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Macrophages and the Recovery from Acute and Chronic Inflammation

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Abstract

In recent years, researchers have devoted much attention to the diverse roles of macrophages and their contributions to tissue development, wound healing, and angiogenesis. What should not be lost in the discussions regarding the diverse biology of these cells is that when perturbed, macrophages are the primary contributors to potentially pathological inflammatory processes. Macrophages stand poised to rapidly produce large amounts of inflammatory cytokines in response to danger signals. The production of these cytokines can initiate a cascade of inflammatory mediator release that can lead to wholesale tissue destruction. The destructive inflammatory capability of macrophages is amplified by exposure to exogenous interferon-γ, which prolongs and heightens inflammatory responses. In simple terms, macrophages can thus be viewed as incendiary devices with hair triggers waiting to detonate. We have begun to ask questions about how these cells can be regulated to mitigate the collateral destruction associated with macrophage activation.

Keywords

cytokines; transcription; prostaglandin; adenosine; TLR; NF-κB

MACROPHAGES CONTRIBUTE TO INFLAMMATION

Macrophages are major producers of inflammatory mediators during autoimmune and autoinflammatory diseases. The kinase-dependent signaling pathways that emanate from ligated pattern recognition receptors on macrophages are well described. These pathways activate latent transcription factors, including NF-κB, CREB, AP-1, and interferon-regulatory factors (IRFs), to initiate cytokine gene transcription. However, recent studies suggest that the pattern of cytokine production by macrophages in response to inflammatory stimuli is more complex than simply the result of the activation of transcription factors (1). Macrophages are poised to rapidly respond to pathogen-associated molecular patterns (PAMPs) with the production of tumor necrosis factor (TNF) because RNA polymerase II is

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already positioned on the TNF gene in the basal state, capable of initiating transcription (2). Furthermore, the TNF promoter has relatively low histone occupancy, and the histones that are associated with the TNF promoter contain the activation marks H3K4me and H3K9Ac (3). Thus, signal-dependent transcription factor activation following receptor ligation, in concert with the positioning and covalent modification of nucleosomes adjacent to the TNF promoter, contributes to the rapid stimulus-dependent transcription of this cytokine gene.

In macrophages, the production of TNF usually precedes and generally promotes the carefully orchestrated release of many other inflammatory mediators, including interleukin (IL)-6, IL-12/23(p40), and the type I interferons. Chemokines, lipid mediators, and antimicrobial peptides are similarly released from stimulated macrophages. Many cells can change their primary transcriptional program in response to danger signals, such as lipopolysaccharide (LPS), but it is the production of these subsequent inflammatory mediators downstream from TNF that makes the macrophage such an important contributor to the inflammatory response. Macrophages are uniquely adept at producing a cascade of inflammatory cytokines and mediators in response to LPS exposure. This is largely due to the presence of lineage-specific transcription factors, such as PU.1 and C/EBP, which decorate enhancer regions upstream from cytokine genes and are thought to hold chromatin in an open configuration, allowing stimulus-dependent transcription factors to bind and promote gene expression (4). Thus, lineage-specific enhancers upstream from many inflammatory genes confer macrophage-specific responsiveness to inflammatory stimuli, and they define the anatomical specificity of macrophage responses in different tissues (5). The second wave of genes produced in response to stimulation usually also depends on enhancer activation, chromatin remodeling, and nucleosome repositioning to allow new transcription factor access. These processes can take time; therefore, one typically sees distinct temporal waves of gene expression following stimulation, sometimes designated as primary and secondary genes (2). Thus, cooperation between lineage-specific and stimulus-activated transcription factors, along with the repositioning of nucleosomes and covalent modifications to histones, determines how a given macrophage population will respond to inflammatory stimuli. Consequently, the chromatin in macrophages residing in the liver and the lung will be differentially “marked,” and therefore these two cells will not produce identical responses to a given stimuli.

INTERFERON-γ, A MAJOR MEDIATOR OF MACROPHAGE INFLAMMATION

Virtually all of these inflammatory responses are accentuated by the presence of interferon-γ (IFN-γ), which dramatically increases the production of inflammatory mediators by macrophages. This increase in cytokine production by IFN-γ-primed macrophages is due primarily to the activation of STAT1. The binding of interferons to their cognate receptors triggers tyrosine phosphorylation of STAT1, its localization to the nucleus, and DNA binding, leading to the transcription of at least 200 genes (6). Among these are mostly inflammation-associated genes, such as GM-CSF, IL-12p40, TNF-α, and IL-6. STAT1 binding increases macrophage inflammatory responses by many diverse mechanisms. It induces covalent modifications to histones to assume an active conformation, allowing the assembly of transcription complexes on inflammatory cytokine genes (7). IFN-γ priming can synergize with Toll-like receptor (TLR) signaling to enhance NF-κB-dependent gene

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expression and increase the stability of mRNAs encoding inflammatory mediators (8). Recent work indicates that IFN-γ treatment of macrophages also results in a more efficient translation of inflammatory mediators (9). Finally, IFN-γ-mediated STAT1 signaling potentiates inflammatory signaling by inhibiting IL-10 secretion. IFN-γ suppresses many of the key molecules required for IL-10 secretion, such as SOCS3, the TLR2-induced activation of the PI3K-Akt pathway (10), and the transcription factors CREB and AP-1 through a glycogen synthase kinase 3 (GSK3)-dependent mechanism (11).

IFN/STAT1 signaling can enhance the pathogenesis of septic shock (12), mediate pouchitis in inflammatory bowel diseases (13), and increase auto-antibody production in systemic lupus erythematosus (14). Recently, dominant gain of function *STAT1* (GOF-S1) mutations in humans, characterized by the hyperphosphorylation of STAT1, were associated with an autoimmune phenotype with IPEX-like syndrome and a heightened susceptibility to fungal pathogens (15).

The increased production of TNF-α, IL-6, and IL-12 by macrophages following IFN-γ exposure is associated with an enhanced capacity for macrophages to kill intracellular pathogens and the development of long-lasting cellular immunity. IFN-γ is thus a hallmark cytokine of Th1 immune responses, and individuals with deficiencies in IFN-γ signaling are susceptible to a variety of intracellular infections (16). However, macrophages with interferon or STAT1 signatures were observed in a number of autoimmune diseases where they are the primary contributors to immunopathology. In rheumatoid arthritis, synovial macrophages express numerous IFN-γ inducible genes (17) and exhibit evidence of STAT1 activation. Increased levels of IFN-γ were also detected in the skin of lupus patients (18). Similarly, IFN-γ production by Th1 cells contributes to neurodegeneration in a murine model of Alzheimer's disease (19). In psoriasis, a Th17 disease, serum IFN-γ levels are elevated and correlate with disease severity (20). In multiple sclerosis (MS), another Th17 disease, the role of classically activated macrophages and IFN-γ production appears to be somewhat more complex and can vary depending on the disease phase (21). Early studies identified IFN-γ-producing Th17 cells in lesions (22), and in progressive MS, patients typically have higher serum levels of IFN-γ and TNF (23). However, in the murine model of MS, called experimental autoimmune encephalomyelitis (EAE), mice lacking IFN-γ are more susceptible to severe EAE, and the administration of antibodies to IFN-γ actually exacerbates disease (21). One explanation for this is that IFN-γ production by astrocytes rather than microglia may preserve neuronal integrity (24), but the other obvious explanation is that IFN-γ may be protective by virtue of its well-described inhibition of (pathological) Th17 responses. Regardless of the mechanism of induction, activated macrophages are key contributors to autoimmune pathology in all of the aforementioned diseases.

Fortunately, several types of molecular controls work to downregulate the inflammatory responses of activated macrophages. These include inhibitors of signaling components associated with pattern recognition receptors, the activation of transcription factors that can repress gene expression, epigenetic silencing of gene expression, and the production of miRNAs to diminish inflammatory responses. These regulatory controls are the focus of this review.

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NEGATIVE REGULATION OF TOLL-LIKE RECEPTOR SIGNALING

A variety of regulatory mechanisms have evolved to protect the immune system from aberrant TLR signaling. Most of these inhibitory proteins are inducible, suggesting a negative feedback on TLR signaling pathways. Some of these known negative regulators with their mechanism of action and their relevance to disease are listed in Table 1. The negative regulation of TLR responses is particularly important in the gut where it prevents unwanted immune responses to the normal flora. Because sustained signaling of TLRs can result in autoimmune and chronic inflammatory diseases, the tight regulation of the TLR signaling pathway is crucial not only to suspend the pathway when danger is removed but also to maintain homeostasis in those anatomical areas of constant exposure to normal flora. Negative regulation of TLR signaling can happen at any step in TLR signaling, from surface sensing of ligands to the transcription of the downstream effector molecules.

Regulators of Ligand Binding

Members of this class either prevent the binding of ligand to membrane TLRs or displace them when bound. This family includes the soluble TLR receptors (sTLRs) sTLR2 and sTLR4 that act as decoy receptors, sCD14 that binds directly to LPS, and radioprotective 105 (RP105), a TLR homolog that prevents TLR4 from binding to LPS. All are negative regulators of TLR signaling. The binding of soluble forms of TLRs to their free ligands in plasma prevents ligand binding to surface TLRs. The high levels of sTLRs in human breast milk to prevent colitis in infants represent one of the best examples of this type of inhibition. The soluble form of coreceptor sCD14 binds to and diverts LPS from membrane-bound CD14. sCD14 also promotes LPS efflux from cell-surface CD14, transferring it to plasma lipoproteins. The role of RP105 may be cell type specific, but in myeloid cells, RP105/MD-1 interacts directly with TLR-4/MD-2 to inhibit the ability of this signaling complex to bind LPS (25). Triad3A, a RING finger protein, acts as an E3 ubiquitin-protein ligase and enhances ubiquitination and proteolytic degradation of TLR4 and TLR9. Thus, it renders receptors inaccessible to their ligands (Figure 1).

Regulators of Adaptor Complexes

TLR signaling includes five adaptors known as MyD88, MAL (TIRAP), TRIF, TRAM, and SARM, all of which contain a Toll/Il-1 receptor (TIR) domain (26). Both MyD88 and TRIF bind directly to TLRs, whereas MAL (adaptor-like MyD88) and TRAM act as recruiting adaptors to bring MyD88 and TRIF to TLRs, respectively. MyD88-mediated signaling results in NF-κB activation, whereas TRIF-mediated signaling results in IRF activation. More inhibitory mechanisms exist for the MAL-MyD88 adaptor complex compared to the TRIF-TRAM complex, as the former is utilized by almost all TLRs (except TLR3), and the latter complex is utilized only by TLR3 and TLR4. Inhibitors to these adaptors include (*a*) negatively regulating adaptors, (*b*) variants of the adaptor proteins, (*c*) proteins that directly bind to and prevent adaptor recruitment or functions, and (*d*) proteins that target adaptor proteins for degradation. SARM is the only negative TIR domain containing an adaptor that negatively regulates TRIF-dependent TLR signaling and MyD88-mediated AP-1 activation. The presence of this adaptor is implicated in endotoxin tolerance. The splice variants of MyD88, termed MyD88s, and of TRAM, known as TAG (TRAM adaptor with GOLD

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domain), are two variants of the adaptor complexes that negatively regulate the two signaling arms of TLR (27, 28). Proteins such as ST2L, TRAF1, and TRAF4 directly bind to the adaptor molecules and negatively regulate their functions. Whereas ST2 prevents the recruitment of MyD88 to TIRAP, TRAFs prevent the association of TRIFs to TLRs. Cbl-b ubiquitin ligase targets adaptors of both pathways via MyD88 and TRIF for ubiquitination, thereby negatively regulating them. Although SOCS1 does not directly mediate ubiquitination, binding of MAL to SOCS1 triggers its polyubiquitination and degradation.

Regulators of Signaling Molecules

This class of molecules inhibits TLR signaling pathway components that lie between the adaptors and the transcription factors. These include IRAKs, TRAF3, TRAF6, TBK1, and TAB2/3. Of these molecules, TRAF6 is of primary importance, as almost all TLR pathways converge at TRAF6 for subsequent signaling. Most of the negative regulation at this level occurs by targeting TRAF6 for ubiquitination/deubiquitination, resulting in varying mechanisms of ubiquitination-dependent inhibition. Interaction of TRAF6 with β-arrestin 2 prevents its oligomerization, autoubiquitination, and subsequent activation of NF-κB and AP-1 (29). In contrast, deubiquitination of TRAF6 by A20 prevents its binding to TAK1 and subsequent formation of the Iκκ complex (30). TRIM38, a tripartite motif-containing molecule, promotes TRAF6 ubiquitination by directly binding to it and targeting it for proteasomal degradation. The observations that multiple negative regulators exist for TRAF6 indicate the essential and central role of this protein in TLR signaling. The inhibition of IRAK kinases can occur through the competitive binding of the splice variants IRAK1c, IRAK2c, IRAK2d, and IRAKM, which competitively bind to adaptor molecules and inhibit IRAK signaling. The Src homology 2 domain-containing proteins tyrosine phosphatase-1 (SHP-1) and phosphatase-2 (SHP-2) suppress the IRAK1 and IRAK2 activity.

Regulators of Transcription

Sensing of PAMPs and danger-associated molecular patterns by the TLR family leads to a cascade of events, most of which converge at transcription factors NF-κB and IRF3/7. The NF-κB family of transcription factors is central to the macrophage inflammatory response. This family of transcription factors is a target for regulation owing to the large number of genes it transcribes. The negative regulation strategies include preventing the binding of NF-κB to target genes, regulating the assembly of its members and inhibiting their transcription, and averting post-translational modifications and protein degradation (31).

B cell lymphoma 6 (Bcl-6) is an important negative regulator of NF-κB signaling. Nearly one-third of the LPS-induced genes are regulated by Bcl-6 (32). This transcriptional repressor also binds to IRF7 loci to restrain antiviral responses. The transcription of key inflammatory chemokine genes in macrophages is also inhibited by Bcl-6 (33). Consequently, Bcl-6-null mice have a lethal inflammatory phenotype. The activation of peroxisome proliferator-activated receptor delta (PPARδ) results in increased macrophage Bcl-6 levels to decrease their inflammatory potential.

In LPS signaling, SOCS1 was shown to decrease NF-κB signaling through the ubiquitination complex known as ECS-SOCS1. This complex, which is triggered by

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COMMD1, is composed of multimeric ubiquitin ligases, containing elongins B and C, Cul2, and SOCS. Through this complex, COMMD1 accelerates the ubiquitination and degradation of NF-κB subunits (34). Activating transcription factor 3 (ATF3) is a type I IFN-inducible protein that recruits histone deacetylases to alter chromatin structure and restrict access to NF-κB and AP-1 to the promoter regions of proinflammatory cytokines (35).

Inhibition of IRF functions in TLR signaling can occur through ubiquitination-dependent IRF degradation or via negatively regulating IRFs. Stimulation of TLRs with dsRNA can lead to Ser339-Pro340 phosphorylation of IRF3. Peptidyl prolyl isomerase 1 interacts with this phosphorylated form of IRF3 and polyubiquitinates the transcription factor, targeting it for proteasome degradation. Negatively regulating IRFs include IRF4 that competitively inhibits IRF5 from binding to TLR, thereby inhibiting inflammatory responses.

OTHER TRANSCRIPTIONAL REGULATORS

Among the transcription factors that are associated with TLR signaling, the STATs play a particularly important role. Whereas STAT1, STAT2, STAT4, and STAT5 signal and mediate proinflammatory responses, STAT3 and STAT6 transcribe anti-inflammatory genes. The best studied is STAT3, which is a key transcriptional regulator of IL-10, a major anti-inflammatory cytokine. This transcription factor can uniquely induce both anti-inflammatory and proinflammatory responses within the same cell. When signaled via IL-10, it induces anti-inflammatory responses, and when stimulated by IL-6, it drives inflammation. In macrophages, STAT3 combines with a specific set of transcription factors such as PU.1, CEBPA, and CEBPB, which are prebound to sites of future STAT3 recruitment (36), thus mediating anti-inflammation. The reciprocal cross talk between STAT3 and the p38 mitogen-activated protein kinase (MAPK) pathway is critical in macrophages to balance the induction, propagation, and resolution of LPS signaling. The IL-10 production that occurs later during TLR signaling triggers STAT3 phosphorylation and activation. STAT3 then drives the expression of DUSP1, which is a negative regulator of the p38 MAPK, thus closing the feedback loop (37). The importance of STAT3 in anti-inflammation is evident from the clinical manifestations associated with STAT3 mutations in humans. Loss-of-function STAT3 in humans results in hyper–immunoglobulin E (IgE) syndrome, which is characterized by both primary immunodeficiency and a severe autoimmune phenotype with high proinflammatory gene transcripts (38).

The binding of cytokines IL-4 and IL-13 to their receptors triggers STAT6 phosphorylation, leading to the alternate activation of macrophages. Phosphorylated STAT6 translocates to the nucleus and transcribes several genes required for alternate activation of macrophages, such as resistin-like α (Retnlα, Fizz1) and chitinase 3-like 3 (*Chi3l3, Ym1*), peroxisome proliferator-activated receptor γ (PPARγ), mannose receptor (*Mrc1*) (39), and genes that are involved in β oxidation of fatty acids. STAT6 also synergizes with Krüppel-like factor 4 and PPARγ to inhibit the inflammatory genes TNF-α, Cox-2, CCL5, and iNOS (40). In AA-Mφ, IL-4 induces the immune inhibitory ligand and the programmed death ligand 2 via STAT6, the surface expression of which leads to the inhibition of Th2 proliferation (41). Numerous studies have demonstrated the important protective role of AA-Mφ and thus STAT6 signaling in host immunity to helminthic infection and in wound healing (42).

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microRNA REGULATION OF MACROPHAGES IN INFLAMMATION AND AUTOIMMUNITY

The levels of several TLR signaling molecules are regulated at the transcriptional level by noncoding RNAs, such as microRNA (miRNA). miRNAs are small, approximately 20–24-nucleotide-long RNAs that generally bind to 3 untranslated regions of mRNAs. They have emerged as critical regulators of macrophage functions and polarization. Studies have profiled miRNA expression in polarized macrophages to better understand their biological function. In general, miRNA profiling shows significant differences between human and mouse macrophages. For example, stimulation of murine bone marrow derived macrophages with IFN-γ+LPS upregulates miR-127-3p, miR-155-5p, miR-181a, miR-204-5p, and miR-451, whereas in human macrophages, miR-27a, miR-29b, miR-125a, miR-146a, and miR-155 are upregulated. Similarly, AA-Mϕ of mouse upregulate miR-125-5p, miR-143-3p, miR-145-5p, and miR-146a-3p, whereas human AA-Mϕ upregulate a different set of miRNAs, including miR-26a and miR-193b (43, 44).

Classification of miRNAs as pro- and anti-inflammatory is difficult owing to wide heterogeneity in miRNA functions even among members of the same family. Whereas some miRNAs, such as miR155, have a clear-cut proinflammatory role, others have dual roles depending on the cell type, stimulus, and experimental conditions. Nevertheless, considerable evidence demonstrates the important roles for some of these molecules in regulating macrophage inflammatory activity.

miR-124 is associated with decreased macrophage inflammation. The anti-inflammatory role of this miRNA was first demonstrated in microglial cells during EAE (45). In this model, the overexpression of miR-124 led to direct inhibition of the transcription factor CCAAT/enhancer-binding protein-α and its downstream target PU.1, resulting in the deactivation of microglial cells. Furthermore, treatment with miR-124 reduced the severity of EAE and the percentage of activated macrophages. The same group studied the role of miR-124 in allergy and asthma, demonstrating that IL-4/IL-13 induced the expression of miR-124 in human macrophages, which led to increased allergic inflammation. In human patients with allergies and bronchial asthma, $CD14^{+}CD16^{+}$ intermediate monocytes expressing miR-124 increased in number (46). The miRNA-146 family of miRNAs, consisting of miRNA-146a and miRNA-146b, consists of negative feedback regulators of TLR signaling. miRNA-146a targets IRAK1, TRAF6, IRAK2, and IRF5, and miRNA-146b targets TLR4, MyD88, IRAK1, and TRAF6 (47). miRNA-146b is an IL-10-dependent miRNA that regulates the LPS-mediated proinflammatory cytokine secretion of TNF-α, IL-6, and IL-8 and chemokines CCL2, CCL3, and IP-10 (48). miRNA-147 regulates inflammatory responses in murine macrophages, and in breast cancer, it suppresses the AKT/mTOR signaling pathway (49). Its relevance in human inflammatory diseases has yet to be uncovered. miR-187 is directly induced by IL-10 signaling. It recruits *TNFA* mRNA to the RISC complex to promote its degradation. It also negatively regulates IL-6 and IL-12p40 production in TLR4-stimulated monocytes (50). Due to its ability to favor an M2 phenotype, miR-187 is overexpressed in long-term asthma and allergic rhinitis in humans (51). The miR-373-3P is located in an intronic region of PPARγ-coactivator-1β, a protein involved in

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the alternative activation of macrophages. Thus, this miRNA is conceivably involved in the alternate activation of macrophages (52).

THE REGULATION OF INFLAMMATION BY ENDOGENOUS REGULATORY MOLECULES

The wave of inflammatory mediators released by macrophages in response to pathogen products is an important innate response to invading pathogens. However, the return of macrophages to homeostasis is equally as important, because when left unchecked, macrophage activation can lead to destructive tissue pathology. In the late stages of some infections, macrophages can be exposed to molecules that not only mitigate inflammation but actively return the macrophage to a resting state. Many of these molecules are produced by macrophages themselves, and we propose that the synthesis and response to these endogenous mediators are what typically induce a transient state of macrophage activation. This transient activation is a way that macrophages can respond to danger and yet still contribute to homeostasis in the host. The release of endogenous mediators into the extracellular milieu by macrophages represents a practical way for macrophages to control their own activation state. These signals act in an autocrine fashion by binding to receptors on the macrophage cell surface to terminate the inflammatory process. Some of the more well-studied endogenous inhibitors include purinergic molecules, such as adenosine, and lipid mediators, including prostaglandins, resolvins, and lipoxins, among others. In this section, we review the involvement of these endogenous mediators in macrophage biology and their role in the systemic resolution of inflammation.

Purinergic Molecules

Purinergic signaling molecules, such as adenosine, are released following metabolic alterations, cell death, or tissue damage, but can also be generated in close proximity to macrophages themselves in response to infection. Adenosine is derived from adenosine triphosphate (ATP), which is generated and released by macrophages following activation. A range of stimuli, including TLRs and type I interferons, induce a metabolic switch in macrophages from oxidative phosphorylation to glycolysis, which results in the generation of intracellular ATP (53). The product of ATP catabolism, adenosine, also enhances glycolysis in TLR-activated macrophages, which suggests a positive feedback loop in the production of ATP that helps maintain the restorative functions of macrophages (54). Upon stimulation, ATP-enriched vesicles in the cytoplasm of macrophages are released by exocytosis (55). Other mechanisms of ATP release involve its conduction through pannexin-1 channels on apoptotic cells or stimulated macrophages (56).

The ATP released from macrophages is catabolized on the plasma membrane in a two-step process (57). The macrophage surface ectoenzyme CD39 (E-NTPDase1) is able to hydrolyze ATP to adenosine monophosphate (AMP) in a Ca^{2+}- and Mg^{2+}-dependent manner (58). Next, the surface enzyme CD73 (Ecto5 NTase) quickly converts AMP to adenosine (59). The coordinated action of these two surface enzymes leads to a rise in the concentration of adenosine directly surrounding the macrophage. Our lab recently demonstrated that this process takes place extremely rapidly, as extracellular ATP is virtually

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undetectable in wild-type macrophages, whereas ATP remains at relatively high levels for up to 24 h in supernatants of macrophages deficient in CD39 (57). To further highlight the importance of this catabolic process, we also demonstrated that macrophage hydrolysis of endogenous ATP via CD39 allows the cell to terminate the synthesis of inflammatory cytokines (57).

Coupled to TLR stimulation, adenosine changes the phenotype of macrophages and promotes an immunoregulatory rather than an inflammatory response (60). Macrophages respond to adenosine via four transmembrane G-protein coupled receptors, all expressed on the cell surface at different levels: A1R, A2aR, A2bR, and A3R (61). The A2a and A2b receptors are involved in promoting anti-inflammatory effects due in part to their coupling to Gαs, which leads to increases in intracellular cyclic AMP (cAMP). Macrophages stimulated with LPS selectively upregulate A2aR and A2bR (62). This upregulation enhances macrophage sensitivity to adenosine, allowing the macrophage to transition toward an immunoregulatory phenotype. The addition of adenosine to macrophages leads to the increased production of IL-10 and decreased production of TNF-α and IL-12 (57, 63). Studies using cells from knockout mice demonstrate that this suppression of TNF-α and IL-12 is enacted through both A2aR and A2bR (64, 65). The anti-inflammatory effects of adenosine are not limited to cytokine production, as studies have elucidated its role in suppressing superoxide and nitric oxide production in macrophages (66). Additionally, high-throughput RNA sequencing from our lab demonstrated that macrophages stimulated with LPS and adenosine potently downregulated many genes involved in inflammation relative to LPS alone (39).

Adenosine receptors are known to modulate a number of diseases containing inflammatory components. In vivo and in vitro studies demonstrate that adenosine promotes the resolution of tissue injury through the A2aR-specific induction of vascular endothelial growth factor production by macrophages (67). A role for adenosine was also shown in atherosclerosis, in which adenosine has widespread protective effects in the vasculature, including the inhibition of foam cell formation and reduced systemic inflammation (68). The transition of macrophages from an inflammatory to an immunoregulatory phenotype is crucial in controlling the harmful effects of endotoxemia (57).

Prostaglandin E_2

Found in a wide range of tissues, prostaglandins are bioactive lipids that are implicated in many processes, including proliferation, angiogenesis, and inflammation. They are part of the prostanoid family of lipids, which are synthesized from essential fatty acids in a multistep process. First, phospholipases hydrolyze membrane phospholipids, liberating arachidonic acid from membrane lipids. Arachidonic acid is oxidized into PGG_2 and reduced to PGH_2 by the cyclooxygenase (COX) enzymes COX-1 and COX-2, the latter of which is highly upregulated throughout the immune system in response to proinflammatory signals, including LPS, IL-1β, and TNF-α (69). Lastly, PGH_2 is converted into PGE_2 via three distinct synthases: mPGES-1, mPGES-2, and cPGES. PGE_2 levels are regulated by both its synthesis and degradation. 15-PGDH and 13-PGR are catabolic enzymes that can rapidly remove PGE_2 from the cellular environment.

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The involvement of PGE_2 in acute inflammation is well documented, but paradoxically, PGE_2 along with the other prostanoids PGD_2 and PGJ_2 also play an important role in immune suppression (70). Cells, including macrophages, sense PGE_2 via four transmembrane G-protein-coupled receptors (GPCRs): EP1–4. EP2 and EP4 are coupled to Gαs proteins, which stimulate the release of intracellular cAMP. In conjunction with TLR stimulation, PGE_2 suppresses IL-12 and TNF-α and partially suppresses IL-6 production in macrophages (71). At the same time, PGE_2 increases IL-10 release from macrophages in a PKA-dependent manner (72). PGE_2 suppresses inflammasome activation in macrophages via the EP4 receptor, attenuating the release of IL-1β (73). Additionally, IL-17 increases in the presence of PGE_2, leading to the formation of M2 macrophage microenvironments (74). Our lab previously demonstrated through functional studies and high-throughput RNA sequencing that murine macrophages stimulated with LPS and PGE_2 adopt an immunoregulatory phenotype (39).

PGE_2 plays a role in regulating the activation of many immune cells, especially those of the innate immune system. For example, PGE_2 is known to suppress natural killer cell activity by dramatically increasing intracellular cAMP (75). Lung inflammation in response to allergens and pollutants, as well as colonic inflammation, is also attenuated by PGE_2 through the EP4 receptor on macrophages (76, 77). Additionally, PGE_2 downregulates MHC class II expression on dendritic cells once they have migrated to lymphoid organs to suppress antigen presentation (78). A PGE_2 dose dependently inhibits bacterial killing by alveolar macrophages (79). Moreover, many bacteria and intracellular parasites, such as *Leishmania donovani*, have developed mechanisms to induce PGE_2 production by macrophages to suppress inflammation and better survive within the host (80). *Mycobacterium tuberculosis* (*Mtb*) can stimulate the production of PGE_2 by macrophages through the TLR2 MAPK pathway to prevent cell necrosis (81). They also showed that EP4 antagonism in *Mtb*-infected macrophages inhibited the expression of COX-2 and mPGES-1, demonstrating a positive feedback loop for endogenous PGE_2 production (81).

COX inhibitors are common nonsteroidal anti-inflammatory drugs (NSAIDs) used to inhibit inflammation in the host. However, chronic inhibition of COX enzymes in macrophages drives them to become more inflammatory, in part due to decreased production of PGE_2 (82). Additionally, it is thought that these classic inhibitors of prostaglandin synthesis may prolong chronic inflammation when taken during the resolution stage (83).

Resolvins and Lipoxins

Similar to prostaglandins, resolvins and lipoxins are lipid mediators that prevent uncontrolled inflammation and promote the resolution of inflammation. Resolvins are a class of molecules biosynthesized from two omega-3 fatty acids: eicosapentaenoic acid and docosahexaenoic acid, leading to E resolvins (RvEs) and D resolvins (RvDs), respectively (84). RvE1 signals through chemokine receptor-like 1 and leukotriene B4 receptor, whereas RvD1 signals through GPR32 and formyl-peptide receptor (FPR2) (85). These receptors are expressed on a variety of cells, including macrophages. One function of resolvins appears to be halting the infiltration of poly-morphonuclear leukocytes to sites of infections once they are no longer needed (86). A recent study demonstrated that macrophages stimulated in the

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presence of resolvins, specifically RvD1, had decreased production of IL-1β, IL-6, and IL-8 (87). This was due to lower caspase-1 activation and signaling through GPR32 on the macrophage surface (87, 88). Additionally, RvD1 attenuates Th1 cytokine production by classically activated macrophages and upregulates arginase I expression (89). Both resolvins and lipoxins potently inhibit TNF production by primary human macrophages in response to purified LPS but not to live *Escherichia coli* (90).

Lipoxins are lipoxygenase-derived eicosanoids, and their synthesis is aspirin triggered. Both resolvins and lipoxins depend on 5-lipoxygenase-activating protein for synthesis. Two lipoxins, LXA4 and LXB4, are derived from arachidonic acid following an inflammatory signal. Lipoxins are able to interact with a number of receptors on the cell surface, including leukotriene receptor CysLT1 and the N-formyl peptide receptor FPR2. Lipoxins enhance the phagocytosis of apoptotic and necrotic cells and are important components of the resolution process.

The production of endogenous molecules that contain resolving properties is critical for the macrophage to control its activation. Adenosine, prostaglandins, and the group of lipid mediators discussed above all have one commonality: They are sensed by GPCRs on the cell surface. GPCRs have long been of interest in drug development due to their widespread expression and therapeutic potential. GPCRs have profound effects on regulating macrophage function through the actions of cAMP and Ca^{2+} (91). Luckily, many tools are available for the manipulation of these receptors to study their roles in inflammation and its resolution. Scholars have long thought that the return to homeostasis by macrophages is a passive process, but in the following section we try to illuminate the active and programmed mechanisms that macrophages initiate to control their activation state.

THE REGULATION OF MACROPHAGES BY EXOGENOUS INHIBITORS

The regulation of macrophage inflammatory responses can occur through the autocrine mechanisms described above, but it can also occur via the addition of numerous exogenous signals to the macrophage microenvironment. Some of these signals turn off inflammatory responses, some turn on anti-inflammatory responses, and some of these signals result in cell death to terminate inflammatory responses.

Turning Off Inflammation

The growing base of knowledge regarding the synthesis and control of inflammatory mediators is leading to the development of inhibitors of these molecular responses. For example, the central role that TNF-α plays in initiating the inflammatory cascade has led to the remarkably successful development of humanized monoclonal antibodies to TNF-α to treat inflammatory diseases (92). Similarly, our improved understanding of the role of inflammasomes in cytosolic host defense has led to the development of drugs to target IL-1β, its receptors, and the host molecules responsible for the secretion of IL-1β and IL-18 (93). Other approaches to drug development are less specific and generally try to inhibit inflammation by targeting the production of inflammatory cytokines or lipid mediators in general. As described above, during inflammation, phospholipase A2 becomes activated and membrane phospholipids release arachidonic acid to promote eicosanoid production

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(primarily prostaglandins, leukotrienes, and thromboxane). NSAIDs were among the earliest synthetic inhibitors of inflammation, which generally work by inhibiting COX enzymes and reducing eicosanoid production to counteract situations in which prostaglandins might be inflammatory (82).

Glucocorticoids are also a general class of macrophage modulators that can reduce the production of a number of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-12, by activated macrophages (94). Glucocorticoid receptors (GRs) have multiple modes of action that help reduce inflammation. First, the complex of ligand and receptor can migrate into the nucleus where it binds to specific response elements. This leads to chromatin remodeling that can initiate or inhibit transcriptional machinery (94). Interference of NF-κB and AP-1 activity, among other inflammatory mediators by GRs, can also occur through direct physical interaction (95). More rapid effects of glucocorticoids include activation of or interactions with MAPKs, adenylyl cyclase, protein kinase C, and heterotrimeric guanosine triphosphate-binding proteins (96). Glucocorticoids also induce synthesis of IκB, which compensates for its degradation during inflammation, decreasing the amount of NF-κB that translocates into the nucleus (97). Through these mechanisms, glucocorticoids inhibit the transcription of proinflammatory cytokines, reduce antigen presentation, and decrease mRNA stability, while increasing the phagocytosis of apoptotic cells leading to anti-inflammatory transforming growth factor-beta (TGF-β) production by macrophages (60, 98). Exogenous addition of naturally made steroid hormones such as glucocorticoids has proven to be effective in the treatment of autoimmune diseases such as MS, rheumatoid arthritis, psoriasis, and many more (94).

Kinase Inhibitors

The initiation of inflammatory pathways following receptor ligation relies heavily on the activation of kinase cascades. General and specific inhibition of such kinases is a widely applied field, focused mainly on the Janus activated kinases (JAKs), spleen tyrosine kinase (SYK), and the MAPKs. All are involved in signaling via TLRs and many cytokine receptors. The JAK/STAT pathway initiates cytokine receptor signaling by phosphorylating tyrosine residues on the receptor and on STATs, activating downstream immune response genes. Interruption of JAK activity prevents the production of IFN-γ and IL-6 and has proven to be effective in treating arthritis, ulcerative colitis, and psoriasis (99). By associating with receptors containing immunoreceptor tyrosine-based activation motifs, SYK plays an important role in a growing number of immune pathways, including cellular adhesion, pathogen recognition, and inflammasome activation (100). Inhibition of this kinase reduces inflammatory degradation of bone in rheumatoid arthritis (101). Research in various disease models shows reduced inflammatory pathology due to SYK inhibition. For example, in rheumatoid arthritis patients, SYK inhibitors demonstrated a reduction in inflammatory IL-6 and MMP-3, whereas SYK inhibition in mast cells prevented degranulation via IgE receptor signaling (100). The well-conserved MAPKs play an important role in proinflammatory signaling cascades, especially p38 kinases and Jun amino-terminal kinases (JNKs) (102). Phosphorylation of JNKs leads to increased c-Jun activation, an important component of the AP-1 complex involved in cytokine gene expression (103). The p38 MAPKs regulate expression of IL-1β, TNF-α, IL-6, IL-8, and

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even COX-2 (104). In the Crohn's disease model, inhibition of both MAPKs resulted in the improved clinical status of patients (105). The development of specific inhibitors of these kinases continues to show great promise in the regulation of inflammatory responses.

Receptor-Mediated Inhibition

Just as many pattern recognition receptors on macrophages can induce inflammatory responses, many macrophages can initiate inhibitory signals to counter macrophage activation and reestablish homeostasis. Signaling through these receptors can reduce inflammatory cytokine production and inhibit free radical production. The signal regulatory protein α on macrophages, acting through tyrosine-based inhibitory motifs, binds CD47 and produces negative signals to prevent phagocytic activity, the production of superoxide, and inflammatory cytokines. This process is mediated through the activation of SHP-1 and SHP-2, phosphatases that counteract kinase activity (106). Similarly, a variety of cell types express CD200, and its interaction with CD200R on myeloid cells produces inhibitory signals. This interaction serves as a constitutive restraint on inflammatory stimulation of macrophages, limiting autoimmune pathology (107). Acting through the downstream kinase, CD200R signaling inhibits Ras, subsequently reducing PI3K signals and inhibiting cytokine production (108). The engagement of CD200 to its receptor leads to reduced responses to IFN-γ and IL-17 stimulation in mouse peritoneal macrophages and decreased IL-5 and IL-13 production following tetanus toxoid stimulation in human peripheral blood mononuclear cells (109). Receptors such as TREM2 can also regulate inflammation. The expression of TREM2 on recently differentiated macrophages entering into sites of inflammation acts in cooperation with DAP-12 to reduce macrophage production of TNF-α and IL-6 (110). The ligands for TREM2 are still under investigation but include endogenously produced molecules and also exogenous compounds such as LPS (111). These and other receptors share the common goal of preventing the overproduction of inflammatory signals by macrophages.

Finally, naturally produced molecules at a given anatomical site can locally dampen macrophage inflammatory responses. For example, intestinal mucosal surfaces help to protect epithelial cells against potentially harmful contents within the intestinal lumen, using phospholipids to establish a hydrophobic surface that can inhibit excess inflammation (112). Phosphatidylcholine, one of the most prevalent phospholipids in the mucosa, can inhibit kinase activation, NF-κB transport, and proinflammatory gene production. Similarly, pulmonary surfactants play an important role in reducing inflammatory responses in the lungs (113). Phosphatidylcholine also decreases phagosome assembly and the killing of *Mtb*, an infection requiring Th1 inflammatory responses for clearance (112).

Turning On Anti-Inflammation

The resolution of inflammatory responses by macrophages is not simply dependent on turning off inflammatory signal production but also by increasing the production of anti-inflammatory mediators. The most common of these mediators are IL-10 and TGF-β. IL-10 signals through the IL-10R on macrophages, inhibiting inflammatory cytokine production and frequently decreasing the killing of intracellular pathogens (114). Many cells produce IL-10, and genetic alterations that result in a failure to produce this cytokine are invariably

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associated with inflammatory im-munopathology. However, the potent immunoregulatory activity of IL-10, which was repeatedly observed by many investigators using a number of inflammatory model systems, has generally not led to the utilization of recombinant IL-10 to treat autoimmunity. Unfortunately, the administration of recombinant IL-10 is typically not able to substantially reverse inflammatory pathology in patients suffering with autoimmunity, including psoriasis and Crohn's disease (115). One reason may be that IL-10 is frequently diverted to the many different cells that have receptors for IL-10, preventing its delivery to inflamed areas. The local induction of IL-10 may be more effective in regulating inflammation. Whether produced by macrophages or other cell types, IL-10 release depends on the strength of stimulation (115). In macrophages, increased signaling that occurs through a combination of stimuli results in high levels of IL-10 production. For example, the addition of LPS in combination with the ligation of macrophage Fcγ receptors (FcγRs) by high-density immune complexes can potently downregulate IL-12 production and induce high levels of IL-10 production (116). This reciprocal alteration in these two key cytokines results in a macrophage population that can mitigate inflammation and provide protection against acute endo-toxicity. These macrophages also exhibit increased susceptibility to intracellular pathogens (117). Inflammation induced by a variety of stimuli, including virtually all TLR ligands, CD40:CD40L, and low molecular weight hyaluronic acid, was modulated when coupled with macrophage FcγR ligation. The physiological relevance of this phenotypic alteration was demonstrated in an infectious disease model in which the intracellular parasite *Leishmania* sp. uses this pathway to induce macrophage IL-10 to promote its survival within macrophages (117).

Although TGF-β exhibits a wider range of roles in cellular processes when compared to IL-10, its ability to modulate inflammation and influence disease progression renders it an extremely important immune modulator. The abundance and activity of TGF-β ligands, the presence of SMAD cofactors, and epigenetic modifications all contribute to the intensity and type of signal created from TGF-β signaling (118). SMAD proteins, activated via the TGF-β receptor, form complexes with each other and with other cofactors to activate or inhibit gene transcription. The addition of exogenous TGF-β to LPS-stimulated macrophages decreased the release of proinflammatory cytokines, suggesting paracrine functions of the signaling (119). In mouse models, TGF-β treatment prevented disease in collagen-induced arthritis and prevented relapse in rheumatoid arthritis, consistent with its anti-inflammatory activity (120). Use of TGF-β as a therapeutic in mouse models for multiple sclerosis (i.e., EAE) showed improved clinical status and decreased nervous system damage, indicating its promise (121). However, the multifaceted effect of TGF-β production is illustrated by its contribution to fibrotic diseases, showing the need to continue studying this complex pathway (122).

Although the functional regulation of the inflammatory response is important in homeostatic maintenance, numerous organisms exploit these pathways to establish a successful infection and to survive in macrophages. *Leishmania* spp. can interfere with MAPK signaling to decrease IL-12 production and promote IL-10 production (123). *M. tuberculosis* relies on an inhibition of IFN-γ and promotion of IL-10 production to survive and replicate in macrophages (124). Intestinal and urogenital bacteria such as *Lactobacillus rhamnosus* can promote anti-inflammatory responses from macrophages. The commensal bacteria's

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secretome was able to induce granulocyte colony-stimulating factor production by macrophages, leading to STAT3 activation, JNK inactivation, and TNF-α suppression in LPS- or *E. coli*-activated macrophages (125). Various nematodes and helminths can powerfully regulate the immune response, decreasing inflammatory Th1/Th17 responses and promoting Th2 response environments (126).

Cell Death and Clearance

Cell death can contribute to a decrease in inflammation. The induction of apoptosis is an important process used to remove inflammatory macrophages (127). The rapid removal of macrophages helps to prevent chronic inflammation and decrease fibrosis. In the lungs, the macrophages recruited to the site of inflammation exhibit high levels of Fas, and the induction of apoptosis via Fas-activating antibodies leads to clearance (128). Endoplasmic reticulum stress in combination with pattern recognition receptor activation may also contribute to macrophage apoptosis (129). Additional mechanisms of macrophage cell death and the importance of these processes in the regulation of inflammation are important research areas that remain under investigation.

The clearance of dead or dying cells by macrophages is a central role of these cells. The uptake of apoptotic cells by macrophage is characterized, and the link to anti-inflammatory responses is well described. Generally, macrophage-mediated clearance of apoptotic cells induces the production of lipoxins, resolvins, and protectins that work in autocrine and paracrine fashion to promote the resolution of inflammation (98). During the immune response, one of the most commonly phagocytized apoptotic cells is the neutrophil. Neutrophils are among the first responders to pathogens, and the potentially toxic molecules released by neutrophils must be tightly regulated. Their clearance subsequent to apoptosis is mediated primarily by macrophages that recognize specific changes on the cell surface (130). Recognition of the common apoptotic marker phosphatidylserine promotes TGF-β production by macrophages to limit inflammation (131). Annexin A1 is exported by apoptotic and necrotic cells. It can bind to phosphatidylserine on the outer membrane, helping to enhance phagocytic uptake (132). Apoptotic cell clearance by macrophages is especially important in the continuous process of cellular turnover as organisms develop and grow (98).

MACROPHAGE ACTIVATION SYNDROME: A FAILURE TO REGULATE

Macrophage activation syndrome (MAS) is a relatively rare autoimmune complication that can follow chronic infectious diseases, T cell immunotherapy, or rheumatologic diseases, especially in children. Persistent fever, coagulopathies, lymphadenopathy, and hepatosplenomegaly are frequently associated with this disease, and hemophagocytosis and hyperferritinemia are striking features of MAS (133). The secretion of IFN-γ seems to be an early event in this disease that leads to a cytokine storm characterized by myriad proinflammatory cytokines and chemokines from macrophages, including IL-1β, IL-6, and TNF-α (134). A central role for activated T cells, especially CD8 T cells, is generally accepted, and in humans there is a strong correlation between IFN-γ production from these cells and clinical pathology. In experimental animal models, recurrent TLR activation has

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been used to induce disease (135), but in humans there is also strong evidence for the activation of inflammasomes, including NLRC4, in this disease (136). The use of anakinra (an IL-1 receptor antagonist) to treat a subset of patients with MAS supports a role for macrophage-derived IL-1 in disease pathology (137). IL-18 is another cytokine that depends on caspase 1 cleavage for secretion, and elevated IL-18 may contribute to MAS pathogenesis, perhaps by inducing additional IFN-γ production (138). One interesting hypothesis is that the failure of activated macrophages to die in a timely manner is in part responsible for this autoinflammatory syndrome. Indeed, this disease and the closely related hemophagic lymphohistiocytosis are associated with mutations that interfere with efficient cytotoxicity (133). The inability of cytotoxic cells to induce apoptosis of activated macrophages may allow these cells to persist and produce inflammatory mediators for prolonged periods of time.

The first line treatment for MAS is corticosteroids, with cyclosporine added if necessary. High-dose intravenous immunoglobulin in combination with corticosteroids is also used as a treatment option when corticosteroids alone prove to be insufficient. Humanized monoclonal antibodies to IL-1 or IL-6 are now frequently added. Because of the accepted practice of treating this disease with drugs that target macrophage cytokine production, one can consider MAS in the simplest terms to be a failure to efficiently regulate macrophage activation in response to inflammatory stimulation. MAS illustrates the enormous pathological potential of activated macrophages and the pressing need to develop new ways of regulating macrophage activation. Current therapies are largely directed at blocking inflammatory cytokine production or preventing the binding of cytokines to their cognate receptors. This therapeutic approach transiently diminishes clinical symptomatology but does not reverse disease or provide long-term protection from recurrences. New ways are needed to reprogram macrophages to change their activation state or to educate them to produce immunoregulatory molecules rather than immunostimulatory molecules.

SUMMARY

Although macrophages represent a potentially lethal source of inflammatory cytokines, they are floating in a sea of regulators that can dampen inflammation and prevent autoimmune sequelae. These regulators prevent cytokine overproduction by a variety of different mechanisms. They include transcriptional repressors, epigenetic silencers, inhibitors of signaling kinases, mechanisms to degrade signaling molecules, and small regulatory RNAs. All of these regulators work to maintain the delicate balance of a healthy homeostatic system of host defense.

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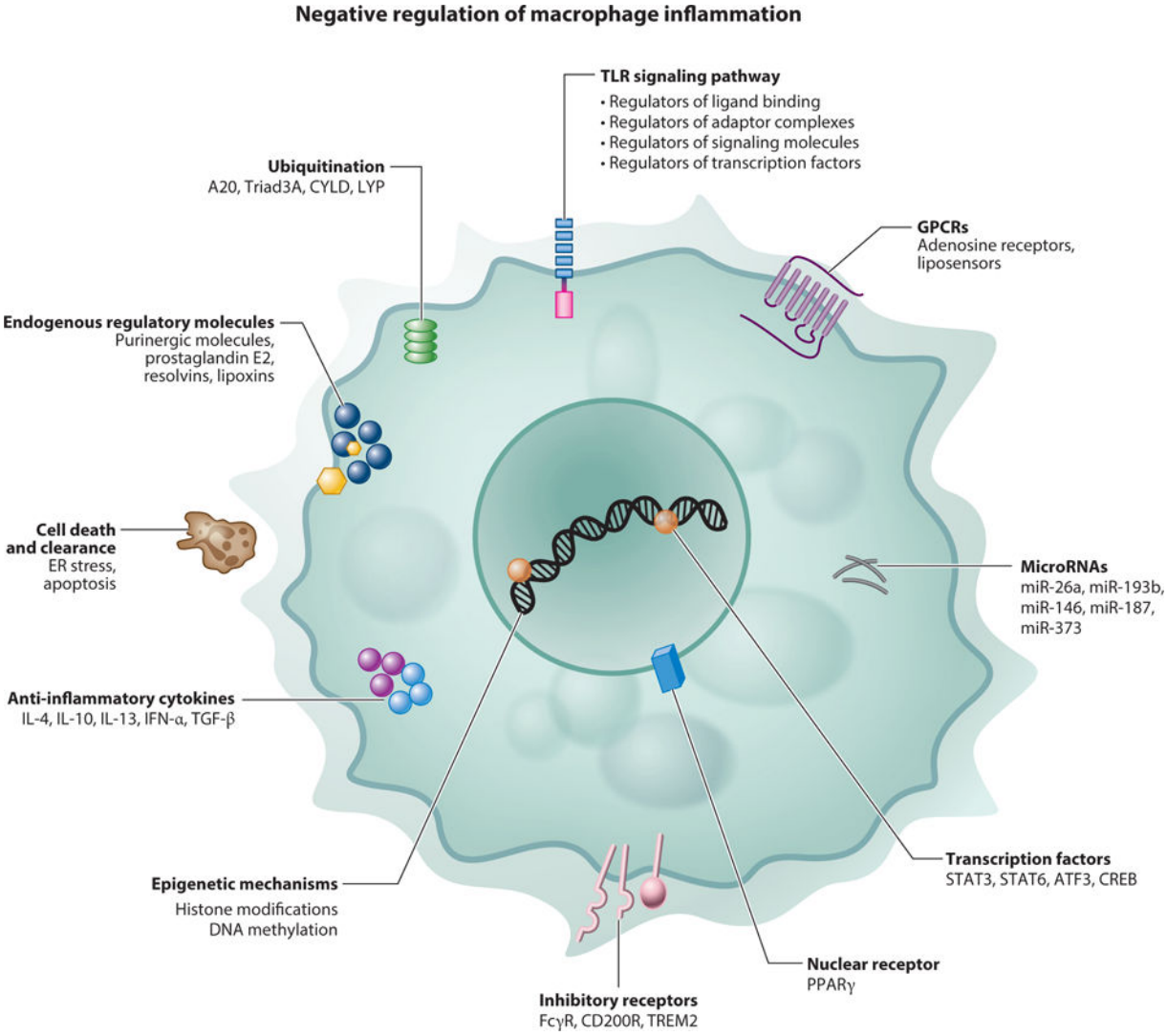

Figure 1.

Inflammation can be negatively regulated by a multitude of cellular molecules and mechanisms. The initiation of TLR signaling pathways leading to inflammation can be negatively regulated at various levels from ligand binding to transcription. Whereas primary or secondary signaling through certain GPCRs, nuclear receptors, or inhibitory receptors can cross-regulate the inflammatory pathways, cellular mechanisms such as epigenetic regulation, ubiquitination, and cell death can operate at both micro- and macrolevels to inhibit inflammation in macrophages. Such receptor-mediated signaling pathways and cellular mechanisms can give rise to anti-inflammatory cytokines that can mitigate proinflammatory cytokines; transcription factors that turn on mRNA transcription of anti-inflammatory molecules; and microRNAs that mediate post-transcriptional silencing of mRNAs encoding inflammatory proteins. Abbreviations: CYLD, cylindromatosis; ER, endoplasmic reticulum; FcγR, Fcγ receptor; GPCR, G-protein coupled receptor; IFN-α, interferon-α; IL, interleukin; LYP, lymphocyte tyrosine phosphatase; PPARγ, peroxisome

proliferator-activated receptor γ; TGF-β, transforming growth factor-β; TLR, Toll-like receptor.

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Table 1

Negative regulators of Toll-like receptor (TLR) signaling with their mechanism of action and relevance to disease

Negative regulators	Mode of action	Diseases implicated	Reference(s)
Regulators of ligand binding			
sTLR2 (soluble form of TLR2)	Prevents lipopeptide binding to TLR2 through competitive binding	In human milk sTLR2 protects infants from enterocolitis and sepsis	139
sTLR4 (soluble form of TLR4)	Prevents LPS binding to TLR4 through competitive binding	Elevated levels of sTLR4 are found in human endotoxemia and auto-inflammatory syndromes	140
sCD14 (soluble CD14) also known as presepsin	Diverts LPS from membrane-bound CD14 and promotes LPS efflux from cell-surface CD14	Marker of inflammatory diseases and sepsis	141, 142
RP105 (radioprotective 105)	RP105-MD1 complex binds to TLR4-MD2 complex and prevents LPS binding	Increased expression in primary biliary cirrhosis	25, 143
Triad3A	E3 ubiquitin-protein ligase that enhances ubiquitination and degradation of TLR4 and TLR9	Overexpression reported in diabetic kidneys	144, 145
Regulators of adaptor complexes			
SARM (sterile alpha and armadillo motif-containing molecule)	Negatively regulates TRIF-dependent TLR signaling and MyD88-mediated AP-1 activation	May mediate endotoxin tolerance	146
Splicing variant of MyD88 (MyD88s)	Splice form is unable to activate NF-κB	None	27
TAG	Splice variant of TRAM, negatively regulates MyD88-independent TLR4 pathway	None	28
ST2 (single immunoglobulin IL-1R-related molecule) ST2L: membrane bound ST2: soluble form	Prevents the recruitment of MyD88 and TIRAP (MAL) to TLR2, -4, and -6 by sequestering them	Prevents renal epithelial immunity in diabetes	145, 147
TRAF1 (TNF receptor-associated factor)	Inhibits TLR3 mediated NF-κB activation and IFN-β signaling by interacting with TRIF	TRAF1 polymorphisms are associated with autoimmune thyroid disease	148, 149
TRAF4	Physically interacts and functionally counteracts with TRAF6 and TRIF	Implicated in human IBD	150, 151
Cbl-b (casitas B-lineage lymphoma, an E3 ubiquitin ligase)	Mediates ubiquitination of MyD88 and TRIF	Causes dysregulation of macrophage activation in obesity-induced insulin resistance	152, 153
CYLD (deubiquitinase)	By deubiquitinating K63-linked polyubiquitination of MyD88, a form required for TLR signaling	Brooke-Spiegler syndrome	154, 155
SOCS1	Phosphorylated TIRAP (MAL) is bound to SOCS1 which results in TIRAP polyubiquitination and degradation	Psoriasis, MS, viral infections, and many others	156
Regulators of signaling molecules			
Splicing variants of IRAK1 (IRAK1c), IRAK2 (IRAK2c, 2d)	Splice forms were unable to activate NF-κB	None	157, 158
IRAKM	Lacks intrinsic kinase activity and hence prevents dissociation of IRAK4 and IRAK1 from MyD88	*IRAKM*+22148 G>A allele associated with risk of developing chronic relapsing pouchitis	159, 160
SHP-1 and SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase-1 and -2)	SHP-1 suppresses IRAK1 and IRAK2 activities and SHP-2 negatively regulates TRIF-dependent type I IFN production	*Leishmania* spp. promote SHP-1 binding to IRAK1 to suppress innate response	161, 162

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Negative regulators	Mode of action	Diseases implicated	Reference(s)
SIGIRR (single immunoglobulin interleukin-1 receptor-related)	Interacts with and blocks the activation of IRAKs and TRAF6	SLE, dominant negative forms reported in human colon tumors	163
A20 (deubiquitinase)	Deubiquitinates TRAF6 and prevents its binding to TAK1 and subsequent IκκComplex formation	*TNFAIP3* gene (that codes for A20) polymorphisms are reported in RA, SLE, psoriasis, Crohn's disease, and other autoinflammatory diseases	30, 164
β-Arrestin 2	Interacts with TRAF6 and prevents its oligomerizationNegatively regulates TLR4 signaling by targeting p38 MAPK and IL-10; regulates TLR4-mediated apoptotic signaling through GSK3α	Several fibrotic diseases	165, 166
TRIM38 (tripartite-motif containing 38)	Binds to TRAF6 and promotes K48-linked polyubiquitination, and proteasomal degradation	Primary Sjögren's syndrome	167, 168
Lyp (lymphocyte tyrosine phosphatase)	Potentiates type I IFN from TLR signaling through interaction with TRAF3 and promoting its K63 ubiquitination	SNP in *PTPN22* gene that encodes Lyp results in autoimmune risk allele (Lyp620W) and hence associated with several autoimmune diseases	169
TRIM30α (tripartite-motif containing −30α)	Negatively regulates NF-κB activation by targeting TAB2 and TAB3 for degradation	DSS-induced colitis mouse model	170
Regulators of transcription factors			
ATF3 (activating transcription factor 3; a member of ATF/DREB family)	Restrict access to NF-κB and AP-1 to the promoter regions of proinflammatory cytokines IL-6 and IL-12b	Type 2 diabetes, cardiac hypertrophy, osteoarthritis	35, 171
NOD2	NOD2 signaling inhibits TLR2 driven activation of NF-κB subunit c-Rel	Crohn's disease, sarcoidosis	172, 173
PI3K	Suppresses p38 MAPK and NF-κB through PKB; PI3K can also directly inhibit IL-12 production	E.g., fibrosis, diabetes, COPD, IBD, RA, SLE, MS	10, 174
Pin1 (peptidyl prolyl isomerase)	Mediates ubiquitination and subsequent proteasome-dependent degradation of IRF3	Overexpressed in several cancers; implicated in allergic pulmonary inflammation	175, 176
IRF4	Competitively inhibits IRF5 binding to TLR	SLE	177, 178
RAUL (RTA-associated ubiquitin ligase)	Catalyzes ubiquitination of IRF3/7 and negatively regulates type I IFN responses	Kaposi sarcoma herpes virus stabilizes RAUL by preventing its degradation and thereby downmodulating antiviral response	179

Abbreviations: COPD, chronic obstructive pulmonary disease; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IFN, interferon; IRF, interferon regulatory factor; LPS, lipopolysaccharide; MAL, MyD88 adapter-like; MS, multiple sclerosis; PKB, protein kinase B; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; TAG, TRAM adaptor with GOLD domain; TIRAP, Toll-like receptor adaptor protein.