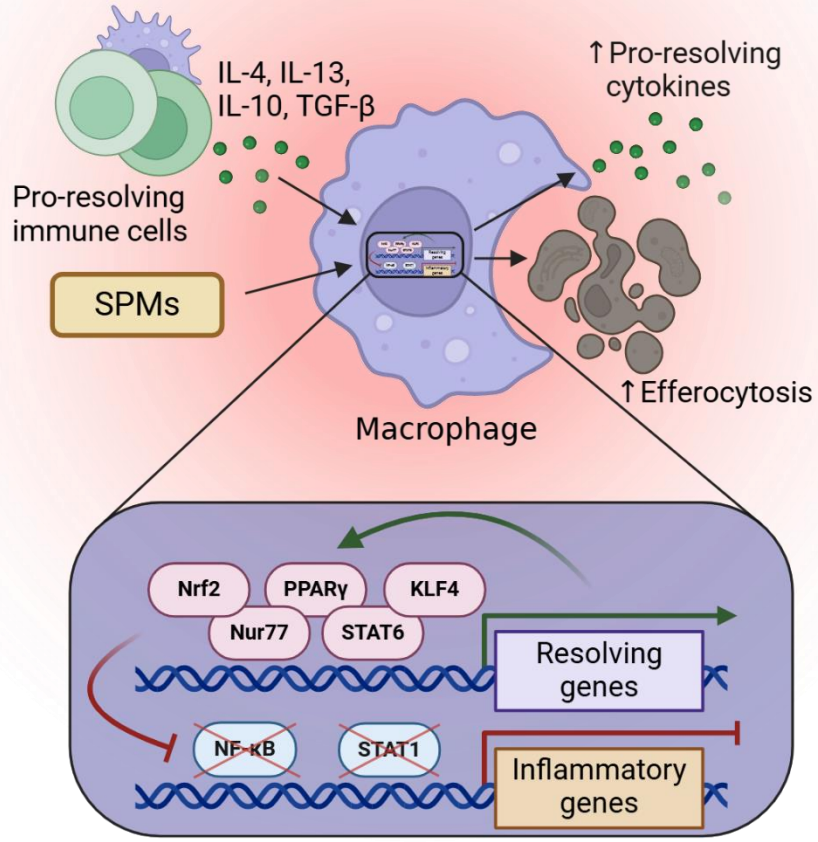
# Guiding macrophages to resolution: insights into the transcriptional pro-resolving mechanisms




Writing assignment

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## Abstract

The inflammatory response is a tightly regulated process, which is essential for maintaining immune homeostasis. When this regulation is out of balance, persistence of the inflammatory response can lead to the development of (chronic) inflammatory diseases. Macrophages play a central role in this balance due to their phenotypic plasticity, allowing them to promote both pro-inflammatory and pro-resolving processes. Current therapeutic strategies predominantly focus on how to dampen the inflammatory response to induce resolution of inflammation. However, resolution is not just the disappearance of inflammatory signaling, but rather an actively regulated process. Here, we review the current understanding of pro-resolving signaling pathways in macrophages and how negative regulation of pro-inflammatory pathways promote resolution. Furthermore, we highlight the importance of the environment and discuss the role of specialized pro-resolving lipid mediators to promote the macrophage conversion towards a more pro-resolving phenotype. This knowledge functions as basis for the development of new therapeutic strategies to treat inflammatory diseases by promoting pro-resolving signaling in macrophages. Nanoparticles present as promising carrier for these pro-resolving drugs to target specifically macrophages at sites of inflammation, restoring the macrophage disbalance and promoting immune homeostasis.

## Layman summary

Inflammation is a natural process that protects organs and tissues from infections. It directs immune cells to the affected site, where they work to fight off the invaders. Once the infection is under control, the inflammatory response must be shut down to prevent persistent activation of the inflammatory response. The overactivation namely causes damage to healthy tissue and leads to the development of chronic inflammatory diseases, like arthritis, atherosclerosis, and inflammatory bowel disease.

Macrophages, a type of immune cell, play a central role in controlling the inflammatory response. When the body needs a strong inflammatory response, macrophages switch to a pro-inflammatory state to help fight the infection. However, when the infection is cleared, they switch to a pro-resolving state to repair the inflamed site and help the body return to normal.

Current therapies mainly dampen the inflammatory response to treat chronic inflammatory diseases. While these treatments help a lot of people, they broadly reduce the inflammatory response, often leading to serious side effects, including a high risk of infection and even cancer. Resolving inflammation is more than just removing the inflammatory response but rather an active process in which resolving processes have to be turned on. Therefore, we discuss the current knowledge on these processes in macrophages that help convert them towards a pro-resolving state. Additionally, we show how external signals, like pro-resolving molecules and specialized pro-resolving mediators, can activate these pro-resolving processes in macrophages.

By better understanding these pro-resolving processes in macrophages, we hope to stimulate the development of new therapies for patients with chronic inflammatory diseases. One of these exciting developments is the use of nanoparticles, which are tiny carriers modified to deliver pro-resolving drugs to macrophages at inflamed site. This targeted approach could stimulate macrophages to restore balance in the immune system, offering a promising alternative for treating patients with chronic inflammatory diseases.

# Abbreviations

| | |
|---|---|
| Bcl6 | B cell lymphoma 6 |
| C/EBP | CCAAT/enhancer binding protein |
| CISH | Cytokine-inducible SH2 containing protein |
| CREB | cAMP-response element binding protein |
| CXCL | Chemokine (C-X-C motif) ligand |
| DHA | Docosahexaenoic acid |
| DNMT1 | DNA methyltransferase 1 |
| EPA | Eicosatetraenoic acid |
| HDAC3 | Histone deacetylase 3 |
| IKK | IκB kinase |
| IL | Interleukin |
| IFN | Interferon |
| IRF8 | Interferon regulatory factor 8 |
| IκB | Inhibitor of NF-κB |
| JAK | Janus kinase |
| KLF4 | Krüppel-like factor 4 |
| LDTF | Lineage-determining transcription factor |
| LGR6 | Leucine-rich repeat-containing G-protein-coupled receptor 6 |
| LOX | Lipoxygenase |
| MAPK | Mitogen-activated protein kinase |
| MaR1 | Maresin 1 |
| MCPIP | MCP-1-induced protein |
| NCoR | Nuclear receptor corepressor |
| NF-κB | Nuclear factor kappa B |
| NO | Nitric oxide |
| NP | Nanoparticle |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| Nur77 | Nuclear receptor subfamily 4 group A member 1 |
| PAMP | Pathogen-associated molecular pattern |
| PD1 | Protectin D1 |
| PDX | Protectin DX |
| PGC-1 | Peroxisome proliferator-activated receptor gamma coactivator 1 |
| PIAS | Protein inhibitor of activated stat |
| PPARγ | Peroxisome proliferator activated receptor gamma |
| RvD1 | Resolvin D1 |
| RvE1 | Resolvin E1 |
| RXR | Retinoid X receptor |
| SDTF | Signal-dependent transcription factor |
| siRNA | Small interfering RNA |
| SMRT | Silencing mediator of retinoid and thyroid hormone receptor |
| SOCS | Suppressor of cytokine signaling |
| SPM | Specialized pro-resolving mediator |
| STAT | Signal transducer and activator of transcription |
| TAK1 | Transforming growth factor β-activated kinase 1 |
| TF | Transcription factor |
| TGF-β | Transforming growth factor beta |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TRAF6 | TNF receptor associated factor 6 |
| Treg | Regulatory T cell |

# Index

# Introduction

Since their discovery, macrophages are an intensively studied cell type, because of their plasticity, variety of functions, and involvement in multiple diseases. Macrophages reside in many organs and travel through the cardiovascular tract to maintain immune homeostasis. Where in some organs, like the brain, the majority of macrophages originate from embryonic precursors, in other organs, like the intestine, these embryonic precursors are almost completely replaced by bone marrow monocyte-derived macrophages (1). Extracellular cues, like cytokines, but also cellular interactions and mechanical extracellular matrix interactions further diversify the macrophage phenotype based on the organ-specific current needs. For example, retinoic acid induces expression of the transcription factor (TF) GATA6, regulating the functional polarization of peritoneal macrophages (2). Additionally, macrophages respond to environmental signals, which shapes their activation state and function. During bacterial infections, macrophages recognize pathogen-associated molecular patterns (PAMPs), such as LPS, which drives them to a pro-inflammatory phenotype to produce the cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF) and enhances their phagocytic capacity (3). Conversely, during resolution of inflammation, macrophages display a more resolving phenotype, which aids in apoptotic cell clearance (efferocytosis) and initiates wound healing responses (3).

The conversion from a pro-inflammatory to a pro-resolving macrophage phenotype is a transcriptionally controlled process directed by both extrinsic and intrinsic regulatory mechanisms. For example, signaling of pro-inflammatory cytokines (e.g., IL-1β and interferon (IFN)-γ) activates inflammatory TFs in macrophages, such as nuclear factor κB (NF-κB) and signal transducer and activator of transcription (STAT)1 (4). Subsequently, these TFs are transcriptionally, translationally, and post-translationally regulated to influence their activity (5). Conversely, secretion of anti-inflammatory and modulatory cytokines (e.g., IL-4 and IL-10) activates pro-resolving TFs, like STAT3 and STAT6, which are on their turn intrinsically regulated (6). This balance in the regulation of pro-inflammatory and pro-resolving TFs is a dynamic process, determining the macrophage phenotype.

Excessive activation of inflammatory pathways in combination with insufficient pro-resolving signaling contributes to the development of chronic inflammatory diseases, including atherosclerosis (7). Classically activated (M1-like) macrophages progress these diseases by producing pro-inflammatory cytokines, generating ROS-induced tissue damage, and impairing wound healing responses (3,8). To shift this macrophage phenotype towards a pro-resolving state, it is essential not only to downregulate the pro-inflammatory response, but also to stimulate pro-resolving signaling pathways. This approach enables macrophages to both terminate their pro-inflammatory actions and actively engage in tissue repair and inflammation resolution processes (9). However, current treatments for inflammatory

diseases predominantly focus on suppressing pro-inflammatory signaling, often overlooking the need to actively activate pro-resolving processes. Hence, this review aims to gain more insight into the transcriptional regulatory mechanisms to induce pro-resolving macrophages. We summarize the transcriptional regulation of macrophage differentiation, outline the most important resolving signaling pathways, and discuss how inflammatory pathways are negatively regulated to promote resolution of inflammation. Thereafter, we highlight the role of pro-resolving immune cells in the environment and specifically focus on one type of pro-resolving molecule, called specialized pro-resolving mediators (SPMs), and how they mechanistically enhance the pro-resolving macrophage state conversion. A deeper molecular understanding of macrophage-directed resolution provides a foundation for developing new drugs aimed at stimulating pro-resolving signaling in macrophages. The use of nanoparticles presents a promising approach for delivering these pro-resolving drugs, as they can be modified to target specifically macrophages at inflamed sites. Thus, this review discusses how to guide macrophages towards resolution through activation of pro-resolving signaling pathways, presenting an alternative approach for treating (chronic) inflammatory diseases.

# Chapter 1: Transcriptional regulation of macrophage differentiation

## From hematopoietic stem cell to macrophage

Specific TFs, known as lineage-determining transcription factors (LDTFs), guide the differentiation of hematopoietic stem cells into various immune cells, including macrophages. Among these, PU.1 is a key LDTF that regulates macrophage differentiation (10). The dosage of PU.1 critically influences the fate of progenitor cells. High levels of PU.1 drive macrophage differentiation, while moderate PU.1 levels direct granulocyte-macrophage progenitors towards a neutrophil lineage. At earlier stages, such as in multipotent progenitors, moderate PU.1 levels can even stimulate B cell differentiation (11). PU.1 exerts its effect by interacting with lineage-determining factors to shape a cell-specific cistrome by binding to the distal region of promotors. Mechanistically, PU.1 binds near binding sites of other LDTFs and induces histone H3K4 monomethylation, which supports chromatin accessibility and marks active promotors (12). After this chromatin remodeling, other LDTFs and signal-dependent TFs (SDTFs) can bind to the *cis*-regulatory elements to aid in macrophage differentiation and responses.

This chromatin remodeling ability of PU.1 is also important for inducing both pro-inflammatory and pre-resolving macrophage responses. For example, in lipopolysaccharide (LPS)-stimulated murine macrophages, PU.1 binds to *Nfkb1* and *Il12b* enhancers to facilitate accessibility for SDTFs (13,14). In the absence of PU.1, heterochromatin forms in the region, restricting the binding of inflammatory-specific TFs. Similarly, PU.1 is essential for transcription of pro-resolving genes, such as *Arg1*, *Ym1*, and *Fizz1*, highlighting the role of PU.1 in both inflammatory and resolving macrophage responses (15). Collectively, this positions PU.1 as a master regulator of macrophage differentiation, with subsequent macrophage responses modulated by more specialized polarizing TFs.

In addition to its independent functions, PU.1 collaborates with other LDTFs, such as CCAAT/enhancer binding proteins (C/EBPs) and interferon regulatory factor 8 (IRF8), to drive macrophage differentiation. C/EBPα is primarily active in the initial stages of myeloid differentiation. Consequently, C/EBPα inactivation by C/EBPγ results in immature macrophage and neutrophil colonies (16). In contrast, C/EBPβ directs the later stages of macrophage differentiation by binding proximal to target genes (17). IRF8 forms a heterodimer with PU.1, further promoting the transcription of macrophage-specific genes by binding to distal promotor regions and inducing histone H3K4 monomethylation (18). Notably, IRF8 interacts with C/EBPα to inhibit neutrophil differentiation, thereby promoting macrophage differentiation (19). Thus, the balance of these key LDTFs determines progenitor cell fate. Disruptions in this LDTF balance could result in hematopoietic disorders, such as leukemia.

# Chapter 2: Pro-resolving macrophage signaling pathways

After the initial differentiation from hematopoietic stem cell to macrophage, macrophages reside in a resting state to support tissue homeostasis or polarize towards a pro-inflammatory or pro-resolving phenotype in response to inflammation or the need for tissue repair. The macrophage exhibits remarkable plasticity, meaning that pro-inflammatory macrophages can still polarize to pro-resolving macrophages and vice versa, depending on environmental cues (4). Within these pro-inflammatory and pro-resolving polarized states, macrophages exhibit a variety of functions, suggesting that a spectrum of macrophage subtypes exists. Indeed, transcriptional data from patients with inflammatory bowel disease show multiple macrophage subsets within the pro-inflammatory and pro-resolving state and even identify a macrophage population likely in the transitional phase from pro-inflammatory towards pro-resolving (20). To get a better understanding of the triggers in the conversion from a pro-inflammatory to pro-resolving macrophage, the major players in this conversion will be explained below.

## Initial stage of pro-resolving signaling cascades

The macrophage conversion from a resting or pro-inflammatory to a pro-resolving state is initiated by the secretion of pro-resolving cytokines (e.g., IL-4, IL-13) by immune cells, including T helper 2 and innate lymphoid type 2 cells (21). Additionally, immunomodulatory cytokines, such as IL-10 (mainly produced by T helper 2 cells and resolving macrophages) and transforming growth factor beta (TGF-β) (mainly produced by regulatory T cells (Tregs) and resolving macrophages) further guide macrophage polarization (21,22). These cytokines activate characteristic signaling pathways, including TGF-β/Smad3, Janus kinase (JAK)/STAT3, and JAK/STAT6 signaling, which generally leads to the activation of resolving processes. For example, activation of the canonical TGF-β pathway initiates wound healing responses by macrophage-mediated deposition of matrix components and activation of myofibroblasts (23). However, TGF-β can also initiate inflammatory signaling by activating NF-κB via the non-canonical pathway, making TGF-β a complex target to influence macrophage polarization (24).

Activation of the pro-resolving JAK/STAT signaling pathways starts with binding of the cytokines IL-4, IL-13, and IL-10 to the IL-4/IL-13 and IL-10 receptor, respectively. Upon IL-4/IL-13 receptor activation, JAK1 and JAK3 are recruited to the receptor and undergo autophosphorylation. Then, the JAKs phosphorylate the IL-4/IL-13 receptor to create a docking site for STAT6. Consequently, STAT6 gets phosphorylated, forms a homodimer, and translocates to the nucleus to function as TF (4). Similarly, IL-10 binds to the IL-10 receptor, whereafter JAK1 is recruited to activate STAT3. Again, STAT3 homodimerizes and translocates to the nucleus to induce expression of resolving macrophage genes (*Arg1* and *Ym1*) to alleviate inflammation (25).

## Pro-resolving functions of STAT6

STAT6 further alters the macrophage phenotype by suppressing pro-inflammatory signaling, while stimulating the transcription of pro-resolving mediators. For example, STAT6 attenuates the inflammatory response by transcriptional repression of a select LPS-induced enhancer set in murine macrophages, which attenuates the inflammasome activation and decreases the production of IL-1β (26). On the other hand, peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1)β and PGC-1-related coactivator, proteins of the PGC family, function as coactivator of STAT6 to enhance expression of pro-resolving genes (*Arg1*, *Ym1*, and *Fizz1*) (27,28).

STAT6 further orchestrates the conversion towards pro-resolving macrophages by transcribing and activating other pro-resolving TFs (Figure 1). STAT6 namely induces binding of retinoid X receptor (RXR) to *de novo* enhancers and upregulates expression of peroxisome proliferator activated receptor gamma (PPARγ), another pro-resolving TF (29). Once upregulated, PPARγ forms a heterodimer with RXR, further enhancing the pro-resolving macrophage polarization via several mechanisms. For example, PPARγ inhibits NF-κB signaling via transrepression – a process based on inhibition through direct protein-protein interactions – reducing the secretion of inflammatory cytokines, such as TNF and IL-6 (30). PGC-1β aids in this process by acting as transcriptional corepressor of NF-κB. PGC-1β also interacts with TAB1, a protein upstream of the NF-κB pathway, to prevent transforming growth factor β-activated kinase 1 (TAK1) and eventually IκB kinase (IKK) activation (29). Moreover, the PPARγ:RXR heterodimers create transcriptional memory by recruiting the accessory proteins P300 and RAD21. As a result, secondary stimulation of IL-4 in macrophages after four days of initial stimulation enhances the induction of the pro-resolving macrophage marker *Arg1* (31). Together, these findings demonstrate that STAT6, in collaboration with proteins of the PGC family and PPARγ, orchestrates a robust transcriptional network that primes macrophages towards a pro-resolving state by inhibiting inflammatory and activating resolving signaling pathways.

Moreover, pro-resolving TFs, including STAT6, induce transcription of the pro-resolving TF Krüppel-like factor 4 (KLF4) (Figure 1). Knockdown of KLF4 induces production of pro-inflammatory mediators in human macrophages, suggesting that KLF4 plays a role in steering macrophage polarization (32). Specifically, KLF4 inhibits the production of TNF, preventing kidney damage and fibrosis (33). Moreover, KLF4 induces expression of resolving macrophage markers, which is under negative regulation of NF-κB activity (34). STAT6-mediated KLF4 expression also induces expression of MCP-1-induced protein (MCPIP). MCPIP suppresses NF-κB signaling and stimulates resolving macrophage polarization by enhancing resolving marker expression and inducing expression of the pro-resolving TF PPARγ (35). Thus, KLF4 is yet another STAT6-induced key TF that promotes resolving macrophage polarization by suppressing inflammatory and enhancing resolving marker and PPARγ expression.

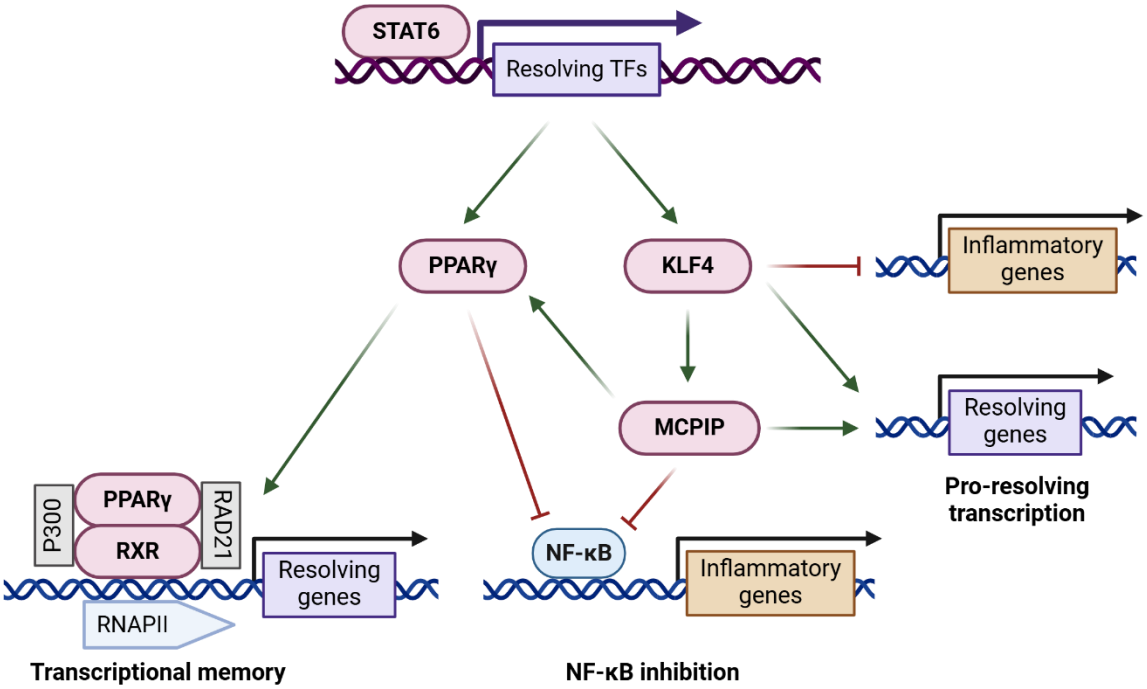


**Figure 1: STAT6 induces pro-resolving signaling in macrophages.**
STAT6 induces expression of the pro-resolving TFs PPARγ and KLF4. PPARγ heterodimerizes with RXR and recruits the accessory proteins P300 and RAD21 to create transcriptional memory of pro-resolving genes. KLF4 inhibits transcription of inflammatory genes, promotes pro-resolving gene transcription, and induces expression of MCPIP. MCPIP promotes expression of pro-resolving genes and PPARγ, and together with PPARγ they inhibit NF-κB signaling, with PPARγ inhibiting NF-κB via transrepression.
Abbreviations: STAT6, signal transducer and activator of transcription 6; PPARγ, peroxisome proliferator activated receptor gamma; KLF4, Krüppel-like factor 4; MCPIP, MCP-1-induced protein; NF-κB, nuclear factor kappa B; RNAPII, RNA polymerase II.

## Pro-resolving macrophages shape the environment

The activation of these pro-resolving TFs does not only affect macrophage polarization, but also shapes the environment by exhibiting immunomodulatory and tissue repair properties. For example, IL-4/IL-13-stimulated and IL-10/TGF-β-stimulated murine macrophages secrete pro-resolving cytokines and suppress the proliferation of $CD4^+$ T cells to create an immunomodulatory environment (36,37). Additionally, IL-10/TGF-β-polarized murine macrophages reduce the inflammatory status of IFN-γ/TNF-stimulated macrophages and induce Treg development by B7-H4-dependent cell-cell interactions (37). After the initial inflammation, resolving macrophages promote tissue repair by secreting proangiogenic factors, depositing extracellular matrix components, and inducing myofibroblast activity (38). These macrophages also produce SPMs which will be discussed later in this review. Thus, the activation of pro-resolving TFs in macrophages not only directs their polarization but also orchestrates a broader immunomodulatory and reparative response in the environment which is essential for effective inflammation resolution and tissue healing.

## Feedback loops to sustain pro-resolving expression signature

To accelerate the conversion of macrophages from a pro-inflammatory to a pro-resolving phenotype and to sustain this pro-resolving signaling, multiple feedback loops exist. These feedback loops consist of pro-resolving mediators which further stimulate pro-resolving signaling or inhibit pro-inflammatory signaling. As mentioned previously, STAT6 promotes the transcription of additional pro-resolving TFs, including PPARγ and KLF4. Additionally, PPARγ and MCPIP negatively regulate NF-κB signaling. Furthermore, STAT6 induces transcription of the IL-4 receptor, which stimulates its own pathway (39). Finally, microRNAs play a significant role in regulating macrophage polarization but are beyond the scope of the review (reviewed in (40)).

# Chapter 3: Transcriptional regulation of inflammatory signaling in macrophages

Enhancing the activation of pro-resolving signaling pathways is crucial in the conversion of macrophages from a pro-inflammatory to a pro-resolving phenotype. To further promote this conversion and prevent excessive inflammation, pro-inflammatory signaling is tightly regulated. Two key inflammatory signaling pathways in macrophages are the PAMP-induced and pro-inflammatory cytokine-induced signaling pathway (4). These signaling pathways primarily activate the TF NF-κB and inflammatory JAK/STAT signaling, which are under intrinsic control via various negative regulatory mechanisms to prevent excessive inflammation and tissue damage, which will be discussed below.

## Negative regulation of NF-κB signaling

PAMP-induced PRR signaling, such as toll-like receptor (TLR)4 activation by LPS, initiates the NF-κB pathway. In this pathway, several kinases phosphorylate the IKK complex, which promotes the degradation of inhibitor of NF-κB (IκB) from the NF-κB/IκB complex, enabling NF-κB to translocate to the nucleus and drive the transcription of inflammatory genes (5).

To prevent continuous activation of this pathway, NF-κB signaling has built-in negative feedback mechanisms (Figure 2). For example, NF-κB induces *de novo* synthesis of IκB as primary response gene, which inhibits further nuclear translocation of NF-κB (41). Subsequently, IKK again directs IκB for proteasomal degradation, allowing NF-κB to translocate back into the nucleus, leading to oscillatory NF-κB signaling until removal of the initial stimulus (41). Notably, only certain stimuli induce this oscillatory behavior, whereas other stimuli promote persistent NF-κB signaling by reprogramming the epigenome through activation of latent enhancers, while inducing similar inflammatory gene activation (42). This discrepancy suggests that oscillation of NF-κB signaling prevents inflammatory latent enhancer activation, thereby dictating the long-term macrophage response towards different stimuli. Moreover, this oscillatory behavior facilitates macrophages to fine-tune their response based on changing external cues to different phases of infection (43).

Another key negative regulator of NF-κB signaling, also synthesized *de novo* in response to NF-κB signaling, is the deubiquitinase A20. A20 destabilizes the IKK complex by deubiquitinating its activators (Figure 2). This destabilization prevents IκB proteasomal degradation, halting NF-κB nuclear translocation (44). A knockdown study shows that A20-deficient human macrophages express higher levels of pro-inflammatory macrophage markers and lower levels of resolving macrophage markers after stimulation with LPS/IFN-γ, highlighting the role of A20 in affecting macrophage polarization (45). Consequently, A20 protects against both acute and persistent inflammatory damage, such as acute

lung injury and colitis by steering macrophage polarization (46,47). In autoinflammatory diseases, such as SLE, monocytes express low levels of A20, resulting in augmented NF-κB signaling and chronic inflammation (48). Thus, negative feedback loops, such as the synthesis of IκB and A20, are essential in preventing prolonged NF-κB activation and promoting the transition to a pro-resolving macrophage phenotype.

Another negative feedback mechanism involves the upregulation of nuclear receptor subfamily 4 group A member 1 (NR4A1, also known as Nur77) expression in response to NF-κB signaling (49). Studies show that Nur77 knockout mice are prone to develop systemic inflammation by an increase in pro-inflammatory cytokine production (50). This observation suggests that Nur77 plays a critical role in negatively regulating inflammatory pathways, such as the NF-κB pathway. Indeed, Nur77 inhibits NF-κB signaling via various mechanisms (Figure 2). One key mechanism involves Nur77 binding to TNF receptor associated factor 6 (TRAF6), an upstream activator of NF-κB, thereby preventing TRAF6 oligomerization and subsequent autoubiquitination, which are essential steps for activating the NF-κB pathway (51). Additionally, Nur77 upregulates expression of the NF-κB inhibitor IκB, while reducing IKKβ expression, a component of the NF-κB activating complex IKK (52). Furthermore, Nur77 prevents DNA binding of NF-κB via transrepression, thereby lowering the expression of pro-inflammatory genes (53). Nur77 also induces an anti-inflammatory metabolic state in murine macrophages by reducing tricarboxylic acid cycle activity, which leads to reduced production of nitric oxide (NO) and pro-inflammatory cytokines (54). Consequently, knockout of Nur77 polarizes macrophage towards a pro-inflammatory phenotype, characterized by elevated *Il12b* and *Nos2,* and reduced *Arg1* expression (55). Thus, Nur77 plays a critical role in regulating inflammation by acting as negative regulator of NF-κB signaling in macrophages.

Finally, NF-κB signaling promotes transcription of nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT). Generally, NCoR and SMRT function as corepressors by binding near gene transcripts and recruiting histone deacetylase 3 (HDAC3), leading to gene silencing (Figure 2). Here, SMRT preferably binds to proximal promotor regions of genes, while NCoR and common NCoR and SMRT genomic sites are mostly found in intron or far distal genomic regions (56). For example, both NCoR and SMRT interact with B cell lymphoma 6 (Bcl6) to suppress NF-κB signaling. The NCoR-Bcl6 and SMRT-Bcl6 cistromes are namely strongly enriched for inflammatory signaling pathways in murine macrophages with nearly 60% correspondence with the NF-κB cistrome (57). However, upon TLR4 stimulation, NCoR rather functions as coactivator of NF-κB by forming a complex with HDAC3 and PGC1β, which exemplifies the impact of environmental cues and available interaction partners on the heterogenic functions of coregulatory proteins regarding their transcriptional regulation (58).

In conclusion, NF-κB signaling is a powerful and impactful inflammatory pathway subject to various regulatory mechanisms. Targeting the expression or activity of these regulatory molecules in macrophages, within the right biological context, may encourage the macrophage conversion towards a pro-resolving phenotype.

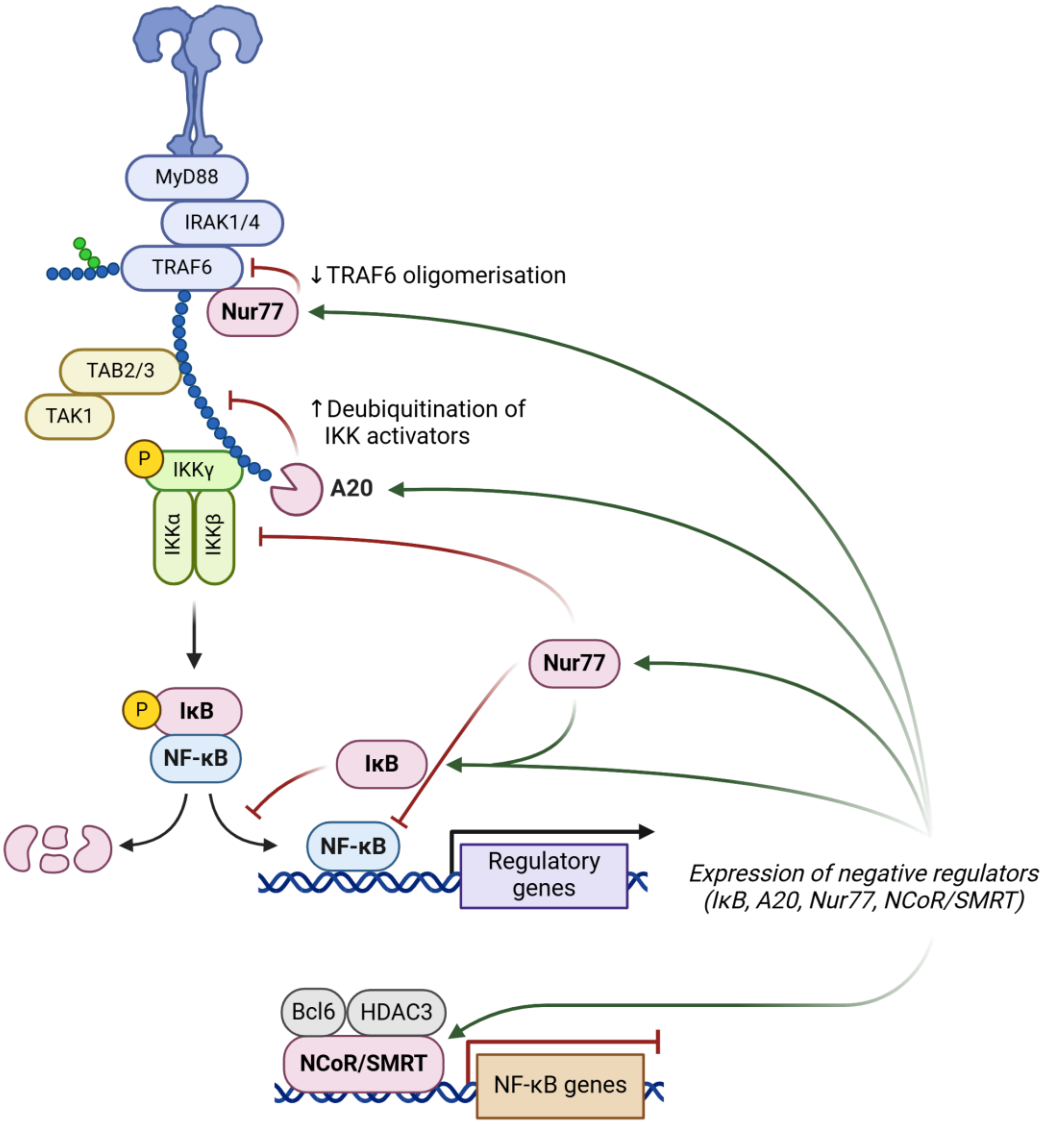


**Figure 2: Negative regulatory mechanisms of NF-κB signaling.**
NF-κB signaling induces *de novo* synthesis of the regulatory genes encoding for IκB, A20, Nur77, and NCoR/SMRT. IκB prevents nuclear translocation of NF-κB to inhibit the expression of inflammatory genes. Nur77 enhances IκB expression, while suppressing expression of IKKβ. Additionally, Nur77 prevents TRAF6 oligomerization and subsequent autoubiquitination and NF-κB DNA binding via transrepression. A20 removes the ubiquitination of protein complexes upstream of NF-κB. NCoR/SMRT reduces the expression of inflammatory genes by competing with NF-κB for DNA binding sites through recruitment of Bcl6 and HDAC3 to induce gene silencing.
Abbreviations: MyD88, myeloid differentiation primary response 88; IRAK1/4, interleukin-1 receptor-associated kinase 1/4; TRAF6, TNF receptor associated factor 6; IKK, IκB kinase; IκB, inhibitor of NF-κB; NF-κB, nuclear factor kappa B; Nur77, nuclear receptor subfamily 4 group A member 1; NCoR, nuclear receptor corepressor; SMRT, silencing mediator of retinoid and thyroid hormone receptor; Bcl6, B cell lymphoma 6; HDAC3, histone deacetylase 3.

## Negative regulation of JAK/STAT signaling

JAK/STAT signaling is primarily activated by cytokine-cytokine receptor interactions, with the cytokines determining whether the cascade drives the transcription of pro-inflammatory or pro-resolving genes. Mechanistically, different cytokines activate and dimerize specific STATs, which translocate to the nucleus to function as TF. For example, IFN-γ binding to its receptor induces dimerization of the receptor, leading to autophosphorylation of JAK1 and JAK2. Subsequently, JAK2 phosphorylates the receptor, creating a docking site for STAT1, which, after phosphorylation, forms a homodimer and translocates to the nucleus to induce transcription of IFN-stimulated genes (59). Other cytokines and growth factors, such as type I IFNs and granulocyte-macrophage colony-stimulating factor, induce the formation of different STAT dimers, such as the STAT1-STAT2 heterodimer and STAT5 homodimer, respectively, leading to transcription of pro-inflammatory macrophage-associated genes and increased production of pro-inflammatory cytokines (4).

To regulate this inflammatory signaling, STATs also induce transcription of suppressor of cytokine signaling (SOCS) family genes, consisting of cytokine-inducible SH2-containing protein (CISH) and SOCS1 through SOCS7, which function as key negative regulators of JAK/STAT signaling, forming a negative feedback loop (6). SOCS proteins modulate JAK/STAT signaling through several mechanisms: (i) SOCS1 and SOCS3 inhibit the kinase activity of JAKs, (ii) CISH and SOCS2-5 compete with STATs for binding sites on cytokine receptors, (iii) SOCS6 and SOCS7 prevent nuclear translocation of STATs, and (iv) all SOCS proteins promote proteasomal degradation of STATs (60). The activity of SOCS proteins plays a crucial role in regulating macrophage polarization. For example, SOCS1 knockdown promotes macrophage polarization towards a pro-inflammatory phenotype and affects cytokine expression by reducing IL-4 and IL-10, while enhancing TNF and IFN-γ production (61). Additionally, some bacteria exploit this regulatory mechanism by inducing SOCS1 expression during initial stages of infection, enhancing their infective capacity (62). Thus, SOCS family proteins serve as critical inhibitors of JAK/STAT signaling, ultimately suppressing pro-inflammatory macrophage polarization.

Additionally, protein inhibitor of activated STAT (PIAS) plays a significant role in negatively regulating JAK/STAT signaling. In mammals, the PIAS protein family consists of four proteins, PIAS1 through PIAS4, of which PIAS1 is the most extensively studied for its role in inhibiting JAK/STAT activity. PIAS1 knockout mice display enhanced protection against viral infection by regulating a subset of IFN-γ genes, suggesting that PIAS1 moderates the pro-inflammatory JAK/STAT signaling (63). Indeed, PIAS negatively regulates the activity of STATs by inhibiting STAT phosphorylation and thereby the translocation to the nucleus (64). This inhibition reduces pro-inflammatory signaling in murine adipocytes and macrophages by decreasing IL-1β, IL-6, TNF, and NO production (65,66). While no studies directly link PIAS to macrophage polarization, these findings suggest that PIAS likely contributes

to a pro-resolving macrophage phenotype by dampening the inflammatory signaling pathway of JAK/STAT.

In conclusion, JAK/STAT signaling is tightly regulated by both SOCS and PIAS proteins, which function as critical inhibitors of the pathway. By modulating STAT activity, these proteins limit pro-inflammatory macrophage polarization while promoting a resolving, anti-inflammatory phenotype.

# Chapter 4: Effect of neutrophils and Tregs on resolving macrophage phenotype

In the microenvironment of infection and inflammation, the polarization of macrophages is heavily influenced by interactions with other immune cells, including neutrophils and Tregs. This dynamic interplay affects the response of macrophages during inflammation, thereby orchestrating their conversion towards a more resolving phenotype.

## Neutrophils as resolution initiator for macrophages

During the initial stages of infection, primarily neutrophils are recruited to the affected site, followed by macrophages. Once the infection subsides, macrophages need to activate pro-resolving pathways to initiate wound healing responses. Recent studies highlight the crucial role of the recruited neutrophils in orchestrating this resolution of inflammation by inhibiting pro-inflammatory and stimulating pro-resolving signaling pathways in macrophages. For example, both apoptotic and viable neutrophils inhibit NF-κB signaling in human primary macrophages by blocking activation of TAK1 and IKKβ, which act upstream of NF-κB (67). Moreover, anti-inflammatory neutrophils promote expression of the immunomodulatory cytokines IL-10 and TGF-β, and increase transcription of the pro-resolving TFs PPARγ, KLF4 and Nur77 in human macrophages (68). In a myocardial infarction mouse model, these neutrophils generate resolving macrophages with increased efferocytosis capacity (69). Once macrophages engulf these apoptotic neutrophils, they stimulate macrophages to secrete anti-inflammatory cytokines and SPMs. These findings reveal the dual role of neutrophils, not only as first responders in infection causing inflammation but also as key regulators in initiating resolution via macrophages.

## Tregs promote pro-resolving signaling in macrophages

Tregs also play an essential role in modulating the macrophage phenotype towards a pro-resolving phenotype by secreting anti-inflammatory signaling molecules. For example, Treg-derived IL-10 induces a pro-resolving macrophage phenotype by upregulating expression of *Arg1*, *Ym1*, and the pro-resolving marker *Cd206*, while reducing TNF secretion (70). Additionally, IL-10 and TGF-β secreted by Tregs enhance resolving arginase activity and efferocytosis capacity of murine macrophages (70). Mechanistically, Tregs activate the JAK/STAT3 signaling pathway, while inhibiting NF-κB signaling in macrophages by secreting immunomodulatory proteins, which further reinforces their pro-resolving state conversion (70,71). Tregs do not only communicate to macrophages via cytokines, but also via exosomes. Exosomes are DNA, RNA, protein, and lipid-containing vesicles that are exchanged between cells to convey messages and alter biological functions of target cells. For example, Treg-derived

exosomes improve cardiac function of mice with acute myocardial infarction by inducing macrophage polarization towards a pro-resolving phenotype (72). These pro-resolving macrophages subsequently promote the generation of additional Tregs, creating a positive feedback loop to sustain the pro-resolving macrophage activities.

In conclusion, the crosstalk between neutrophils and Tregs with macrophages drives tissue repair after the initial inflammatory response. This cell-cell communication is crucial in orchestrating resolution of inflammation, emphasizing the complexity for developing targeted therapies in inflammatory diseases.

# Chapter 5: Effect of SPMs on macrophage phenotype

SPMs play a pivotal role in resolving inflammation. These mediators, derived from docosahexaenoic acid (DHA) and eicosatetraenoic acid (EPA), are biosynthesized by the enzymatic action of 5-lipoxygenase (5-LOX) and 15-lipoxygenase (15-LOX) into a variety of SPMs, including resolvins, protectins and maresins (73). In general, SPMs promote the resolution of inflammation through enhancing efferocytosis, modulating cytokine production, and inducing pro-resolving immune signaling in macrophages (Table 1). In this chapter, the role of resolvin D1 (RvD1), resolvin E1 (RvE1), maresin 1 (MaR1), protectin D1 (PD1), and protectin DX (PDX) in these processes will be highlighted.

**Table 1: Effects of SPMs on macrophage signaling and phenotype.**

| SPM | Targeted signaling/TFs | Effect on macrophage phenotype |
|---|---|---|
| RvD1 | ↑ PPARγ (74) | ↑ M2 polarization (75–77) |
| | ↑ Nrf2 (78) | ↑ Efferocytosis of apoptotic PMNs (75,76) |
| | ↑CREB signaling (79) | ↑ Phagocytosis of microbial particles (80) |
| | ↓ NF-κB (75,78,79) | ↑ Anti-inflammatory signaling (79) |
| | ↓ MAPK signaling (78) | ↓ Pro-inflammatory cytokine secretion (76,79,80) |
| | | ↓ Chemotactic migration (80) |
| | | ↓ Neutrophil chemotaxis (75,79,80) |
| RvE1 | ↑ Nrf2 (81) | ↑ M2 polarization (82,83) |
| | ↓ NF-κB (83,84) | ↑ Efferocytosis of apoptotic PMNs (85,86) |
| | ↓ MAPK signaling (83) | ↑ Phagocytosis of microbial particles (82) |
| | | ↓ Pro-inflammatory cytokine secretion (83,84) |
| | | ↓ Chemotactic migration (82) |
| | | ↓ Neutrophil chemotaxis (83) |
| MaR1 | ↑ PPARγ (87) | ↑ M2 polarization (87–89) |
| | ↑ Nrf2 (88,89) | ↑ Efferocytosis of apoptotic PMNs (90) |
| | ↑CREB signaling (79) | ↑ Phagocytosis of microbial particles (90) |
| | ↓ NF-κB (79,88,89) | ↑ Anti-inflammatory signaling (79,89) |
| | ↓ MAPK signaling (88) | ↓ Pro-inflammatory cytokine secretion (79,87–89) |
| | | ↓ Neutrophil chemotaxis (79) |
| | | ↓ Inflammasome activation and pyroptosis (88) |
| PD1 | | ↑ Efferocytosis of apoptotic PMNs (85) |
| | | ↑ Phagocytosis of zymosan (85) |
| PDX | ↑ PPARγ (91,92) | ↑ M2 polarization (91) |
| | ↓ NF-κB (92) | ↑ Phagocytosis of fluorescent beads (91) |
| | | ↓ Pro-inflammatory cytokine secretion (91,92) |
| | | ↓ Neutrophil chemotaxis (91,92) |

## SPMs enhance macrophage-mediated efferocytosis and phagocytosis

SPMs induce pro-resolving macrophage polarization by enhancing their ability to clear apoptotic cells (efferocytosis). For example, RvD1 and RvE1 stimulation improves efferocytosis of neutrophils by murine macrophages following reperfusion (75). Similarly, RvE1 and PD1 enhance efferocytosis of apoptotic neutrophils by macrophages during acute inflammation (85). In a murine model of severe aplastic anemia – a condition marked by persistent inflammation – intraperitoneal injection of RvE1 promotes macrophage-mediated efferocytosis by downregulating the inhibitory receptor signal regulatory protein alpha (86). Additionally, MaR1 stimulates efferocytosis of apoptotic neutrophils by human macrophages through activation of leucine-rich repeat-containing G-protein-coupled receptor 6 (LGR6) (90). This enhanced efferocytosis by SPMs influences macrophage polarization. Specifically, efferocytosis induces myc-dependent proliferation of non-inflammatory macrophages. These macrophages display a pro-resolving phenotype by enhanced IL-10 and TGF-β production and promote plaque regression in an atherosclerotic mouse model (93). Additionally, SPM-induced efferocytosis reduces pro-inflammatory cytokine secretion (85). This shift in cytokine profile further enhances the efferocytotic capacity of macrophages, creating a positive feedback loop, which is called continual efferocytosis.

In addition to enhancing efferocytosis, SPMs also improve the phagocytic capacity of macrophages. For example, RvD1 and RvE1 enhance the phagocytosis of microbial particles by LPS-stimulated human macrophages (80,82). MaR1 also stimulates phagocytosis of *E. coli* through LGR6 activation (90). Furthermore, RvE1 and PD1 increase the phagocytic capacity of murine macrophages against zymosan, an inflammatory compound that activates TLR2 and TLR6 (85). For PDX, an isomer of PD1, a study shows enhanced phagocytosis of fluorescent beads by murine macrophages (91). In summary, SPMs indirectly polarize macrophages to a pro-resolving state by promoting efferocytosis of apoptotic neutrophils and phagocytosis of microbial particles.

## SPMs promote pro-resolving chemokine and cytokine secretion by macrophages

SPMs also promote a pro-resolving macrophage phenotype by modulating macrophage chemokine and cytokine production, shifting the immune response from a pro-inflammatory to a pro-resolving phase. For example, RvD1 impairs neutrophil chemotaxis by downregulating expression of the chemoattractants chemokine (C-X-C motif) ligand (CXCL)1 and CXCL2 in murine macrophages after reperfusion (75). Similarly, RvD1 and MaR1 inhibit the secretion of CXCL8 in LPS-stimulated macrophages, further limiting recruitment of inflammatory immune cells (79,80). In an LPS-induced heart injury mouse model, intraperitoneal injection of RvE1 reduces neutrophil infiltration into the heart, improving cardiac function (83). Similarly, intraperitoneal injection of PDX limits neutrophil

infiltration into the peritoneum in a septic mouse model (91). RvD1 and RvE1 also impair the chemotactic migration of LPS-stimulated macrophages themselves, presumably reducing the recruitment of additional pro-inflammatory macrophages to the site of inflammation (80,82).

Additionally, SPMs modulate the balance of pro-inflammatory and anti-inflammatory cytokine production. For example, RvD1 and MaR1 reduce secretion of the key pro-inflammatory cytokines IL-1β and TNF, both *in vitro* and *in vivo* (76,79,80,87–89). Similarly, RvE1 and PDX lower the expression and secretion of IL-1β, IL-6, and TNF in inflamed tissues of the colon, heart, and lung (83,84,91). Conversely, RvD1 and MaR1 enhance the secretion of the anti-inflammatory cytokine IL-10 in LPS-stimulated human macrophages (79). The effect of PD1 on cytokine expression of macrophages should be investigated further since current data is inconclusive. Thus, SPM-stimulated macrophages exhibit reduced neutrophil infiltration and display a less pro-inflammatory cytokine repertoire, contributing to a more pro-resolving immune environment.

## SPMs activate pro-resolving signaling cascades and induce macrophage polarization

SPMs promote a pro-resolving macrophage phenotype by activating pro-resolving signaling cascades in macrophages. For example, LPS-stimulated human macrophages treated with RvD1 or MaR1 upregulate cAMP-response element binding protein (CREB) signaling, which enhances the secretion of the anti-inflammatory cytokine IL-10 (79). Additionally, RvD1, RvE1, and MaR1 activate the pro-resolving TF nuclear factor erythroid 2-related factor 2 (Nrf2) in murine macrophages and inflamed tissues (78,81,88,89). Nrf2 reduces inflammatory signaling and activates expression of antioxidant proteins by binding proximal to these genes in macrophages (94). Consequently, MaR1 inhibits inflammasome activation and proptosis of murine macrophages through Nrf2, preventing additional liver injury, breaking the cycle of inflammation (88). Moreover, RvD1, MaR1, and PDX stimulate PPARγ expression in murine macrophages from various tissues (74,87,91). Subsequently, PPARγ enhances IL-10 and TGF-β signaling, while reducing IL-6 and TNF secretion, underscoring PPARγ as a key TF in SPM-induced resolution (87).

SPMs also inhibit pro-inflammatory signaling pathways to promote resolution in macrophages. For example, RvD1, RvE1, MaR1, and PDX prevent the nuclear translocation of NF-κB, reducing the expression of pro-inflammatory mediators (79,84,92). Additionally, SPMs suppress mitogen-activated protein kinase (MAPK) signaling, which is associated with pro-inflammatory macrophage polarization by enhancing inflammasome activation and the secretion of pro-inflammatory cytokines and NO (95). Specifically, RvE1 and MaR1 inhibit MAPK activation *in vitro* in murine macrophages and *in vivo* in myocardial tissue, respectively, upon LPS stimulation (83,88). Additionally, intraperitoneal injection of RvD1 dose-dependently reduces MAPK signaling in a murine steatohepatitis model (78). Subsequently,

this upregulation of pro-resolving signaling and inhibition of pro-inflammatory signaling influences macrophage polarization. RvD1, RvE1, MaR1, and PDX namely increase expression of the pro-resolving macrophage markers *Arg1*, *Ym1*, and the pro-resolving marker *Cd206*, while inhibiting expression of *Nos2* and costimulatory markers (75,83,88,91). Consequently, intramucosal injections of RvE1-containing nanoparticles stimulate intestinal epithelial would repair by inducing a more pro-resolving macrophage phenotype (96).

In conclusion, SPMs play an important role in regulating macrophage phenotype. By enhancing efferocytosis and phagocytosis, modulating chemokine and cytokine production, and activating pro-resolving signaling pathways, SPMs promote the transition of macrophages from a pro-inflammatory to a pro-resolving phase, aiding in the process of inflammation resolution.

# Chapter 6: Therapeutic options to treat inflammatory diseases

## Current treatment options

The current treatments for inflammatory diseases are mostly based on suppressing the inflammatory response and can be roughly divided into three categories: general immune suppressants, biologicals, and small-molecule inhibitors. General immune suppressants, such as methotrexate and the corticosteroid prednisone were developed at the end of the twentieth century but are often still used as first-line treatments in inflammatory diseases. Treatment with these mediators is successful, due to their anti-proliferative and gene modulatory properties (97,98). Since these drugs have such widespread effects on proliferation, metabolism, and immune cell function, treatments are accompanied by many side effects including stomatitis, osteoporosis, and increased susceptibility to infections.

With more understanding of disease mechanisms and the identification of causative inflammatory pathways, biologicals and small-molecule inhibitors were developed to target cytokines, their receptors, and intracellular signaling molecules. The first approved antibody-based therapy targeting cytokines is infliximab, which targets TNF in rheumatoid arthritis (99). Thereafter, antibodies targeting other cytokines and integrins, such as ustekinumab and vedolizumab, were developed to prevent inflammatory signaling and migration of immune cells. Although these cytokine-targeting therapies narrowed the range of side effects, cytokines are often redundant and still activate a wide variety of signaling pathways. To circumvent these restrictions, small-molecule inhibitors were developed against specific proteins in inflammatory pathways of which JAK inhibitors are used the most in current practice. JAK inhibitors are approved for the treatment of many inflammatory diseases and each inhibitor preferentially blocks a singular or multiple JAKs, which provides a wide range of treatment options for different inflammatory diseases and heterogenic patient populations (100). However, these JAK inhibitors still come with gastrointestinal side effects, cytopenias and often have a black box warning for malignancy and serious infections.

In summary, we have come a long way in narrowing the target specificity to treat inflammatory diseases from general immune suppression to targeting specific JAKs. Although these treatments have been proven to be very effective, they are accompanied by many side effects, which asks for alternative treatment approaches.

## Future treatment strategy: stimulate resolving processes

One such promising treatment approach for inflammatory diseases involves not only suppressing inflammatory signaling but also actively promoting resolution processes. Rather than solely aiming to inhibit inflammation, which is the current procedure, this approach focusses on enhancing pro-resolving pathways, preferably in macrophages.

A natural way to stimulate resolution is through a diet rich in SPMs. These SPM-based treatments hold potential to improve quality of life for patients with chronic pain and inflammation. For example, oral supplementation of SPM-enriched marine oil emulsion increases detection of SPMs in plasma and serum in healthy individuals. (101). While inflammation biomarkers in the blood remain unchanged, there is some evidence that the emulsion reduces pain in patients suffering from chronic pain (102). Additionally, synovial and plasma SPM levels negatively correlate with the erythrocyte sedimentation rate – a common marker for inflammation – and synovial RvE2 levels inversely correlate with pain in arthritis patients (103). Moreover, supplementation of EPA and DHA reduces *ex vivo* expression of pro-inflammatory cytokines, like TNF, in monocytes of patients with chronic inflammation (104). To establish the full therapeutic potential of SPMs, future studies should prove the causal effect of SPMs on reducing inflammation in patients with inflammatory diseases and explore the long-term outcomes of direct and indirect SPM supplementation. Notably, macrophages respond dose-dependently to SPMs, indicating that a sufficient SPM concentration is required for proper conversion of macrophages from a pro-inflammatory to pro-resolving state (79). This observation highlights the importance of identifying an ideal dosage for oral supplementation to maximize the therapeutic benefits of SPM-based treatments.

Another potential approach to promote resolution is to enhance the expression of pro-resolving TFs. For example, the pro-inflammatory effect of DNA methyltransferase 1 (DNMT1) depends on its ability to methylate DNA at promotor regions, thereby suppressing the expression of KLF4 (105). Inhibition of DNMT1 reduces ROS and NO production, as well as *Nos2* expression in macrophages, suppressing their pro-inflammatory phenotype. Additionally, myeloid deficiency of DNMT1 reduces the inflammatory response in plaques and slows the progression of atherosclerosis, suggesting that methyltransferase inhibitors could be effective in resolving inflammation by enhancing the expression of pro-resolving TFs (105).

To effectively promote the anti-inflammatory capabilities of macrophages that already express pro-resolving TFs, like Nur77 and PPARγ, targeted strategies are needed to enhance the activity of these TFs. As discussed before, Nur77 activation effectively reduces inflammation through several mechanisms. For example, the Nur77 agonist cytosporone B inhibits NF-κB nuclear translocation by

preventing IκB degradation, decreasing expression of pro-inflammatory cytokines (106). Nur77 also directly interacts with NF-κB, blocking its DNA-binding capacity and consequently reducing the transcription of inflammatory genes. However, this inhibitory effect is hampered by the LPS-induced expression of MAPK p38. p38 namely phosphorylates Nur77, preventing its binding and blocking effect on NF-κB. To counter this effect, the compound *n*-pentyl 2-[3,5-dihydroxy-2-(1-nonanoyl) phenyl]acetate competes with p38 for binding to Nur77, which effectively restores the inhibitory effect of Nur77 on NF-κB (53). In LPS-induced peritonitis, PPARγ is also expressed, but not involved in pro-resolving activities due to the lack of an activating ligand. The PPARγ agonist rosiglitazone stimulates PPARγ activity, which in turn attenuates inflammation by inhibiting NF-κB activation, presumably by the improved capacity of PPARγ to inhibit NF-κB via transrepression (30,107). Thus, enhancing the activity of Nur77 and PPARγ is a viable strategy to stimulate pro-resolving and suppress pro-inflammatory processes, which should be further explored for modulating the macrophage phenotype towards a more resolving state.

Additionally, researchers identified many compounds that promote the conversion of IFN-γ/TNF-stimulated human primary macrophages towards a pro-resolving phenotype using a phenotypic screen (108). These compounds target a variety of biological processes, such as signaling, proliferation, and metabolic processes and should be individually investigated for their application in the treatment of inflammatory diseases.

In conclusion, promoting resolution processes in macrophages offers a promising approach to treat chronic inflammatory diseases. Strategies such as SPM-rich diets and enhancing the expression and activation of resolving TFs can help shift macrophages to a pro-resolving state (Figure 3). Future research should refine these interventions and identify additional compounds to maximize the therapeutic potential and long-term benefits in treating patients with inflammatory diseases.

## Nanoparticles as carriers of macrophage modulating compounds

Macrophages play a dual role in maintaining homeostasis and contributing to autoimmune and inflammatory diseases, making them a prime therapeutic target for suppressing inflammation and promoting resolution (109). As described above, there are many natural and chemical compounds aiding in this process. However, the compounds still have to reach the macrophages at a high enough concentration to be effective. Therefore, many studies tackle the question of how to target macrophages specifically, which will be discussed below.

Nanoparticles (NPs) are the most investigated route for delivery to macrophages. These NPs attract macrophages through various coatings, enabling macrophage uptake and release of the therapeutic

agents inside the cell (Figure 3). Some NPs target inflammatory macrophages specifically to repolarize them to a resolving phenotype, while other NPs prevent the recruitment of additional inflammatory macrophages. For example, folic acid- and hyaluronic acid-coated NPs bind the folate receptor and CD44 on inflammatory macrophages, delivering immunomodulatory agents which reduce expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF) and prevent progression of inflammation in rheumatoid arthritis mouse models (110,111). Alternatively, IgGγ-opsonized NPs utilize Fc receptors on inflammatory macrophages to promote Fc receptor-mediated endocytosis of the NP to deliver the anti-inflammatory compound (112).

To minimize off-target effects and maximize efficacy, NP are engineered to increase their concentration at inflamed sites (Figure 3). For example, ROS-sensitive NPs use arylboronic ester bonds as structural component, which release their content in high oxidative, inflammatory environments. These NPs suppress the secretion of pro-inflammatory cytokines (IL-1β, IL-6, and TNF) and reduce the plaque area in atherosclerotic mice (113). Similarly, pH-responsive coatings, such as polyethylene glycol, shed from the NP in more acidic, inflammatory environments, revealing its macrophage-attracting coat. Using this technique, NPs present phosphatidylserine at the inflamed site to mimic apoptotic bodies, which induces resolving macrophage polarization through enhancing efferocytosis via phosphatidylserine receptors, such as TIM-4 (114). Furthermore, clustering of the mannose receptor by NPs coated with a carbohydrate-presenting substrate induces a pro-resolving macrophage phenotype in bone marrow-derived macrophages and improves overall survival in an inflammatory bowel disease mouse model (115). Thus, many NPs are currently developed to target macrophages of different phenotypes at the inflamed site and are therapeutically effective in inflammatory disease models.

NPs also enable targeted delivery of nucleic acid-based treatments, facilitating polarization towards pro-resolving macrophages (Figure 3). For example, IL-10 plasmid-loaded NPs decrease *Nos2* and *Il12b*, but increase *Arg1*, *Cd206* and *Il10* mRNA levels in LPS-stimulated macrophages, reducing inflammation in arthritis and peritoneal inflammatory mouse models (116,117). Additionally, NPs with microRNA-223, which suppresses NF-κB and inflammatory STAT signaling, repolarize macrophages by decreasing pro-inflammatory cytokine secretion of peritoneal macrophages (118,119). Alternatively, small interfering RNAs (siRNAs) are packed into NPs to more specifically target pro-inflammatory pathways. For example, NPs containing siRNAs against TNF downregulate the level of TNF, increasing the survival of LPS-challenged mice (120). Additionally, siRNA targeting $Ca^{2+}$/calmodulin-dependent protein kinase γ – a necrosis driver in atherosclerosis – decreases the necrotic area, while improving the efferocytosis capacity of macrophages in an atherosclerotic mouse model (121). These innovations highlight the role of NPs in delivering nucleic acid-based therapies for chronic inflammatory diseases by specifically targeting macrophages to promote resolution.

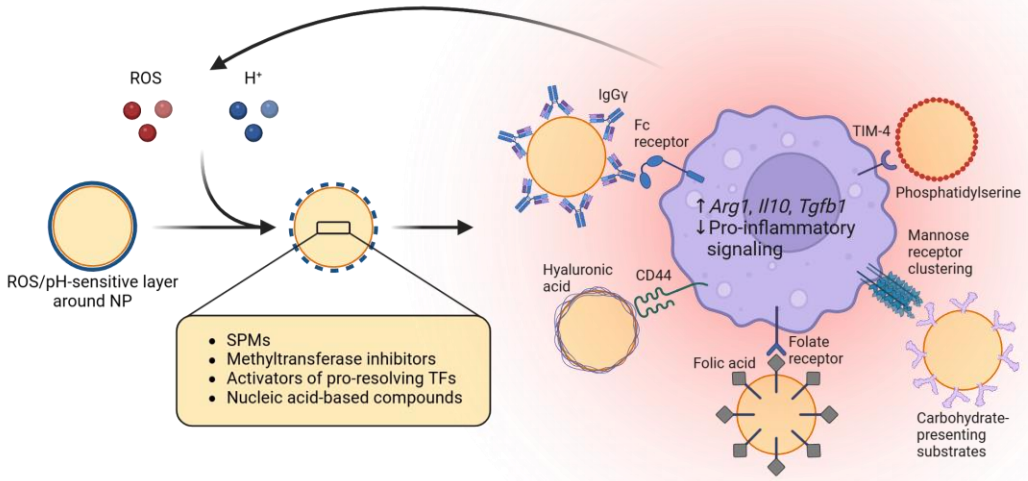


**Figure 3: NPs as carriers for delivering pro-resolving factors to pro-inflammatory macrophages at inflamed sites.**

In inflamed environments with elevates ROS and $H^+$ levels, the ROS/pH-sensitive outer layer of the NPs degrades, exposing their mechanisms to enhance macrophage targeting. For example, NPs are coated with IgGγ, hyaluronic acid, folic acid, carbohydrate-presenting substrates, or phosphatidylserine which are recognized by macrophages. Upon uptake by macrophages, the immunomodulatory/pro-resolving content of the NPs reduces pro-inflammatory signaling and increases pro-resolving markers, shifting the macrophages towards a more pro-resolving phenotype.

Abbreviations: ROS, reactive oxygen species; NP, nanoparticle; SPM, specialized pro-resolving mediator; TF, transcription factor; TIM-4, T cell immunoglobulin mucin receptor 4.

# Chapter 7: Concluding remarks and future perspectives

Macrophages play a pivotal role in maintaining immune homeostasis by their involvement in both pro-inflammatory and pro-resolving processes. We here discussed how regulators drive macrophages towards a pro-resolving phenotype. Activating pro-resolving TFs, such as STAT6, PPARγ, KLF4, Nur77, and Nrf2, while suppressing pro-inflammatory TFs, like NF-κB and STAT1, in macrophages remains key in achieving resolution of inflammation. The immune environment, SPMs, and negative regulation of pro-inflammatory pathways are instrumental in initiating these pro-resolving pathways. Through positive feedback loops, activation of additional pro-resolving TFs, and active repression of pro-inflammatory TFs, macrophages undergo a phenotypic shift, underscoring resolution as an actively regulated process rather than a passive outcome.

Expanding our knowledge on how to actively promote pro-resolving signaling in macrophages – beyond simply suppressing inflammatory responses – is critical for the development of future therapies for chronic inflammatory diseases. To achieve this, we need a deeper understanding of pro-resolving signaling pathways in general and where to intervene in the pathway during the different stages of inflammation. Researchers should also consider that various inflammatory diseases, and even individuals with the same disease, may require activation of different pro-resolving pathways, due to differences in disease etiology, genetic variation, environmental factors, and disease heterogeneity. Activating these pathways restores immune balance by breaking the cycle of inflammation in chronic inflammatory diseases. Moreover, identifying pro-resolving compounds and developing delivery methods to specifically target macrophages are critical to restore immune homeostasis and improve outcomes of patients with inflammatory diseases.

## Declaration of generative AI in the writing process

During the writing process the author used ChatGPT-4 (OpenAI) to enhance the quality of writing by improving sentence and paragraph structure. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the review.

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