,766                                         *)
M2            8,931   *)
                     +---------+---------+---------+---------
                     0         80        160       240


Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  -------+---------+---------+---------+--
1       106,070  (--------*--------)
2       106,831    (--------*-------)
3       114,426                    (--------*--------)
                -------+---------+---------+---------+--
                      105,0     110,0     115,0     120,0
```

A.5.5 ANOVA analysis of TNF-α and CCL18 ELISA results after reversible polarization presented in Figure 4.6

A.5.5.1 ANOVA analysis of TNF-α after 24 and 72 hours



(a) (b)

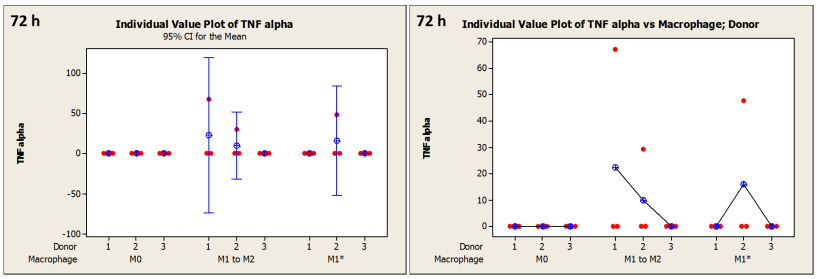


(c)

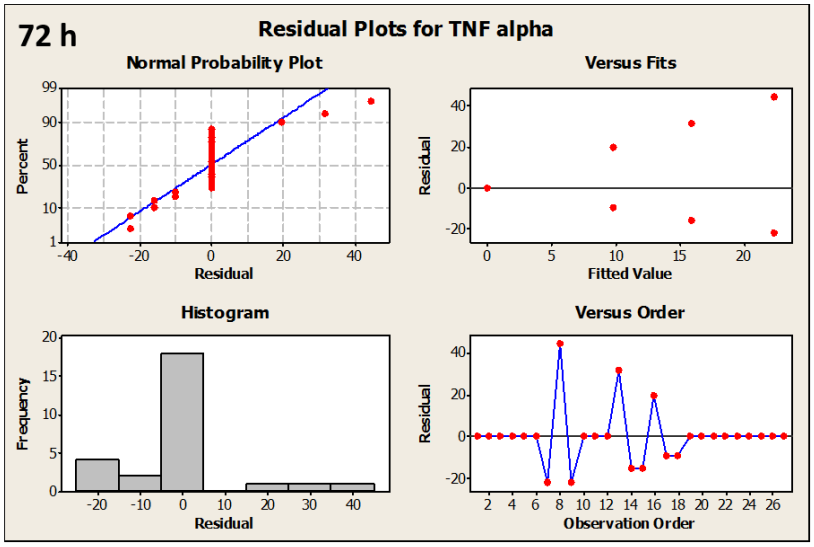
Figure A.23: ANOVA analysis of TNF-α secretion after 24 hours reversible polarization.

```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF       SS        MS       F       P
Macrophage     2  18377,0   9188,52   17,41   0,000
Donor          2   3321,0   1660,48    3,15   0,067
Interaction    4  15266,4   3816,60    7,23   0,001
Error         18   9498,5    527,69
Total         26  46462,9
S = 22,97   R-Sq = 79,56%   R-Sq(adj) = 70,47%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  ------+---------+---------+---------+---
M0           0,0000  (-----*-----)
M1 to M2     63,8911                          (------*-----)
M1*          33,0784               (-----*------)
                     ------+---------+---------+---------+---
                           0         25        50        75
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --------+---------+---------+---------+--
1       47,2247                    (---------*----------)
2       20,6349  (----------*---------)
3       29,1100         (---------*----------)
               --------+---------+---------+---------+--
                       15        30        45        60
```



(a) (b)



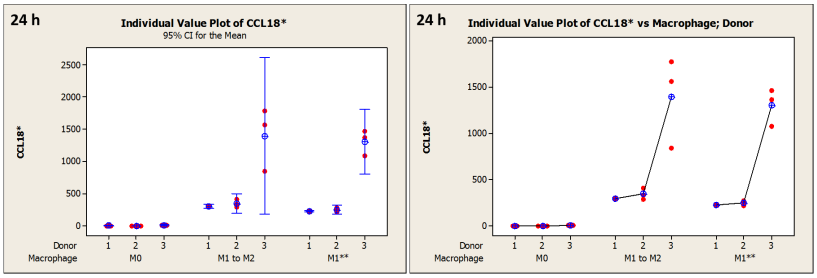
(c)

Figure A.24: ANOVA analysis of TNF-α secretion after 72 hours reversible polarization.

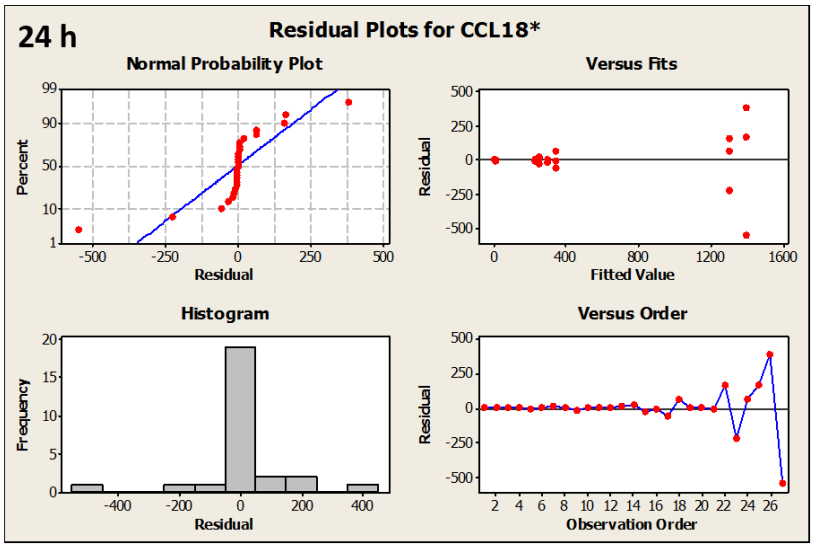
```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF       SS        MS      F       P
Macrophage     2   515,44   257,720   0,91   0,419
Donor          2   389,09   194,543   0,69   0,515
Interaction    4   867,65   216,913   0,77   0,559
Error         18  5079,03   282,169
Total         26  6851,21
S = 16,80   R-Sq = 25,87%   R-Sq(adj) = 0,00%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  --+---------+---------+---------+-------
M0            0,0000  (-----------*-----------)
M1 to M2     10,7022             (-----------*-----------)
M1*           5,2909         (-----------*-----------)
                     --+---------+---------+---------+-------
                       -10         0         10        20
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --+---------+---------+---------+-------
1       7,44800          (----------*----------)
2       8,54511           (----------*----------)
3       0,00000  (----------*----------)
                 --+---------+---------+---------+-------
                  -10         0        10        20
```

A.5.5.2 ANOVA analysis of CCL18 after 24 and 72 hours



(a) (b)

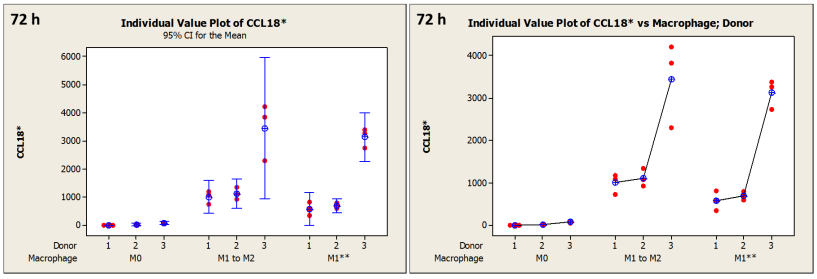


(c)

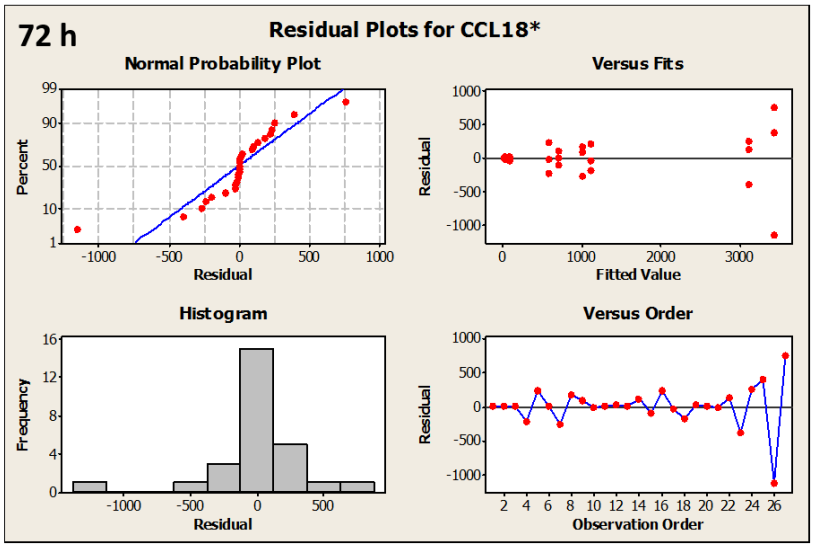
Figure A.25: ANOVA analysis of CCL18 secretion after 24 hours reversible polarization.

```
Two-way ANOVA: CCL18* versus Macrophage; Donor

Source        DF       SS        MS       F       P
Macrophage     2  2446146   1223073   38,87   0,000
Donor          2  3079495   1539748   48,94   0,000
Interaction    4  1507594    376899   11,98   0,000
Error         18   566357     31464
Total         26  7599592
S = 177,4   R-Sq = 92,55%   R-Sq(adj) = 89,24%
```

```
Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  -----+---------+---------+---------+----
M0             2,648  (----*----)
M1 to M2     680,126                            (----*----)
M1*          593,298                          (----*----)
                      -----+---------+---------+---------+----
                           0        250       500       750

Individual 95% CIs For Mean Based on
Pooled StDev
Donor       Mean  --------+---------+---------+---------+-
1        174,995  (----*----)
2        198,300    (----*----)
3        902,777                            (----*----)
                  --------+---------+---------+---------+-
                         250       500       750      1000
```



(a) (b)



(c)

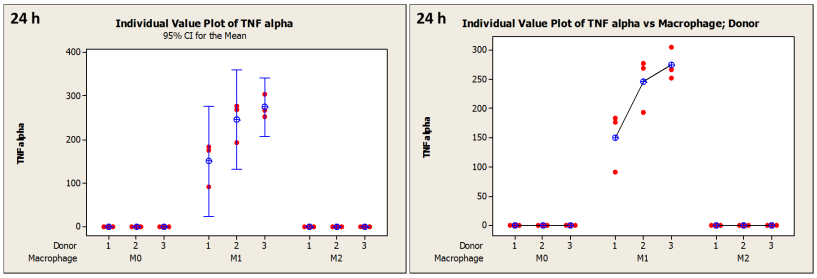
Figure A.26: ANOVA analysis of CCL18 secretion after 72 hours reversible polarization.

```
Two-way ANOVA: CCL18* versus Macrophage; Donor
Source       DF        SS        MS      F      P
Macrophage    2  16530961   8265481  57,23  0,000
Donor         2  16321510   8160755  56,51  0,000
Interaction   4   7513822   1878455  13,01  0,000
Error        18   2599611    144423
Total        26  42965904
S = 380,0   R-Sq = 93,95%   R-Sq(adj) = 91,26%
```

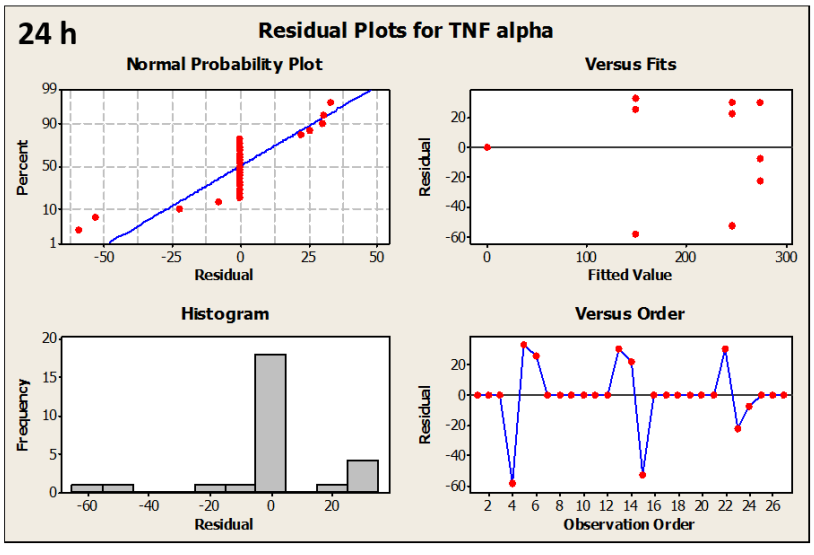
```
Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  ----+---------+---------+---------+-----
M0             31,73  (----*---)
M1 to M2     1850,85                                (----*---)
M1*          1464,03                          (---*----)
                       ----+---------+---------+---------+-----
                           0        600       1200      1800

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ------+---------+---------+---------+---
1        525,20  (----*---)
2        607,34    (---*----)
3       2214,06                              (----*---)
               ------+---------+---------+---------+---
                     600       1200      1800      2400
```

A.5.6 ANOVA analysis of TNF-α and CCL18 ELISA results after culturing in a 24-well plate presented in Figure A.1

A.5.6.1 ANOVA analysis of TNF-α after 24 and 72 hours



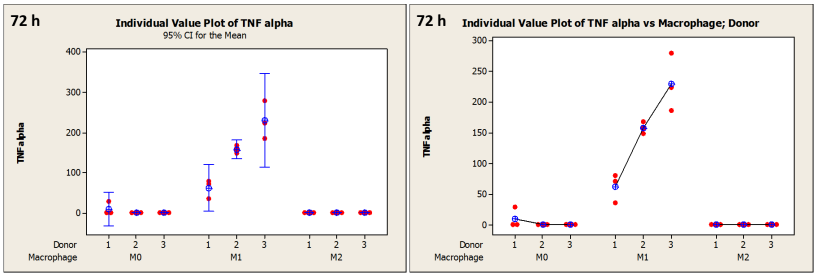
(a) (b)



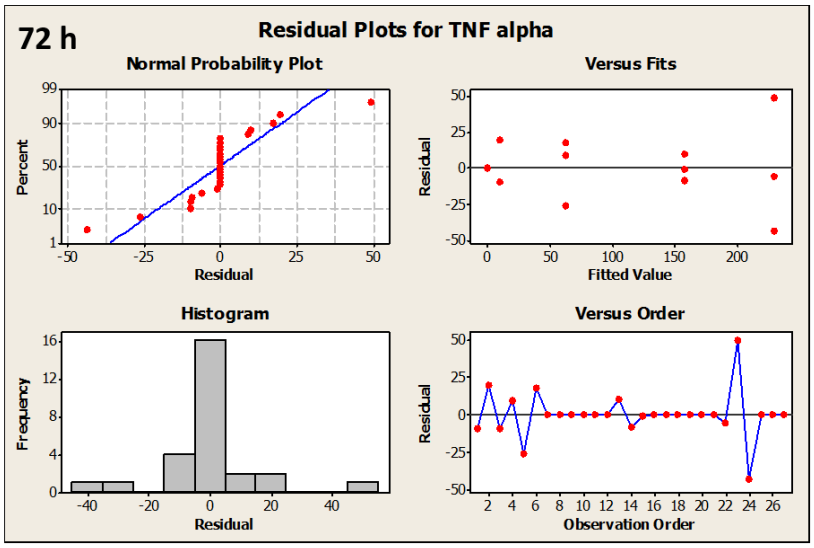
(c)

Figure A.27: ANOVA analysis of TNF-α secretion after 24 hours polarization in a 24-well plate.

```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF       SS       MS        F       P
Macrophage     2   300103   150051   246,80   0,000
Donor          2     8512     4256     7,00   0,006
Interaction    4    17024     4256     7,00   0,001
Error         18    10944      608
Total         26   336583
S = 24,66   R-Sq = 96,75%   R-Sq(adj) = 95,30%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  --+---------+---------+---------+-------
M0             0,000  (-*-)
M1           223,645                                  (--*-)
M2             0,000  (-*-)
                    --+---------+---------+---------+-------
                      0         70        140       210

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ----+---------+---------+---------+-----
1       50,0289  (--------*---------)
2       82,1182                (--------*---------)
3       91,4980                         (--------*-------)
               ----+---------+---------+---------+-----
                   40        60        80        100
```



(a) (b)

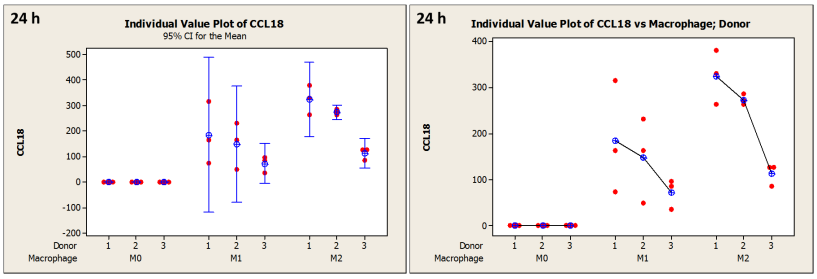


(c)

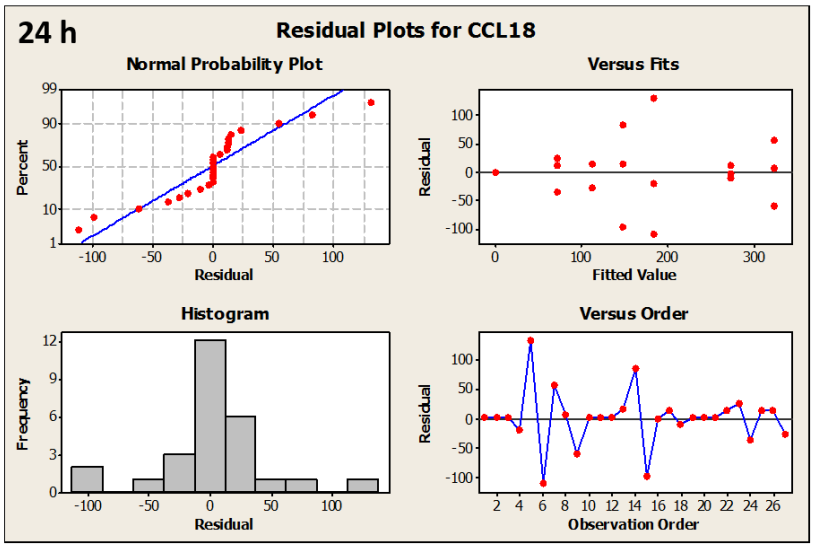
Figure A.28: ANOVA analysis of TNF-α secretion after 72 hours polarization in a 24-well plate.

```
Two-way ANOVA: TNF alpha versus Macrophage; Donor

Source        DF       SS        MS        F       P
Macrophage     2   132002   66000,9   190,97   0,000
Donor          2    12423    6211,6    17,97   0,000
Interaction    4    29976    7494,1    21,68   0,000
Error         18     6221     345,6
Total         26   180622
S = 18,59   R-Sq = 96,56%   R-Sq(adj) = 95,03%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  ---+---------+---------+---------+------
M0             3,254   (--*-)
M1           149,925                                  (--*--)
M2             0,000  (--*--)
                      ---+---------+---------+---------+------
                         0         50        100       150

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ----+---------+---------+---------+-----
1       24,0371  (-----*------)
2       52,6336            (-----*------)
3       76,5089                      (-----*------)
               ----+---------+---------+---------+-----
                   20        40        60        80
```

A.5.6.2 ANOVA analysis of CCL18 after 24 and 72 hours



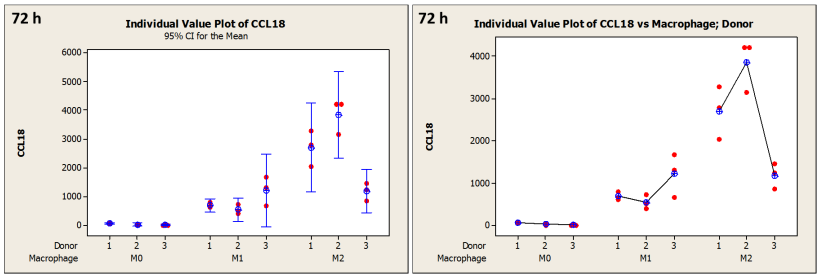
(a) (b)



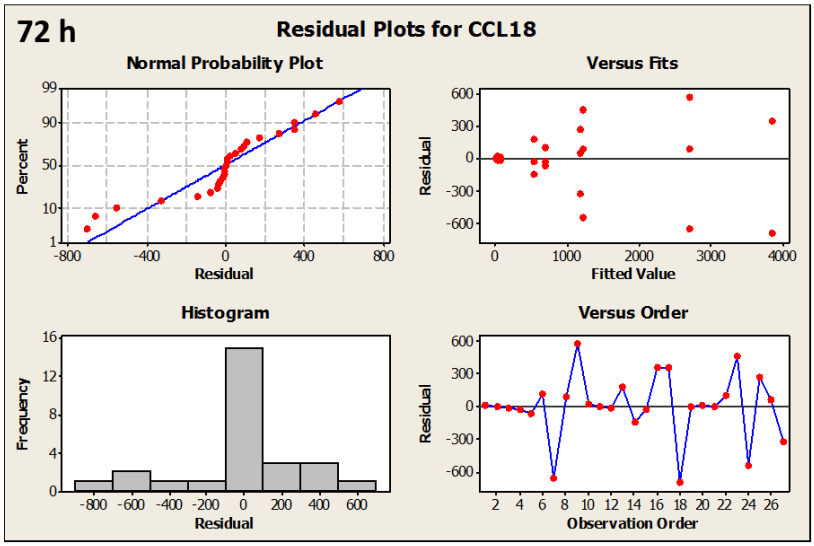
(c)

Figure A.29: ANOVA analysis of CCL18 secretion after 24 hours polarization in a 24-well plate.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source        DF       SS       MS       F       P
Macrophage     2   253069   126534   39,89   0,000
Donor          2    56165    28083    8,85   0,002
Interaction    4    36785     9196    2,90   0,052
Error         18    57096     3172
Total         26   403116
S = 56,32   R-Sq = 85,84%   R-Sq(adj) = 79,54%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage      Mean  -----+---------+---------+---------+----
M0              0,000  (----*----)
M1            134,626                (----*----)
M2            236,384                          (----*---)
                     -----+---------+---------+---------+----
                          0         80        160       240

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  ------+---------+---------+---------+---
1       169,393                          (-------*-------)
2       140,209                 (-------*-------)
3        61,408  (-------*-------)
                 ------+---------+---------+---------+---
                       50        100       150       200
```



(a) (b)



(c)

Figure A.30: ANOVA analysis of CCL18 secretion after 24 hours polarization in a 24-well plate.

```
Two-way ANOVA: CCL18 versus Macrophage; Donor

Source       DF         SS         MS       F      P
Macrophage    2  30578846  15289423  120,03  0,000
Donor         2   2024816   1012408    7,95  0,003
Interaction   4   9517987   2379497   18,68  0,000
Error        18   2292797    127378
Total        26  44414447
S = 356,9   R-Sq = 94,84%   R-Sq(adj) = 92,54%


Individual 95% CIs For Mean Based on
Pooled StDev
Macrophage     Mean  ---+---------+---------+---------+------
M0            30,48  (--*---)
M1           816,09            (--*--)
M2          2575,86                                  (--*--)
                     ---+---------+---------+---------+------
                        0       800      1600      2400

Individual 95% CIs For Mean Based on
Pooled StDev
Donor      Mean  --+---------+---------+---------+-------
1       1150,30              (-------*--------)
2       1471,37                        (-------*-------)
3        800,78  (--------*-------)
                 --+---------+---------+---------+-------
                  600       900      1200      1500
```