CMLS, Cell. Mol. Life Sci. 60 (2003) 2334–2346
1420-682X/03/112334-13
DOI 10.1007/s00018-003-3020-0

CMLS Cellular and Molecular Life Sciences

Review

Regulation of macrophage activation

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Received 16 January 2003; received after revision 14 March 2003; accepted 2 April 2003

Abstract. IFN-γ rapidly primes the macrophage via JAK1/2-STAT1 pathway so that it can subsequently undergo a slower classical type 1 activation upon exposure to T helper (Th)1 cytokines such as IFNγ or other activators, including tumor necrosis factor and lipopolysaccharide, e.g. in intracellular killing of phagocytosed *Mycobacterium tuberculosis*. If instead it is driven by Th2 cytokines interleukin (IL)-4 and IL-13, it undergoes alternate type 2 activation, which enhances endocytotic antigen uptake and presentation, mast cell and eosinophil involvement and type 2 granuloma formation, e.g. in response to parasitic and extracellular pathogens. Particle-induced macrophage activation was shown to differ from classical and alternate activation, showing in DNA microarray experiments (complete linkage/ Euclidean distance metric analysis) upregulation of nonsecreted structural/signaling molecules and lack of secreted proinflammatory cyto- and chemokines. The switch-off (deactivation) of already activated macrophages is an active, controlled process in which IL-10 and corticosteroids play important roles and to which15dPGJ2, PGA1/2 and vasoactive intestinal peptide often contribute.

Key words. Macrophage; classical activation; alternate activation; priming; deactivation.

Introduction

Macrophages were first recognized by their large size and phagocytic properties. Their development starts in the bone marrow (stem cell→colony forming unit granulocyte-macrophage or GM-CFU→monoblast→promonocyte→monocyte, 2–3 days), driven by colony-stimulating factors multi-CSF [interleukin (IL)-3], GM-CSF and M-CSF (CSF-1) with 5×10^9 monocytes being produced for export into the bloodstream per day. After some 18 h in this transit compartment they settle in their target tissue, instructed by the local microenvironment to differentiate to locoregional-specific and heterogeneous cells, such as synovial lining macrophage-like type A cells, Kuppfer's cell of the liver or alveolar macrophage of the lung, and in connective tissue in general just simply to (tissue) macrophages. These resident and long-lived (years) macrophages can locally demonstrate modest proliferative activity and may thus contribute to the maintenance of the resident tissue macrophage pool under homeostasis [1]. After a noxious insult and the first acute wave of neutrophils, cleared by apoptosis, circulating monocytes make an initial tethering endothelial cell contact and roll, adhere, are activated and finally transmigrate to tissue in great numbers to form the mononuclear (round) cell infil-

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trates characteristic of chronic inflammation. Tissue macrophages are often primed before classical activation, or they can undergo so-called alternate activation. Activation is followed by deactivation or cell death.

Regulation of macrophage activation

In innate immunity

Macrophages play a dual role in the host defence. They form the first line of defence as a component of the innate immune response, but they also act as important accessory cells in the adaptive immune response. Macrophages were initially recognized as nonspecific phagocytic scavenger cells, capable of opsonin-independent clearance. This refers to their capacity to phagocytose particles in the absence of immunoglobulin or complement coating (immune opsonization). However, 'nonimmune' molecules can also act as opsonins, e. g. C-reactive protein and serum amyloid P. The receptors utilized in the innate situation include Fc receptors (which then recognize C-reactive protein and serum amyloid P), complement receptors (which then recognize mannan-binding lectin), integrins (fibronectin, vitronectin), scavenger receptors, mannose receptors and Toll-like receptors (TLRs) [2]. This review also contains a section on macrophage activation by microbes, lipopolysaccharide of the outer lipid bilayer of Gram-negative bacteria and peptidoglycan polysaccharide of the bacterial cell wall (capsid) (see below). Microbial phagocytosis triggers production of proinflammatory chemo- and cytokines, activates microbial killing systems and antigen processing and presentation, but suppresses genes encoding molecules involved with bacterial recognition and internalization. This important function to destroy dangerous intruders can eventually cause harm in the case of implants. The conventionally used metal-to-plastic (ultra high molecular weight polyethylene, UHMWPE) gliding surface of a totally replaced joint produces a high number of wear debris particles. Macrophage aggression against such synthetic particles leads to a progressive and perpetuated chronic foreign body inflammation, with periprosthetic bone loss and aseptic loosening as a result. When these particles are too large to handle, macrophages join forces and fuse to foreign body giant cells [3]. This strategy is also utilized in the bone morphogenetic units (BMUs) during the initial stages of the activation-reversal-formation (ARF) cycle when monocytes are activated and fuse to form polykaryons. Finally, if bacteria and/or particles are too difficult to destroy, macrophages differentiate to epitheloid cells and form granulomas, to isolate and confine potentially harmful stimuli.

Adaptive immunity

The most important outcome of macrophage-mediated phagocytosis from the point of view of adaptive immunity is antigen processing and presentation. Acid proteinase-degraded peptides are in the MHC (major histocompatibility complex) class II-containing peptide-loading compartment (MIIC) bound to MHC class II molecules. On the cell surface the MHC class II-antigen peptide complex is presented to the T cell receptor (TCR) of $CD4^+$ T lymphocytes [4]. Two sets of instructive cytokines play a key role in the polarization of the T-helper cell responses. In the presence of IL-12 and IL-18 (produced by e. g. activated macrophages and dendritic cells), a T helper 1 (Th1) response predominates, whereas in the presence of IL-4 (and IL-13) Th2-type response is predominant in a mutually exclusive way [5, 6]. Activated Th1 cells produce IFN-γ, which forms a positive feedback loop by priming/activating more macrophages and by upregulating their MHC class II expression.

Apoptosis

Apoptosis does not lead to macrophage activation, but to removal of inflammatory cells and production of macrophage deactivating stimuli. These include transforming growth factor-β (TGF-β), IL-10 and cyclopentenone prostacyclins (cyPGs) [7, 8]. Native and oxidized phosphatidylserine (PS)/other phospholipids, altered sugar chains and other apoptotic cell surface determinants are recognized by apoptosis receptors. These include phosphatidylserine receptor (PSR), oxidized low-density lipoprotein (LDL) receptors (CD68/macrosialin and lectin-like oxidized LDL receptor-1 LOX-1), scavenger receptors SR-A and SR-BI, endotoxin receptor CD14, asialoglycoprotein receptor and other lectins, as well as CD36 without or with vitronectin receptor $\alpha v\beta 3$ [9]. Newcomers include Mer receptor tyrosine kinase and CD91. Thrombospondin, C1q, $\beta 2$ glycoprotein I, mannose-binding lectin, surfactant protein A and a product of growth arrest-specific gene gas6 can act as opsonins or bridge molecules [10, 11]. Although many of the very same receptors are utilized in microbial phagocytosis, it seems that the engagement of PS-PSR interaction prevents macrophage-mediated production of proinflammatory cytokines and escape of immunogenic material.

Regulation of macrophage activation from the kinetic functional point of view

Priming

Most of the monocyte/macrophages in peripheral tissues, produced by local proliferation or recruited from the blood, die out of apoptosis. If they receive a survival signal, they can differentiate and, eventually, be activated, with or without earlier priming.

Priming is a very effective first step towards full-scale activation. In priming, the cell is prepared or educated by an initial insult [12]. After priming the cell is ready for subsequent second insults. Priming alters or modulates its response to such secondary stimulation. In a sensitized state it responds to subthreshold stimuli and, with an exaggerated stimulus-response ratio, to suprathreshold stimuli. However, the primed cell does not have an activated phenotype, so it does not produce proinflammatory cytokines and microbicidial oxygen or nitrogen intermediates. The most important priming stimulus for macrophages is low-dose IFN-γ. Homodimeric IFN-γ binds with two IFN-γ receptor (IFN-γR) ligand-binding α chains. Dimerized α chains associate with two signaling IFN-γR β chains [13]. These α and β chains of IFN-γR are constitutively associated with Janus kinases JAK1 and JAK2, respectively. Ligand binding induces phosphorylation events, which first lead to binding of latent, cytosolic signal transducer and activator of transcription-1α (STAT1α) followed by its subsequent activation by phosphorylation. Activated STAT1α is released from the IFN-γ/IFN-γR complex and forms a homodimer known as gamma-interferon activation factor (GAF). GAF translocates to the nucleus and binds to gamma activated site (GAS) to initiate transcription [14]. Natural killer (NK) cells form an important first-line defence of the native immunity, ready to respond without need for antecedent sensitization. The primary IFN-γ JAK/STAT response is not dependent on de novo synthesis of transcription factors, because activation of already available, preexisting components mediates it. Finally, subthreshold concentrations of IFN-γ upregulate its own expression in activated NK cells and increase the sensitivity (i.e. prime) of macrophages to a subsequent second stimulus. Such sensitization can cause a problem in rheumatoid synovitis, which is characterized by only low production of T lymphocyte-produced cytokines (e.g. IFN-γ), but ample production of various monocyte-derived cytokines. The low concentration of locally produced IFN-γ may prime the synovial macrophages to a subsequent non-IFN-type stimulus (see below for example tumor necrosis factor-α, TNF).

Classical type 1 macrophage activation

In the presence of Th1 cells/cytokines, macrophages are activated or triggered to become effector cells, e.g. with a greatly enhanced capability for killing intracellular microbes, such as *Mycobacterium tuberculosis*. Apart from lipopolysaccharide (LPS) and peptidoglycans (discussed in a separate section below), IFN-γ seems to be the most important activating stimulus. In addition to the rapid JAK/STAT1α-mediated priming response, in macrophage activation IFN-γ continues by inducing a set of transcription factors known as interferon regulatory factors (IRF-1 to IRF-9, which include IRF-8, formerly known as ICSBP or interferon consensus sequence-binding protein, and IRF-9, formerly known as ISGF3 or interferon-stimulated gene factor 3). IRF-1, by binding to IRF-binding element (IRF-E), induces an antiviral state by upregulating IFN-α and IFN-β and an antibacterial state by upregulating iNOS (inducible nitric oxide synthase) expression, whereas IRF-2 attenuates IRF-E-mediated transcriptional activation [15]. Classically activated 'angry' macrophages are highly microbicidal and proinflammatory.

In most cases IFN-γ acts synergistically with TNF in macrophage activation. TNF trimer signals through its receptors, TNF-R2 and, in particular, TNF-R1. Ligand binding causes dimerization of TNF-R1 and release of silencer of death domain (SODD) proteins from the cytoplasmic part of the ligand-receptor complex [16]. This leads to ordered binding of several adaptor proteins. These are TNR receptor-associated death domain (TRADD), receptor interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) [17–19]. These adapter proteins bind some key enzymes, which can initiate three different signaling arms. These signaling arms are (i) FADD-dependent binding and activation of caspase-8 (apoptosis) [20], (ii) TRAP2-dependent activation of the JNK (c-Jun NH_2-terminal kinase) pathway and (iii) RIP-dependent activation of nuclear factor kappa B (NFκB), which promotes production of proinflammatory mediators and protects against apoptosis [21]. In most cases IFN-γ and TNF synergism is based on ISRE and NFκB sites in the promoters of the proinflammatory genes affected.

Other cytokines, such as IL-1β and GM-CSF, also play a role as modulators of macrophage activation. Binding of IL-1β to IL-1R1 activates both mitogen-activated protein kinase (MAPK)-AP-1 (activator protein-1, Fos and Jun heterodimers) and I-κB kinase (IKK)-NFκB pathways via MyD88 (myeloid differentiation factor 88), IRAK (interleukin-1 receptor associated kinase) and TRAF6 (tumor necrosis factor receptor-associated factor 6). GM-CSF binds to a heterodimeric GM-CSFR, which shares a common β_c signaling chain with IL-3 and IL-5 [22]. This binding activates MAPK-AP-1 and JAK2-STAT5 pathways. The role of IL-12 and IL-18, produced by antigen-presenting cells, in directing T helper cell maturation to Th1 type was already dealt with in the section on the regulation of macrophage activation in adaptive immunity [23].

Alternate type 2 macrophage activation

In the presence of some Th2 cell cytokines, macrophages become activated in an alternative way to combat parasitic and extracellular pathogens (compare with phagocytosis and intracellular killing of *M. tuberculosis*) [24].

Type 2 activation refers to an alternate activation in the presence of IL-4 and IL-13, signaling to macrophages in part through a common receptor chain, IL-4Rα. Antigen-presenting cell recruitment is increased via selectively induced production of macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC). MDC and TARC are produced in type 1 and 2 codominant granulomas. Mannosyl receptor and MHC class II are upregulated to enhance endocytotic antigen uptake and presentation, respectively. Macrophage-mediated production of reactive oxygen species and proinflammatory cytokines is partially inhibited, but not as totally as by IL-10. Production of nitric oxide (NO) is decreased in rodents and not observed in humans, perhaps as a result of induction of arginase type 1. Arginase type 1 is upregulated by IL-4 and IL-13, but suppressed by IFN-γ. NOS uses L-arginine and dioxygen to produce L-citrulline and NO. Interestingly, arginase type 1 hydrolyses the same substrate, L-arginine, to urea and L-ornithine. L-ornithine is used to produce polyamines and proline to promote cell growth and collagen production. Increased arginase type 1/L-ornithine may contribute to the formation of epitheloid cells and granulomatous fibrosis. Indeed, alternative macrophage activation is associated with macrophage fusion and granuloma formation, which are not so prominent or may even be reverted if classical macrophage activation ensues [25]. Similarly, a weak proliferative effect due to autocrine M-CSF secretion and healing are promoted by type II activation. Therefore, the Th1 and Th2 paradigm has received type 1 and 2-activated macrophages and, tentatively, also APC1 and APC2 antigen-presenting cells, at its side.

Deactivation

The switch-off of already activated macrophages is an active and controlled process. It can be achieved with antiinflammatory cytokines, such as IL-10 (see below). Glucocorticosteroids bind to a cytosolic steroid hormone receptor and downregulate gene transcription of iNOS, COX-2 and TNF. In high concentrations glucocorticosteroids are immunosuppressive and inhibit MHC class I and II expression and antigen processing. IL-10 and glucocorticosteroids share a glucocorticosteroid-induced leucine zipper (GILZ) as a transcriptional mechanism of action [26].

Some glucocorticosteroid effects are mediated via transcriptional upregulation of antiinflammatory genes such as annexin 1 (lipocortin 1). Annexin 1 binds to cell membrane substrates of phospholipase A2 and downregulates formation of arachidonic acid. Arachidonic acid is a rate-limiting substrate for the synthesis of the proinflammatory prostaglandins (PGs) and leukotrienes (LTs). Three isoforms of cyclooxygenases (COX-1 to -3) and 5-lipoxygenase, respectively, play important roles in their synthesis. PG and LT production is later, during resolution, switched to antiinflammatory cyPGs (e.g. PGD_2-derived 15dPGJ$_2$ and $PGE_{1/2}$-derived $PGA_{1/2}$) and lipoxins (LXs, e.g. LXA_4 and LXB_4). cyPGs downregulate macrophages and their proinflammatory NFκB, AP-1 and STAT signaling pathways, whereas LXs play a major role in neutrophil deactivation. cyPGs increase macrophage apoptosis and decrease their reactive oxygen and nitrogen intermediate production. 15dPGJ$_2$ was first thought to act as an agonist of peroxisome proliferator-activated receptor γ (PPARγ). Due to 15dPGJ$_2$-mediated modifications of cysteine residues, the function of the IKK-NFκB (IKK is IκB kinase) pathway is attenuated as a result of blocked IKK and NFκB DNA binding [27]. NO participates in eicosanoid production as L-arginine-deficient medium and inhibitors of NOS activity block PGE_2 accumulation. Conversely, a severalfold increase in PGE_2 production is observed in cultures treated with NO [28]. NO and the superoxide anion form the strong oxidizing agent peroxynitrite. Peroxynitrite is an inorganic hydroperoxide that is utilized by the peroxidase activity of COX for prostanoid production. Thus, peroxynitrite stimulates COX, whereas NO or superoxide alone do not appear to do so [29].

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP), neuropeptides also produced by Th2 and mast cells, downregulate TNF, IL-6, IL-12p40, IFNγ and TGFβ secretion and upregulate IL-10 secretion of LPS and/or IFN-γ-stimulated macrophages. Inducible NOS, COX-2 and costimulatory CD80 (B7.1) and CD86 (B7.2) are downregulated. VIP can bind to three different receptors, VPAC1, VPAC2 and PAC1. In an experimental arthritis model the main deactivating effects of VIP seem to be monocyte/macrophage and VPAC1 mediated [30]. Binding of VIP to VPAC1 activates the cyclic AMP (cAMP)/PKA system. According to Ganea and Delgado [31], activation of cAMP/PKA, alone or with cAMP/PKA-independent pathways, leads to downregulation of the (i) IRF-1, (ii) AP-1- and (iii) NFκB-driven proinflammatory responses. Some other important neuropeptide effects on macrophages have been reviewed [32].

Direct cell-to-cell contact may play a deactivating role when a $CD47^+$ target cell ligates and crosslinks signal-regulatory protein-α (SIRPα), which contains an immune receptor tyrosine-based inhibitory motif (ITIM). When phosphorylated, they bind Src homology 2 domain-containing protein tyrosine phosphatases, which interrupt signaling from tyrosine kinase-dependent receptors. This may overcome Fcγ- and/or complement-receptor-mediated signals to phagocytose.

Downregulation can follow from desensitization by internalization and dissociation of the IFN-γ/IFN-R complex. Uptake of apoptotic material generates antiinflammatory

signals. High concentrations of activating stimuli are cytotoxic to macrophages. These stimuli include LPS, TNF and silica particles. The autocrine production of TNF induced by LPS mediates apoptosis in macrophage, and the noninflammatory removal of apoptotic macrophages removes them from the scene.

Regulators of macrophage activation

Microbes

Macrophages can recognize a range of bacteria, virus, parasite and fungi through pathogen-associated molecular patterns (PAMPs) [33]. Microbes have a very limited number of invariant PAMPs (recognition structures), not found in the host cells. They are essential for microbial survival, and even slight changes (of PAMPs) are harmful to microbes. Therefore, they can be safely recognized by a limited set of conserved host receptors that are referred as pattern-recognition receptors (PRRs). PRRs are found intracellularly, on the cell surface and in soluble form in various body fluids. They play major roles in opsonization (e.g. soluble PRRs C-reactive protein and serum amyloid P, already mentioned earlier), phagocytosis and apoptosis.

The conserved lipid-A pattern of LPS of the outer membrane of Gram-negative bacteria is a typical PAMP. LPS (or endotoxin), in complex with LPS-binding protein (a soluble PRR), is recognized by a cell membrane-bound high-affinity LPS PRR known as CD14, acting together with MD-2. CD14 is bound to macrophage cell membrane via a glycosylphosphatidylinositol anchor. To signal, it has to cooperate with a signaling PRR coreceptor, namely TLR4. TLR4 shares a Toll/interleukin-1 receptor (TIR) domain and, therefore, also signal transduction pathway with IL-1 receptor (see above). It also signals via a MyD88-independent, specific pathway. These TLR-specific signaling pathways induce heterogeneity in the ultimate host cell responses [34]. LPS stimulation leads to an effective classical macrophage activation, including IL-12 production. This also includes accessory antigen processing and presenting function, and activation of the slower adaptive immune response to host defence. Some TLRs are relatively monospecific, such as TLR3 for double-stranded RNA (dsRNA), TLR5 for flagellin and TLR9 for unmethylated CpG DNA, whereas others such as TLR2 (acting together with e.g. TLR1 or TLR6) have a broad specificity, including peptidoglycans and yeast zymosan. Fcγ-, complement- and scavenger-receptor ligation, e.g. by opsonized particles or latex microspheres, can even dramatically downregulate IL-12 production. This may confer protection from inadvertent host injury, when the stimulus is rapidly cleared (e.g. opsinized microbes) or when it is nondangerous non-self (e.g. wear debris) [35].

Mature macrophages produce more proinflammatory cytokines upon viral activation than immature monocytes. Influenza A- and Sendai virus-stimulated macrophages produced IL-1β, TNF, IL-18 and antiviral IFN-α/β, whereas monocytes only produced IL-1β or IL-18 in low amounts [36]. IFN-α/β produced by virally infected macrophages induces an antiviral state in fellow macrophages via eukaryotic initiation factor eIF-2 to inhibit protein synthesis and via 2′,5′-oligoadenylate synthetase to degrade viral RNA, which together can prevent a productive viral infection. Virally infected cells can be eliminated via perforin- and granyzme-mediated killing or via Fas, TNF-R1 or DR-mediated apoptosis. Viruses can ‘deregulate’ macrophage activation by producing homologues of cytokines or their receptors, e.g. Epstein Barr virus produces viral IL-10, which deactivates macrophages. Human immunodeficiency virus (HIV) gp 120, engaging CXCR4 and CCR5, activates G proteins, ionic signaling, phosphorylation of p38 and JNK/stress-activated protein kinase (SAPK), mitogen-activated protein kinase (MAPKs) and focal adhesion-related kinase Pyk2, perturbating the macrophage function to such an extent that macrophages (and multinucleated giant cells) form, together with the CD4-lymphocytes, a key HIV target.

Foreign bodies

Literature analysis

The problem with nondigestable debris is particularly prominent with the use of so-called biostable implants used for joint replacement, which is taken as an example. Bulk implants can lead to osseointegration, fibrous capsule formation, regular aseptic loosening or foreign body granuloma reaction [37, 38]. Hydrophobic and cationic substrates increase macrophage adhesion and fusion and decrease their apoptosis [39]. These characteristics also affect biofilm formation. Albumin has a passivating effect, whereas fibrinogen seems to provoke inflammation via engagement of CD11b/CD18 ($\alpha_M\beta_2$ integrin, Mac-1, complement receptor 3bi). Thus, phagocytosis of particles per se does not seem to be necessary for implant material-driven, macrophage-mediated proinflammatory cytokine production. When cytochalasin B was used to reduce phagocytosis of particles by 95%, the release of TNF and IL-6 did not decrease. CD11b/CD18 seems to be enough for this effect by activating tyrosine and serine/threonine kinases, and NFκB and NF-IL-6 [40].

Particle-driven granulomatosis has been considered as an adverse host tissue reaction, characterized by macrophage activation as a key step [41]. The gliding pair of the artificial joint is usually made of UHMWPE-to-metal alloy pair. 4.7×10^5 polyethylene particles are produced at each step from the soft plastic polymer. Most of these particles are of submicron size. Macrophages migrate to par-

ticle accumulation areas in periprosthetic tissue in order to get rid of them. Particles 0.5–10 µm in size are phagocytosed. Particle load, size, shape, rigidity, charge and chemical structure have been considered important for macrophage activation. Implant metal ions (Co, Cr) enhance cytokine release from activated macrophages [42]. Periprosthetic macrophages around loosening joint implants are activated to produce proinflammatory cytokines, which contribute to periprosthetic bone lysis and osteoclast formation [43]. Diminished wear debris formation by the use of diamond coating, ceramics or highly crosslinked UHMWPE could prevent particle-driven macrophage activation.

The host is protected from inadvertent inflammation if bulk or particle phase implant biomaterial induces macrophage apoptosis instead of activation. CoCrMb particles have been considered safer than the less toxic $TiAl_6V_3$ particles because CoCrMb-induced macrophage apoptosis may limit the macrophage-mediated inflammatory response. Similarly, particulate ceramic Al_2O_3 stimulates less TNF and is more apoptotic than UHMWPE [44].

DNA microarray data

To establish the consequences of monocyte-macrophage exposure to nondigestable particle debris, we analysed macrophage activation induced by 0.8–2 µm polymethylmethacrylate particles ('bone cement', a synthetic plastic polymer used to fix joint implants to bone). These particles were used to stimulate peripheral blood mononuclear cells in an LPS-free environment. messenger RNA (mRNA) was isolated at 4, 8, 24 and 72 h and analysed using DNA microarray. In this gene expression study, two types of replications were used: (i) duplicate biological samples were harvested for each experimental condition and time point, and (ii) all samples were run on three to four replicate microarrays on the same day, as described in detail elsewhere [45, 46]. Log transformation of the measured intensities was performed, and outlier intensity values were removed followed by two-step normalization. First, a nonlinear normalization was applied on the replicate miccroarrays for each sample. A second normalization was a sorted nonlinear smoothing spline that was applied across samples. Differential gene expression (ratio) was calculated for the particle-stimulated versus control (nonstimulated) values at each time point. Gene-specific errors (variances) between replicate microarrays were estimated as the pooled variance within each comparison set (after step 1 normalization). This pooled variance and the final normalized ratios of particle-stimulated to control values were used in *t* statistics to calculate *P* values. T statistics were used on normalized data to find those genes which differed at a 0.05 significance level. To reduce the number of genes selected due to random chance, a false discovery rate (FDR) correction [47] was applied. The normalization steps and statistical tests were performed in Splus 6.1 Professional, MathSoft (Seattle, Washington, USA). Genes selected for the final analysis had to satisfy several conditions: (i) differential gene expression (as a result of the particle stimulation) at the *P* value of 0.05, (ii) a significant difference after FDR calculation, and (iii) 1.4-fold change relative to the control for at least one of the time points. For this purpose, gene cluster analysis was performed using OmniViz version 3.5 software (http://www.omniviz.com) to facilitate detection of patterns and to be able to group the

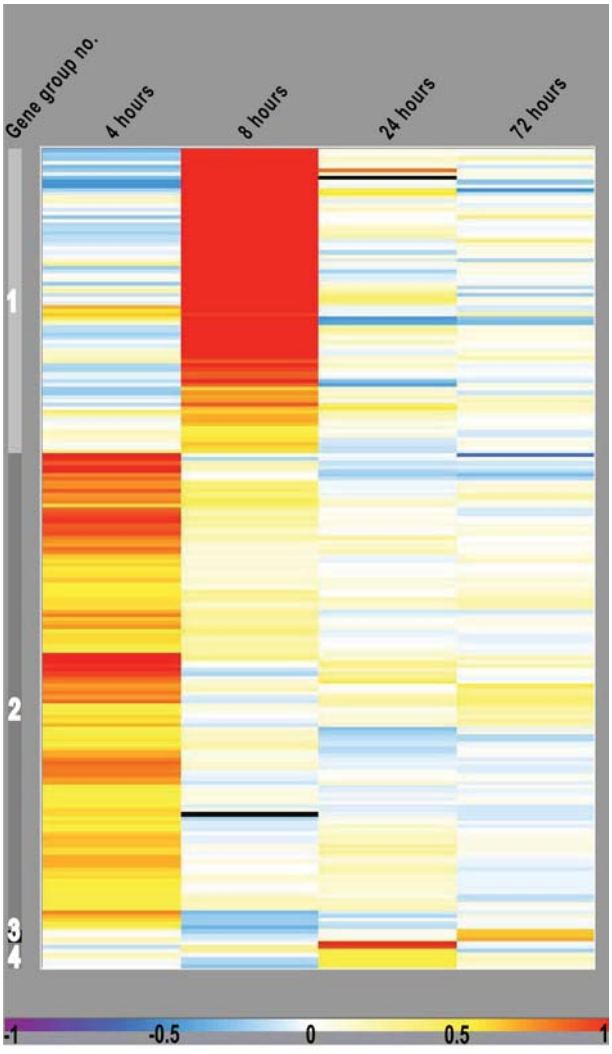

Figure 1. This heat map shows the magnitude and temporal variations of gene expression in monocyte/macrophages as a result of their exposure to polymethylmethacrylate particles (see text for details). Only those genes which were upregulated 1.4-fold or more are shown. Results represent $\log_2$ ratios of nonstimulated controls to stimulated monocyte/macrophages (for values, see color scale below the figure). Genes were grouped (groups 1 through 4) according to the degree of their upregulation. Note that all the genes in group 1 are upregulated at 8 h, the genes in group 2 at 4 h, while the genes of group 3 and 4 mainly at 24 and 72 h, respectively. There is a clear overall dwindling of gene activation at the later time points.

data. An agglomerative hierarchical cluster analysis was performed, using complete linkage and a Euclidean distance metric. Usually, twofold stimulation is used as a cut-off, but in the present experiment polymethylmethacrylate (PMMA) particles were added to monocyte cultures without any further manipulation such as shaking, which would increase the number of particle/cell contacts and could provide additional stress stimulation of the cells. Because the stimulation in our experiment is likely to be mild and transient, we used an unusually low cut-off value.

Figure 1 shows the expression pattern of genes (out of nearly 12,000 genes analysed), which were upregulated 1.4-fold or more and survived the *P*-value false discovery rate calculation. As mentioned, shaking was not used in this experiment, which therefore shows a brisk initial response, that calms down as a function of time. Micromotion has for a long time been considered of importance as a second stimulus for adverse biological host responses against foreign bodies. Table 1 shows the distribution of the upregulated genes across different gene categories. Our microarray data clearly show that most of the upregulated genes were nonsecreted structural proteins rather than secretory products. Confirmatory quantitative studies have been initiated on a relatively small number of the more interesting factors/genes affected. These include genes coding for the integral membrane proteins and receptors, signaling molecules and molecules involved in the metabolic or pharmacological activation of the particle-stimulated macrophages. There seems to be some type of inverse relationship between activation to phagocytosis and secretion [48]. This notion seems to largely agree with the pattern of the gene expression recorded in our experiments, since genes coding for the secretory products such as TNF (and TACE), IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10 (and its receptor), IL-11, IL-12, IL-13, IL-18, CSF-1, M-CSF, GM-CSF, TGF-β, IFF-γ, COX-1, COX-2 and NOS were not induced (not shown) in monocyte/macrophages as a result of particle phagocytosis. Instead, most of the genes induced were rapidly, but only temporarily, upregulated, suggesting monocyte/macrophage priming rather than activation. Thus, it seems that monocyte/macrophages, primed by indigestible particulate matter, require 'regulatory' costimulation by extracellular matrix, LPS and other exogenous soluble, cell-mediated or mechanical stimuli for full-scale activation and/or differentiation.

Foreign body giant cells and epithelioid cells

Activation of macrophages can lead to the formation of foreign body giant cells or osteoclasts. They contain at least three nuclei and are formed as a result of activation, via homotypic fusion aggregation and fusion of mononuclear postmitotic, prefusion cells of the monocyte/macrophage cell lineage. Such cells have a much amplified resorptive capacity against their substrate and occur

Table 1. Distribution of upregulated genes across different gene categories in monocyte-macrophages after exposure to polymethylmethacrylate.*

Gene/gene product category	Time point (%)			
	4 h (N = 125)	8 h (N = 78)	24 h (N = 8)	72 h (N = 3)
By cellular localization				
Localized and structural proteins	42	74	50	66
Secreted and extracellular	14	17	–	–
Cell membrane proteins	41	38	38	–
Receptors	8	26	13	–
Organelle proteins	14	22	38	33
Nuclear proteins	15	21	38	–
By function				
Transmembrane transport	10	12	–	–
Cell signalling (intracellular signal transduction and regulation)	32	54	63	33
Transcription	7	12	25	–
RNA metabolism	9	15	25	
Post-translational protein modification including phosphorylation	17	21	13	67
Cell metabolism	26	40	50	67
Cell growth, development, differentiation and proliferation	17	28	25	–
Protein metabolism, cleavage and degradation	17	21	31	67
Hydrolases	6	6	25	33
Inflammation	5	5	25	–
Immunity	14	19	25	–
Antigen recognition	8	12	–	–

* The number of upregulated genes at a given time point is indicated as *N*. Percentages indicate which proportion of the upregulated genes at a given time point fall into a particular gene category. The same genes/gene products may fall into more than one category.

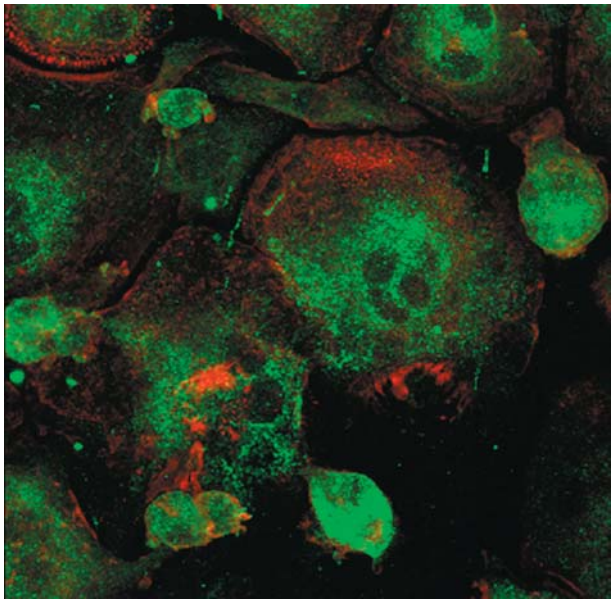

Figure 2. Laser scanning confocal microscope image of human peripheral blood-derived monocyte/macrophages stimulated with M-CSF and RANKL show a phenotype change to large multinucleated cells. They express cathepsin K (green) and begin to polymerize F-actin rings (red). The cells exhibit high motility and capability to form resorption pits when cultured on devitalized cortical bone matrix.

in highly lytic lesions such as osteoclastomas [49] and aseptic loosening of hip implants [50]. Fusion can occur under the influence of either Th2 or Th1 cytokines. Soluble stimuli alone, without particles, such as M-CSF and RANKL (fig. 2) or pseudosynovial peri-implant fluid, can induce osteoclast formation [51]. Another property of many stimuli activating macrophages to polykaryons in vivo seems to be related to large size and/or persistent nature, e.g. parasite, virus, fungus, *M. tuberculosis*, foreign body or bone surface. Rapidly destroyed autologous tissue (debris) in vasculitic diseases may fulfil this requirement. Finally, low pH triggers formation of polykaryons, as has been described around loosening implants [50].

Cell fusion has some important requirements: cell accumulation (density), cell adhesion and aggregation (contact) and membrane fusion (polykaryon formation by fusion). Critical cell density can be achieved in two ways: (i) precursor cells multiply at the site before differentiation and fusion or (ii) cells accumulate to the site from the bloodstream and start to fuse. Chemokine and cytokine production is critical for cell accumulation. It is likely that immigrant cells differentiate and then fuse at the inflammatory site, although resident macrophage proliferation may contribute to this end.

Monocyte adhesion can be mediated by $\beta 2$ integrins, whereas $\beta 1$ integrins are strongly expressed on aggregated/fusing macrophages and foreign body giant cells [52]. However, not all membrane binding results in membrane fusion. In cell-to-cell fusion different donor membrane domains need to be tagged with appropriate adhesion molecules, which are able to act as ligands for their receptors in their acceptor or fusion counterpart membrane. Fusion molecules mediate recognition (adhesion), homotypic binding (aggregation), but above all membrane fusion. They need to overcome the repulsive forces between fusion partners and promote intimate hydrophobic membrane-to-membrane prefusion contacts between the initiator membrane patches. The ligands, receptors and fusion molecules contributing to membrane fusion are collectively referred to as docking molecules. They can be divided into pure binding, pure fusion and binding/fusion proteins [53]. Fusion-specific molecules are induced in polykaryon-directed macrophage activation at the time of fusion, often to disappear from functionally mature cells. Some of them are discussed below.

Proteins belonging to the ADAM (a disintegrin and a metalloproteinase) family are involved in cell-cell fusion processes [54]. Meltrin-α (ADAM 12) participates in myoblast fusion and, most probably, in osteoclast fusion [52]. ADAM 8 increases the number of osteoclasts when present in the fusion stage [55]. ADAMs may bind to integrins via their disintegrin domains, may exist as part of multimeric complexes with binding proteins or they may need other factors to promote fusion. Macrophage fusion receptor (MFR, P84/SHPS-1/SIRPα/BIT) and its ligand CD47 are essential for fusion. MFR probably interacts with CD47 via its immunoglobulin (Ig)V domain, which can be used to block fusion. Soluble extracellular domain of CD44 prevents multinucleation [53].

Osteoblasts (stromal cells) produce RANKL (=osteoclast differentiation factor ODF, osteoprotegerin ligand OPGL, TRANCE). RANKL binds to its receptor RANK, expressed on the surface of (pre)osteoclasts. In the presence of M-CSF, the RANKL-RANK ligation induces osteoclasts. Soluble decoy receptor osteoprotegerin (OPG) binds RANKL and blocks the productive RANKL-RANK interaction. TNF, in the presence of TGF-β, also promotes osteoclast formation, which can be inhibited by IFN-γ [56]. Although foreign body giant cells are morphologically indistinguishable from RANKL-induced osteoclasts, at least avian foreign body giant cells cannot be stimulated with RANKL to resorb bone [53, 57].

Epithelioid granulomas, small and compact nodular collections of chronic inflammatory cells, contain macrophages, foreign body giant cells and epithelioid cells. Macrophage-derived epithelioid cells (i.e. activated macrophages) in the granuloma center are secretory cells. Immunologically active granulomas contain also other cells, mainly T lympocytes. Granulomas are formed in response to protracted stimulation. A classical example of type 1 granuloma is the one seen in tuberculosis. In these instances IL-12 and IFN-γ play important roles as activating stimuli [58]. Usually, evoked and cryptogenic

granulomas are noncaseating. A typical type 2 granuloma is schistosomal antigen elicited and driven by Th2 cytokines IL-4 and IL-13 [59].

Macrophages (autocrine regulation of macrophages by their effector molecules)

There is no agreement on the significance of autocrine macrophage regulation. For example, the proposed autocrine effects of IL-12 and IL-18 on IFN-γ production [60] have been criticized [61]. There seems to be more speculation than direct evidence for autocrine macrophage activation by INF-γ [62] or for existence of a negative autocrine feedback between NO and cytokine production by activated macrophages [63]. Some uncertainty also comes from the lack of agreement whether macrophages can be an efficient source of INF-γ or TGF-β [62] and macrophage heterogeneity. Moreover, there is variation in macrophage regulation and responses to the same mediators, not only within, but also between different species, NO being a good example of a mediator of this type. Nevertheless, autocrine regulation of macrophages by INF-γ, IL-12, IL-10 and MIF may have biological and clinical significance (table 2).

T lymphocytes

T cells regulate activation/deactivation mainly through two types of signals. The first type of signal is to release cytokines (table 3), for example IFN-γ, which is the best-known macrophage-activating factor. The dominant effects on macrophage function of cytokines released by activated Th1 cells are activating, whereas dominant effects of cytokines released by activated Th2 cells are alternatively activating or inhibitory. The second type of signal delivered by CD4 T cells is direct cell-to-cell contact, mainly via CD40-CD154 (CD40 ligand) ligation.

Activated NK cells, Th1 cells and TC1 cytotoxic cells produce IFN-γ. Other effects include strong upregulation of the p40 subunit of IL-12, the key cytokine for the induction of Th1 responses, COX-2, gp91phox component of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and MHC class I and II. The important role of IFN-γ in the regulation of macrophage functions in vivo has been demonstrated by phenotypes of mice with disrupted IFN-γ [64] or IFN-γR genes [65]. These mice showed increased susceptibility to many intracellular pathogens, such as *Listeria monocytogenes* [65] and different viruses [66], which are normally controlled by IFN-γ-mediated cellular responses, including macrophage activation.

IL-10 was first described as a cytokine synthesis inhibitory factor (CSIF), produced by mouse Th2 cells [67]. The ability of IL-10 to inhibit cytokine production by Th1 cells was found to be indirect, via inhibition of monocyte/macrophage function [68, 69]. IL-10 profoundly inhibits a broad spectrum of activated macrophage functions (table 4). In addition, IL-10 enhances expression of interleukin-1 receptor antagonist (IL-1Ra) and soluble p55 and p75 TNF-R by monocytes. IL-10 produced by regulatory T cells may also be involved in regulation of important macrophage functions, e.g. to maintain self-tolerance.

Table 2. Products of macrophages with potential role in their autocrine regulation.

Mediator	Effect*
IFN-γ	stimulation and priming of macrophages
TNF	proinflammatory; regulation of apoptosis; priming
IL-1β	$\uparrow$ of production of G-CSF and IL-1β; regulation of apoptosis
IL-6	$\downarrow$ of GM-CSF and TNF gene expression
IL-8	$\uparrow$ of production of IL-8 through CXCR1/MAPK
IL-10	$\downarrow$ production of GM-CSF; regulation of IL-12 and TNF production; regulatory effect on dendritic cells
IL-12	regulation of TNF, IL-1β, IL-6 and NO production; synergy with IL-18 in stimulation of INF-γ production by BM-derived macrophages
IL-18	augmentation of IL-12-induced INF-γ production by dendritic cells; synergy with IL-12 in stimulating INF-γ production by BM-derived macrophages
IL-15	regulatory effect on other cytokine production
MIF	regulation of TNF and toll-like receptor 4 production; inhibition of p53
HMGB1	proinflammatory
M-CSF	sensitization to ECM proteins (e.g. fibronectin and several integrins)
GM-CSF	regulation of apoptosis
TGF-β	regulation of proinflammatory cytokine production and macrophage differentiation
NO	inhibition of proinflammatory cytokine production
PGE_2	regulation of proinflammatory cytokine production
TXA_2	regulation of TNF and IL-1β synthesis

* A proinflammatory effect is defined as one, few or all of the following: production of proinflammatory cytokines, lipid mediators and reactive oxygen species. $\uparrow$, stimulation; $\downarrow$, inhibition; BM, bone marrow; HMGB1, high mobility group B1; MIF, macrophage migration inhibitory factor; TXA2, thromboxane A2; CXCR1, chemokine C-X-C receptor 1.

Table 3. Dominant effects of T-cell-derived cytokines on macrophage function.

Classical activation effects	
Cytokines	Major T cell source
IFN-γ	Th1
TNF	Th1
TNF-β	Th1
IL-2	Th1
GM-CSF	Th1
IFN-α/IFN-β	T cell
Alternate activation effects	
IL-4	Th2, regulatory T cell subets
IL-13	Th2
Inhibitory effects	
TGF-β*	T cells, regulatory T cell subsets
IL-10	Th2, regulatory T cell subsets

* TGF-β has either a stimulatory or inhibitory effect on monocyte/macrophage function, depending on the other cytokines present and the state of differentiation or tissue origin of the cells. Combinations of cytokines, in situ, may have different effects on monocyte/macrophages than the sum of the parts. For example, IFN-γ and IFN-α/β can antagonize each other, probably by competition for signaling pathways.

Table 4. Inhibitory effects of IL-10 on activated monocyte/macrophage functions.

Inhibition of cytokine production: IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF and PAF
Inhibition of chemokine production:
CC chemokines (MCP1, MCP-5, MIP-1α, MIP-1β, MIP-3α, MIP-3β, RANTES, MDC)
CXC chemokines (IL-8, IP-10, MIP-2, GRO-α)
Downregulation of the expression of MHC class II and costimulatory molecules
CD80 and CD86
Inhibition of production of NO and PGE_2
Inhibition of expression of matrix metalloproteinases: gelatinase and collagenase

LIF, leukemia inhibitory factor; PAF, platelet activating factor; CC and CXC chemokines, refer to the arrangement of their N-terminal cysteine residues; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T-cell expressed and secreted; IP-10, interferon-inducible protein 10; KC, keratinocyte-derived chemokine; GRO = growth-related oncogene.

TGF-β has pleiotropic effects on monocyte/macrophage function, either stimulatory or inhibitory, depending on the local cytokine environment and the state of differentiation or tissue origin of the cells. Effects of TGF-β on peripheral blood monocytes are mainly activating, including production of a variety of cytokines, induction of a chemotactic response, enhancement of phagocytic activity, upregulation of the expression of several cell surface receptors, such as Fc-γ receptor and adhesion receptors, LFA-1 (LFA, lymphocyte function-associated antigen) and VLA-3 ($\alpha_3\beta_1$; VLA, very late activation antigen).

In contrast to the activating effects of TGF-β on monocytes, its actions on tissue macrophages are generally suppressive. This has been attributed to a difference in the expression of receptors for TGF-β on these two cell populations [70]. Suppressive effects of TGF-β on activated macrophages include its ability to modulate the activating cytokines similar to effects of IL-10. One of the most important deactivating effects of TGF-β on macrophages is to limit the production of reactive oxygen and nitrogen intermediates by cells activated by either IFN-γ or LPS.

Preactivation of T cells is required for the activation of macrophages. Ligation of CD40 on the surface of monocytes was determined to be one of the major T-cell-generated signals for the activation of macrophages (table 5). The contact-dependent interactions between T cells and macrophages through CD40-CD154 provide bidirectional stimulatory signals that are important in the activation of specific T cells and in the regulation of activation of macrophages as well. Indeed, cell-to-cell contact via CD40-CD154 interactions is required for production of IFN-γ and IL-12 by macrophages [71, 72]. Furthermore, studies with T cells from CD154-deficient mice also indicate the requirement of CD40-CD154 interaction in activation of macrophages to produce TNF and NO in vivo [73]. Increasing evidence suggests that CD40-CD154 interactions not only have a role in control of infection by activated macrophages, but also play an important role in inflammatory and autoimmune diseases, such as in atherosclerosis. Other costimulatory molecules may play a role in contact-mediated macrophage activation. Jean-Michel Dayer has pointed out that high-density lipoprotein-associated apolipoprotein A-I, a negative acute phase reactant, can inhibit contact-mediated activation of macrophages [74], which has interesting implications for sex steroid effects on macrophage activation. Estrogens upregulate apolipoprotein A-I promoter activity. Androgens are believed to block the actions of estrogens.

Hormones (table 6)

Monocytes/macrophages have classical cytosolic estrogen (ERα and ERβ) and also androgen receptors (ARs) as shown by Maurizio Cutolo [75]. In addition, both estrogens and androgens have unconventional surface receptors, which mediate rapid actions. The promoters of the key inflammatory cytokines lack elements responsive to these ligand-receptor complexes (transcription factors), which seem to regulate by interfering with the action of other transcription factors, such as NFκB and AP-1. Progesterone receptors have not been observed on human macrophages. Many autoimmune diseases are more common in women than in men. Therefore, one could perhaps expect that estrogens and progesterone enhance, and testosterone decreases, monocyte/macrophage activation. However, progesterone, 17β-estradiol and testosterone decrease LPS-induced, human monocyte-mediated IL-1α and IL-1β production [76]. Another study showed that

Table 5. Major effects of CD40-CD154 ligation on macrophage function.

Augmentation of APC function: stimulation of costimulatory function
Induction of cytokine/chemokine production: IL-1α/β, IL-6, IL-8, IL-10, IL-12, TNF, MIP-1α
Stimulation of NO production
Induction of metalloproteinase production
Rescue from apoptosis

Table 6. Hormone effects on macrophage activation.

Hormones	Effects
Epinephrine	+/– macrophage activity
Norepinephrine	+/– macrophage activity
Glucocorticoids	+/– NO, IL-1 production
ACTH	– macrophage activation
PTH	+ osteoclast activation
$1,25(OH)_2D_3$	+ osteoclast activation
PGE_2	– cytokine expression
Estrogen	+ phagocytosis, +/– TNF, IL-1 production
Progesterone	– phagocytosis, +/– TNF, IL-1 production
Testosterone	+/–IL-1 production, + superoxide production
Estradiol	+ IL-1 production, – phagocytosis
Dihydrotestosterone	– phagocytosis, –NO, IL-1 production

+, stimulation; –, inhibition; ACTH, adrenocorticotropin; PTH, parathyroid hormone.

estradiol and progesterone decreased LPS-induced, monocyte-mediated IL-1β messenger RNA (mRNA) in a dose-dependent manner [77]. Human monocytes in this study were harvested from women during the luteal phase of the menstrual cycle. In postmenopausal women physiological concentrations of 17β-estradiol decreased spontaneous (but not LPS-induced) IL-1β, TNF and IL-6 production in whole blood cultures [78]. This action was blocked by the use of the antiestrogen ICI 182780 and not caused by the inactive form of estrogen 17α-estradiol. Therefore, although conflicting data have also been presented (reviewed [79]), it seems that both estrogens and androgens diminish classical monocyte/macrophage activation. Lack of estrogens (menopause) predisposes to osteoporosis, to which probably IL-1β, TNF and IL-6 contribute. Estrogens increased IL-1R2 decoy receptor and decreased the proinflammatory IL-1R1 receptor in osteoclast-like cells [80].

Systemic inflammation, as a stressful stimulus, activates the hypothalamus – pituitary – adrenal (HPA) axis and the sympathetic nervous system. Neural input to hypothalamus in response to stress leads to release of corticotropin-releasing hormone (CRH), which stimulates release of corticotropin (i.e. ACTH) from the pituitary stimulating release of glucocorticosteroids from the adrenal cortex. Neural input to locus coeruleus in response to stress leads to release of norepinephrine from sympathetic nerves and epinephrine from the adrenal cortex. These stress hormones, acting on glucocorticosteroid receptor and β2-adrenergic receptor on monocyte/macrophages, inhibit the production of proinflammatory cytokines TNF and IL-12 [81]. Catecholamines also increase IL-10 production. However, in some tissue compartments the α2-adrenergic component is predominant, and then norepinephrine can increase stimulated macrophage-mediated TNF production. Stress response-induced negative feedback helps to control the inflammatory damage.

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