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## Different pathways of macrophage activation and polarization

### Ścieżki aktywacji i polaryzacji makrofagów

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#### Summary

Monocytes are short-lived cells and undergo spontaneous apoptosis every day. Inflammatory responses may induce dramatic up-regulation of monocyte survival and differentiation. When monocytes are recruited to an area of infection they may differentiate into macrophages. In different microenvironments macrophages polarize into two types. The M1 or classically activated macrophages are characterized by the high ability to produce pro-inflammatory cytokines and the production of NO through the induced synthesis of iNOS. The M2 or alternatively activated macrophages are divided into 3 subtypes, M2 a, b and c, and they have anti-inflammatory properties. Mediators of M1 macrophage TLR-dependent polarization include transcription factors such as NF-κB, AP-1, PU.1, CCAAT/enhancer-binding protein α (C/EBP-α), STAT1 as well as interferon regulatory factor 5 (IRF5), while the transcription factors which promote M2 activation include IRF4, C/EBP-β, Krüppel-like factor 4 (KLF4), STAT6 and PPARγ receptor.

**Key words:** M1/M2 macrophage polarization • circulating monocytes • macrophage surface receptors • macrophage transcription factors

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**Abbreviations:** **AP-1** - activator protein-1, **C/EBP-α** - CCAAT/enhancer-binding protein α, **CLR** - C-type lectin receptor, **c-Maf** - essential transcription factor for IL-10 gene expression in macrophages, **CMFs** - common myeloid progenitors, **CREB** - cAMP-responsive element-binding protein, **DC** - dendritic cell, **GM-CSF** - granulocyte-macrophage colony stimulating factor, **INF-γ** - interferon γ, **iNOS** - induced nitric oxide synthase, **IRF** - interferon regulatory factors, **KLFs** - Krüppel-like factors, **LPS** - lipopolysaccharide, **MAPKs** - mitogen-activated protein kinases, **MARCO** - macrophage receptor with collagenous structure, **MCPs** - monocyte chemoattractant proteins, **M-CSF** - macrophage colony stimulating factor, **MHC** - major histocompatibility complex, **MPS** - mononuclear phagocyte system, **MyD88** - myeloid differentiation primary response protein-88, **NF-κB** - nuclear factor-κB, **NLR** - NOD-like receptor, **NO** - nitric oxide, **oxLDL** - oxidized low-density lipoprotein, **PPARγ** - peroxisome proliferator-activated receptor γ, **PRRs** - pattern-recognition receptor, **pSTAT1** - phosphorylated form of signal transducer and activator of transcription 1, **PU.1** - transcription factor PU.1, **RBP-J** - recombination signal binding protein for immunoglobulin kappa J region, **RIG1** - retinoic acid-inducible gene 1, **RLRs** - retinoic acid-inducible gene 1 (RIG1)-like helicase receptors, **ROS** - reactive oxygen species, **TGF-β** - transforming growth factor β, **TLR** - Toll-like receptor, **TNF-α** - tumour necrosis factor α, **Treg** - regulatory T lymphocytes.

## INTRODUCTION

The medullar mononuclear phagocyte system (MPS) consists of cells which have pleiotropic biological properties responsible mainly for phagocytosis, production of cytokines as well as antigen presentation, characterized by distinct morphology and phagocytic activity [31,40]. The MPS is composed of myeloid medullar precursors, monocytes circulating in blood, tissue macrophages and dendritic cells of monocytic origin [1,31,40]. This system shows particular activity in the inflammation or infection course, during which peripheral blood monocytes move to the tissues where they differentiate into mature macrophages or DCs. Monocytes arise from common bone marrow myeloid progenitor cells (CMPs) [1,31,40,68]. They can transform into two separate cell lineages, i.e. dendritic cells and macrophages. The differentiation process of monocytes is driven by cytokines such as IL-3 (interleukin 3), GM-CSF (granulocyte-macrophage colony stimulating factor) and M-CSF (macrophage colony stimulating factor). The GM-CSF and IL-4 induce the differentiation of monocytes into dendritic cells and under the influence of M-CSF and GM-CSF monocytes become macrophages. Macrophage's activity depends on its localization as well as signals from the surrounding microenvironment [31,40].

## MONOCYTE-DERIVED MACROPHAGES

Macrophages are derived from two various subsets of circulating inflammatory or resident monocytes. In response to infection or inflammation monocytes migrate to the tissues where they form a resident macrophage pool [52]. For many years blood monocyte-derived macrophages served as a valid experimental model of human *in vivo* macrophages. However, comparatively great amounts of blood are usually required from each donor to conduct the experiments [66].

Recent studies have shown differences in gene expression between monocytes and macrophages and their periods of existence. The life span of macrophages is longer than that of circulating monocytes and ranges from months to years [66]. Monocytes are short-lived cells and undergo spontaneous apoptosis every day. In normal conditions, shortly before this process, they circulate in the bloodstream [17].

Inflammatory responses may induce dramatic up-regulation of monocyte survival and differentiation. Therefore, the processes involved in regulating removal and survival of monocytes are significant for population control. The lack of an adequate stimulus causes monocytes to spontaneously undergo apoptosis. When inflammation is absent, the development of monocyte precursors from the marrow is greater than needed to replace normal tissue macrophage numbers [17]. As a result of a complex network of differentiation factors, monocytes flee their apoptotic pathway and differentiate into macrophages with a longer life span, which can be found in nearly every organ [48].

Fully diversified residual macrophages are highly specialized cells and show specificity for many tissues and organs, in which they regulate organ homeostasis and remodelling. They include macrophages located in bones (osteoclasts), interstitial connective tissue (histiocytes), adipose tissue macrophages [19], brain (brain microglial cells in the central nervous system), liver (liver Kupffer cells), lungs (alveolar macrophages), peritoneum (peritoneal macrophages), as well as in heart, skin, gastrointestinal tract, skeletal muscles and kidneys [1,53].

## MACROPHAGE SURFACE RECEPTORS

Macrophages, classified as professional phagocytes, show the expression of many cell surface receptors recognizing signals which do not occur in healthy tissues. Via pattern-recognition receptors (PRRs, including TLRs, CLRs, scavenger receptors, retinoic acid-inducible gene 1 (RIG1)-like helicase receptors (RLRs) and NLRs), they receive alarm signals sent by foreign substances, dead or dying cells and signals connected with invading pathogens [38,40]. Inflammatory cytokine receptors such as TNF- $\alpha$  also intercede in acute macrophages' activation.

Even though proximal signalling by these receptors may differ from each other significantly, they all trigger a core inflammatory signalling program essential for acute activation. The activation of MAPKs (mitogen-activated protein kinases), NF- $\kappa$ B (nuclear factor- $\kappa$ B) and IRFs (interferon regulatory factors), resulting in transient expression of pivotal chemokines, cytokines and inflammatory mediators, is a part of this core response [26]. Several PRRs such as mannose receptor (CD206) or macrophage receptor with collagenous structure (MARCO) play an important role in the phagocytosis and pathogen binding process while such PRRs as TLRs, RLRs or NLRs recognize microbial products and are expressed on the cell surface or in the cytoplasm of cells. This process initiates the activation of transcriptional mechanisms, resulting in phagocytosis and cellular activation. It also leads to release of cytokines, chemokines and growth factors. As a result, the PRRs, which are scavenger receptors, simplify opsonisation of pathogens and removal of apoptotic cells, aged red blood cells and necrotic tissues, as well as toxic molecules from the circulation [21,38].

## MACROPHAGE POLARIZATION

Stimulation-dependent polarization determines definite functions and phenotypes of macrophages. Based on their polarization state they may be divided into classically activated (M1) and alternatively activated (M2 and subtypes: M2 a, b, c) [6,29,31,40]. Due to the ability of linkage of their functions they are well adapted to the changeable environment [40,65]. Macrophages are cells that consist of heterogeneous cell populations with regard to functional as well as phenotypic properties [67]. They have the ability to modify their functional profiles under the influence of activating signals, induced by pathological processes occurring in the organism [31,40]. The diverse

status of macrophages is strongly connected with cytokines produced by the Th cells [6].

### **PRO-INFLAMMATORY M1 MACROPHAGES**

When bone marrow macrophages are grown with GM-CSF, they differentiate into M1 type, classical pro-inflammatory macrophages which secrete IL-12 like cytokines. However, when they are cultured with M-CSF, they differentiate into the M2 type, which secretes anti-inflammatory cytokines such as IL-10 [62].

Macrophages of the M1 phenotype (pro-inflammatory) arise as a result of classical activation by the stimulation of TLR receptors through appropriate ligands such as bacterial LPS (lipopolysaccharide) and some cytokines (mainly INF- $\gamma$ ) [5,40]. They are closely related to the Th1 and Th17 polarization of the immune response [31,40]. They are characterized by the high ability to produce pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, IL-23, chemokines (inter alia CCL5, CCL8, CXCL2 and CXCL4) [40] and the production of NO through the expression of induced synthesis of iNOS. Moreover, they show increased ability to present antigens [15], eliminate infections caused by bacterial, virus or fungal factors [5] as well as kill tumour cells [40].

### **ANTI-INFLAMMATORY M2 MACROPHAGES**

Macrophages of the M2 phenotype show high expression of markers of alternative activation (inter alia arginase-1, chitinase-like Ym1, mannose receptor, receptor CD14, CCL17, CCL22, CCL24 [36,62]). They also play an important role in regulation of the immune response to parasites [5] and in the allergic reactions [15]. Additionally, they take part in tissue remodelling and angiogenesis and may have an influence on tumour progression [62]; they also have anti-inflammatory properties and the ability to induce the Th2 immune response. Furthermore, they are able to induce regulatory T lymphocyte (Treg) differentiation [40]. This process of activation in the direction of M2 involves cytokines such as IL-4, IL-10 and/or IL-13 [67], as well as IL-33, a member of the IL-1 and IL-21 family [36]. M2 macrophages produce IL-10, TGF- $\beta$  and IL-1 receptor antagonist (IL-1RA) and also block iNOS. Unlike M1 macrophages, they do not display any cytotoxic properties [40]. Three subpopulations of cells may be distinguished among M2 macrophages. IL-4 and/or IL-13 induce the development of wound healing M2a macrophages. Immune stimulating complexes of ligands for TLR receptors lead to the development of M2b. However, when exposed to an anti-inflammatory stimulus such as glucocorticoids, IL-10 or TGF- $\beta$ , they differentiate into the M2c subpopulation [10,49].

### **M1/M2 MACROPHAGE SURFACE RECEPTORS**

M1 macrophages are characterized by high expression of MHC II molecules and co-stimulatory CD80/86 molecules, which points to their ability to present antigens [40]. By

contrast, M2 macrophages show the expression of phagocytosis marker CD206 and/or the CD163 (haemoglobin scavenger receptor), whose expression is dependent on IL-10 [65].

The well-characterized F4/80 marker can be used to distinguish monocytes from macrophages. It is expressed at lower levels on monocytes, eosinophils and dendritic cells [64].

Having been polarized, macrophages show increased levels of CD80 and CD64. The study of Ambarus et al. [2] showed that CD80 is the most suitable marker that can be used for identification of INF- $\gamma$  polarized macrophages. When the macrophages are treated with IL-10 it causes induction of CD16, CD32 and CD163. What is more, the IL-4 polarized macrophages downregulate CD14 levels, and upregulate CD200R as well as CD206 [2].

Many types of tissue macrophages are characterized by the expression of CD68 surface marker (an endosomal glycoprotein, also called macrosialin) which is present both in human and mouse [44]. The results of Mário Henrique M. Barros et al. show that CD163 or CD68 in combination with pSTAT1 or RBP-J may serve to identify M1 type of macrophages, whilst the combination with c-Maf may be useful to identify M2-like macrophages [7].

An innovative study conducted by Gian Paolo Fadini et al. [16] showed that the prediabetes period is associated with a high level of M1 peripheral blood monocyte-derived macrophages, which can differentiate into foam cells in atherosclerotic lesions by virtue of surface expression of oxLDL (oxidized low-density lipoprotein), scavenger receptor CD68 and a distinctive cholesterol responsive transcriptional profile [14]. Moreover, in pre-diabetic subjects, the high expression of CD68, responsible for cholesterol loading to the cells, was associated with production of protective markers such as CD163 and CD206, resulting in the protective tissue-cleaning characteristics of M2 macrophages [16].

### **TRANSCRIPTION FACTORS INVOLVED IN MACROPHAGE POLARIZATION**

Mediators of M1 macrophage TLR-dependent polarization include transcription factors such as NF- $\kappa$ B, AP-1 (activator protein-1), PU.1, CCAAT/enhancer-binding protein  $\alpha$  (C/EBP- $\alpha$ ), STAT1 as well as IRF5. Transcription factors which promote M2 activation include IRF4, C/EBP- $\beta$ , Krüppel-like factor 4 (KLF4), STAT6 and receptor PPAR $\gamma$  (peroxisome proliferator-activated receptor  $\gamma$ ) [5,31].

### **NF- $\kappa$ B**

NF- $\kappa$ B is claimed to be a significant transcription factor, which regulates macrophage activation in response to inflammatory cytokines, infection and stress signals [37]. In order to initiate the NF- $\kappa$ B-mediated signalling cascade, cytokines and pathogen-associated molecular patterns (PAMPs) bind cell surface receptors including TLRs [22].

Activated NF- $\kappa$ B drives expression of target genes and is connected with macrophage re-location and activation in the infected area. NF- $\kappa$ B takes part in monocyte recruitment by regulating the expression of inflammatory mediators, differentiation into macrophages and directing their cell fate determination [4,22].

### AP-1

AP-1 mediates gene regulation in response to physiological and pathological stimuli such as growth factors, cytokines, bacterial and viral infections, apoptosis, as well as oncogenic responses [13]. AP-1 together with NF- $\kappa$ B in signal-dependent gene expression, significant for innate immunity, are extremely important in classically activated (M1) macrophages. JunD (similar to Fos) is a member of AP-1 that is constitutively expressed, and its connection with cell protection from oxidative stress as well as the ability to reduce tumour angiogenesis by limiting the production of ROS has been previously shown [25]. Gene knockout studies have demonstrated the primary role for AP-1 in myeloid lineage cells, which is the generation of osteoclasts. In addition AP-1 mediates induction of inflammatory genes such as TNF- $\alpha$ . AP-1 proteins are crucial to elicit the inflammatory effects of MAPKs (mitogen-activated protein kinases), which are able to activate CREB/activating transcription factor, stabilize mRNAs, which encode inflammatory cytokines and lead to phosphorylation of histones at inflammatory gene loci [23].

### C/EBP

The C/EBP (CCAAT/enhancer-binding protein) family of bZIP (basic region leucine zipper) transcription factors plays significant functions in myeloid development and macrophage activation [31]. C/EBPs are considered to be very important in the regulation of gene expression in the monocyte-macrophage population. C/EBP  $\alpha$  and  $\beta$ , which both consist of activation and regulatory domains, permit them to function as activators. Moreover, C/EBPs can regulate the expression of a number of chemokines and cytokines (inter alia IL-6, IL-8 and MCP-1 – monocyte chemoattractant protein-1) [34].

What is more, according to recent studies, when the expression of C/EBP $\beta$  is induced by cAMP-responsive element-binding protein (CREB) (which also is a part of bZIP family transcription factor), it regulates M2-associated genes (including Arg1 and IL-10) [55]. Previous studies have shown that C/EBP- $\alpha$  directly activates transcription of PU.1 in monocytes [9] which, as a result, can cause increased expression of CD11b, CD45, F4/80 and MHC class II [3,42].

### PU.1

PU.1 is a member of the Ets family of transcription factors that is absolutely necessary for normal haematopoiesis [47]. PU.1 is active in controlling proliferation of macrophages by upregulating the receptor for M-CSF [11]. The

expression level of PU.1 is critical for determining cell fate. In case of perturbation of its expression, even modest decreases in PU.1 level can cause lymphomas [54]. Moreover, PU.1 is regarded as a very significant factor in maturation of macrophages from their haematopoietic progenitors by regulating the expression of genes that are involved in differentiation. The lack of PU.1 causes that neither M-CSF-dependent proliferation nor differentiation of monocytes is observed, while PU.1-deficient granulocytes can undergo limited differentiation. Transcriptional regulation of GM-CSF receptor, which is essential for terminal differentiation of macrophages, involves PU.1 [27,35]. PU.1 deficiency is also associated with changes in innate and adaptive immunity and regulates genes such as TLR2 and TLR4 (required for pathogen recognition). Furthermore, recent studies have shown the genome-wide connection of PU.1 with inflammatory signal induced gene expression patterns [20,27,39,59]. Regardless of the great importance of PU.1 in the process of macrophage maturation, the role of PU.1 and its impact on NF- $\kappa$ B activation in matured macrophages lacks direct *in vivo* evidence in a whole animal model [27].

### KLF4

A subclass of the zinc finger family of transcription factors consists of Krüppel-like factors (KLFs). The KLF proteins typically take part in regulation of critical aspects of cellular development, activation and differentiation. Recently, Noti *et al.* [43] discovered that macrophage differentiation factor CD11d can be regulated by KLF4. The research study of Mark W. Feinberg *et al.* [18] showed that pro-inflammatory stimuli such as IFN- $\gamma$ , LPS, and TNF- $\alpha$  are induction factors of KLF4 in macrophages, and the anti-inflammatory growth factor TGF-1 causes their inhibition. The critical role of KLF4 in mediating the pro-inflammatory effects of IFN- $\gamma$  or LPS on the induction of the macrophage activation marker iNOS has also been demonstrated. These findings indicate the key role of KLF4 in mediating pro-inflammatory responses in macrophages [18]. Z.Y. Chen *et al.* [12] demonstrated the interaction between KLF4 and the NF- $\kappa$ B family member p65 to cooperatively induce the iNOS promoter.

### IRF4 AND IRF5

The IRFs constitute a family of genes responsible for regulation of the IFN system. They are involved in the process of regulating the congenital and acquired immune response [32]. As transcription factors, they take part in regulation of the expression of genes responsible for production of IFN type I [57]. Moreover, they are involved in the process of regulation of macrophage (and DC) ontogenesis as well as their polarization towards M1/M2 phenotype [57]. IRF5 and IRF4 have been proposed to be pivotal for M1 and M2 differentiation respectively [52]. Recent studies have shown the role of IRFs in MyD88-dependent TLR signalling [63]. Krausgruber *et al.* [30] identified IRF5 as a lineage-defining factor for macrophages. It is extremely important for the induction of IL-12, IL-23

as well as TNF- $\alpha$  expression and takes part in eliciting Th1 and Th17 responses [30]. Moreover, according to another study, IRF5 strongly contributes to TLR-mediated induction of pro-inflammatory cytokines including IL6 [61]. Differentiation of M1 macrophages is connected with the upregulation of IRF5 levels. The overexpression of IRF5 in M2 polarized macrophages leads to their phenotypic switch. It makes the M2 macrophages functionally similar to M1. In contrast, knockdown of IRF5 in M1 macrophages converts M1 macrophages to the M2 expression profile. Therefore, IRF5 determines macrophage plasticity. It is also clear that in macrophages, IRF5 acts as a negative regulator of IL-10 synthesis [30]. Another member of the IRF family, IRF4, forms a complex with MyD88 like the IRF5 form does. They bind the same region of MyD88 [41]. It is possible that IRF4 may be involved in the negative-feedback regulation of TLR signalling [41]. The activation of IRF5 expression characterizes commitment to the macrophage population by driving M1 macrophage polarization and, according to recently published results on the role of IRF4 in controlling M2 macrophage markers [56], creates a new point of view for macrophage polarization and emphasizes the potential for new therapies via modulation of the IRF5 and IRF4 balance [30].

### STAT 1 AND STAT6

The IFN/STAT network takes part in upregulation of macrophage activation, which corresponds to a local or systemic increase in production of IFN. Regulation by IFN is a property of chemokines linked with IFN- $\gamma$ /LPS-induced M1 proinflammatory macrophage polarization, whereas M2 anti-inflammatory polarization is linked with the production of a different set of chemokines [51].

STATs appear to be key factors in M1 and M2 macrophage polarization. There is a well-established antagonism between STAT6 and STAT1 that has been described for Th1 and Th2 cell polarization by IFN- $\gamma$  and IL-4, respectively [31,46].

Kernbauer et al. [28] underline the significance of the STAT1-mediated macrophages' activation. They show that IFN-activated macrophages are important and required for pathogen clearance during early stages of infection [28].

STAT1 is associated with M1 macrophage polarization occurring in the Th1 immune response, whereas STAT6 is connected with M2 macrophages' activation during the Th2 cell-mediated immune reaction. Both may comprise a crucial factor in M1 versus M2 polarization and a potential tipping point for the modulation of macrophages' polarization for therapeutic purposes [31,46].

Studies of Xudong Liao et al. [33] show that KLF4 cooperates with STAT6 to induce typical M2 targets such as Arg1 following IL-4 stimulation. The Arg1 promoter reveals that intact KLF4 sites are required to achieve the maximal transcriptional activity of STAT6 (and vice versa) [33].

### PPAR $\gamma$

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) is a ligand-activated transcription factor which belongs to the superfamily of nuclear hormone receptors. It constitutes a main regulator of lipid metabolism in macrophages, which inhibit pro-inflammatory gene expression by means of several mechanisms, for example trans repression of NF- $\kappa$ B [50,58]. It is suggested that obesity and/or inflammatory stress may lead to a M2/M1 switch in the phenotype of adipose tissue macrophages (they are constitutively expressed in adipose tissue), causing further inflammation and insulin resistance in the absence of PPAR $\gamma$ -mediated pro-inflammatory gene repression. Pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  derived from macrophages have been shown to be essential mediators of obesity-induced insulin resistance. The expression of PPAR $\gamma$  may also be induced by IL-13 and IL-4, which indicate that the M2 phenotype can also involve the PPAR $\gamma$  receptor. The recent study of Szanto et al. [60] indicated a vital role for STAT6 as a cofactor in PPAR $\gamma$ -mediated gene regulation *in vitro*. The crosstalk between PPAR $\gamma$  and the IL-4-STAT6 axis might take part in controlling the M2 phenotype. The other described pathways for crosstalk involve IL-4-mediated stimulation of PPAR $\gamma$  activation through the synthesis of putative endogenous PPAR $\gamma$  ligands [8,24,31,60].

There is a possibility that KLF4 and STAT6 can cooperate to increase PPAR $\gamma$  expression - which is supported by the inductive effect of KLF4 and STAT6 on the *Pparg* promoter. Recent observations show the possibility that a cascade of inductive and cooperative interactions in the STAT6/KLF4/PPAR $\gamma$  axis may allow M2 activation [45,56].

### CONCLUSION

This paper presents the general characteristics of monocyte-derived macrophages, which are part of the mononuclear phagocyte system. It shows their basic functions and activity, depending on signals coming from the environment, which have an impact on the activation of various transcription factors. All these factors play a role in the process of changing the macrophage phenotype - pro-inflammatory or anti-inflammatory - which in the course of pathological processes can promote the development of tissue damage. The ability to identify the M1 or M2 profile and knowledge about factors affecting it may lead to the development of new methods for their phenotypic switch and modulation of immune response induction. This may lead to development of new therapies of many diseases, i.e. those with autoimmune background, such as diabetes mellitus type 1. Such diseases are becoming increasingly common and affect people of different ages. Conducting this type of research may help to reduce the vascular complications, which can be life-threatening.

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