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REVIEW

Functional Phenotypes of Macrophages and the M1–M2 Polarization Concept. Part I. Proinflammatory Phenotype

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Abstract—Current concepts concerning the main functional phenotypes of mononuclear phagocytes are systematized, molecular mechanisms of their formation are considered, and the functional polarization concept of macrophages is critically analyzed. Mechanisms of macrophage priming activation mediated by pattern recognition receptors TLR, NLR, RLR, and CLR are described, and the features of each phenotype acquired via various pattern recognition receptors are emphasized. It is concluded that there is a huge variety of proinflammatory phenotypes from highly to poorly polarized ones. Thus the widespread notion of "classical activation" of macrophage concerns just a particular case of proinflammatory phenotype formation.

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Key words: macrophage, polarization, phenotype, TLR, NLR, RLR, CLR

Opposing "programs" of functional activity of macrophages are described in a great number of works on macrophage immunobiology: pro- and anti-inflammatory; immunogenic and tolerizing; destructive and reparative [1]. The concept of formation of definitive functional macrophage (Mph) phenotypes, mainly biocidal-destructive or reparative-fibrous, has become widespread and acquired paradigm status. This concept is opposed by the notion of macrophage differentiation and activation as a dynamic continuum of transitory functional states [2]. This review deals with both approaches. This article is the first part of a review and deals with mechanisms of formation and comparative characteristics of proinflammatory phenotypes induced by activation of pattern recognition receptors TLR, NLR, RLR, and CLR.

Abbreviations: AIM2, absent in melanoma 2; ASC, CARD domain containing apoptosis-associated speck-like protein; CARD, caspase activating and recruiting domain; CLR, C-type lectin receptors; CTLL, C-type lectin-like receptors; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, danger/damage-associated molecular patterns; DISC, death-inducing signaling complex; ERK, extracellular signal-regulated kinases; FADD, Fas-associated protein with death domain; HIN200, hematopoietic IFN-inducible nuclear protein with 200-amino-acid repeat; HMGB1, high mobility group box 1 protein; IFI16, γ-interferon-inducible protein 16; IFN, interferon; IL, interleukin; iNOS, inducible NO synthase; IPS1, stimulator 1 of IFN-β promoter; IRAK1, IL-1 receptor-associated kinase 1; IRF, IFN regulatory factor; ISRE, IFN-stimulated response elements; ITAM, immunoreceptor tyrosine-based activation motif; JNK, Jun N-terminal protein kinase; LBP, LPS-binding protein; LPS, lipopolysaccharide; LRR, leucine-rich repeat domain; MAPK, mitogen-activated protein kinases; MD2, myeloid differentiation protein-2; MDA5, melanoma differentiation associated gene 5; Mph, macrophage; NK, natural killer; NLR, nucleotide oligomerization domain (NOD)-like receptors; PAMP, pathogen-associated molecular patterns; PRR, pattern recognition receptors; RIP1, receptor-interacting protein 1; RLR, retinoic acid-inducible gene-I-like receptors; STAT, signal transducer and transcription activator; STING, stimulator of interferon genes; TAB1, TAK1-binding protein; TAK1, TGF-β activated kinase 1; TGF, transforming growth factor; TIR, Toll/IL-1R domain; TIRAP, Toll receptor IL-1R domain-containing adaptor protein; TLR, toll-like receptors; TNF, tumor necrosis factor; TRADD, TNFR1-associated death domain; TRAF, TNF-receptor-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor-inducing IFN-β.

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MACROPHAGE PRIMING AND CLASSICAL ACTIVATION

General characteristics of classical activation. At the beginning of the 1960s, George Mackaness showed that Mph cells of mice infected with facultative intracellular bacteria, in particular with *Listeria monocytogenes*, are capable to attack nonspecifically the infectious agents of different nature [3, 4]. This was the beginning of a burst of works on biology of the phenomenon that Mackaness called "macrophage activation". Although the term "macrophage activation" appeared in the 1960s, the concept of Mph activation emerged a century earlier, when I. I. Mechnikov found that phagocytosing mononuclear cells from animals resistant to certain infections are able to efficiently eliminate these bacteria [5]. Mackaness' concepts of Mph reactivity were further developed in pioneering works of K. Nathan, M. Karnovsky, H. Murray, and other authors who used *ex vivo* and *in vitro* model systems and showed that, depending on conditioning character, mononuclear phagocytes acquire different definitive morphofunctional phenotypes and different ability to respond to stimuli (to be activated) [6]. Mackaness and other authors also showed that intraperitoneal administration of thioglycolate causes influx into the peritoneum of Mph cells devoid of cytotoxicity towards other cells but exhibiting ability to secrete lysosomal enzymes and to adhere to the substrate surfaces more efficiently than the original resident Mph cells [7]. Later these authors showed that Mph cells recruited into peritoneum during T-cell response induced by intraperitoneal introduction of purified derivative of mycobacterial protein not only secrete increased amount of lysosomal enzymes, but that they also acquire high cytotoxicity as well. These and similar experiments resulted in a concept of formation of elicited and activated Mph cells.

At the end of 1970s and the beginning of the 1980s, Karnovsky et al. [8] developed the first classification of Mph phenotypes based on their functional activity (biocidal activity and ability to generate respiratory burst). Four cell conditions were distinguished: quiescent cells, elicited Mph cells, activated Mph cells, and Mph cells conditioned by lymphocyte supernatants.

Quiescent cells are Mph-resident cells weakly responding to stimulus and having low functional activity, while elicited cells are those recruited into an inflammatory focus and acquiring increased locomotor and phagocytic activities, high ability to adhere and spread over a support surface, and to secrete lysosomal enzymes. Together with features characteristic of elicited Mph cells, activated Mph cells are characterized by additional very high biocidal activity, and they efficiently eliminate tumor cells and microorganisms including intracellular parasites (*Protozoa*, *Toxoplasma*, *Leishmania*, etc.). Such mononuclear phagocytes have also been called "armed". Mph cells obtained from inflammation foci with high contamination of T cells or those treated by supernatants of activated lymphocytes were characterized by properties similar to those of elicited Mph cells, but lipopolysaccharide (LPS) or different stimuli caused much more rapid, compared to elicited Mph cells, emergence of features of fully activated Mph cells. The high biocidal ability of activated Mph cells was explained by generation of active oxygen species during respiratory burst [9], but hyperreactivity of these cells after conditioning remained unexplained.

According to current concepts, the original classification of Karnovsky seems quite adequate because it reflects real differentiation conditions of mononuclear phagocytes. Lymphocyte-conditioned Mph cells are now called "primed", and their high reactivity is explained by the sensitizing effect of interferon-γ (IFN-γ) derived from activated Th1 cells, and to a lower extent by the effect of some various cytokines such as CD40L/CD154 [10, 11]. The pronounced biocidal-destructive ability of activated Mph cells proper is now explained not only by the effect of lysosomal enzymes and production of reactive oxygen species, but also by formation of reactive nitrogen species (NO, etc.), and expression and secretion of TNF-α [12, 13] and other cytokines. The TNF-α secretion is considered as an inherent pathognomonic feature of Mph activation, whereas production and secretion of different factors like IL-1β, IL-6, IL-8, CD11b/CD18, eicosanoids, lysosomal hydrolases, reactive O_2 and N species, etc. is typical of activated Mph cells but is not obligatory [14].

So, quiescent Mph-resident cells exhibit moderate ability to response to a stimulus and to be activated. Elicited Mph cells react more efficiently, i.e. they are "sensitized" or "primed", but more efficient priming is carried out in response to the product of activated T-lymphocytes and of NK cells – IFN-γ. In other words, the classical Mph activation requires no less than two consecutive signals, priming (IFN-γ) and triggering. The latter is mediated by conservative molecules immanently associated/typing various classes of microorganisms (pathogen-associated molecular patterns). The Mph recognizes these molecules via toll-like receptors (TLR) specialized for all main pathogen classes. LPS is recognized via TLR4, lipopeptides via TLR1, 2, and 6, flagellin via TLR5, double-stranded RNA and its synthetic analog poly(I:C) via TLR3, and nucleic acids via TLR7, 8, and 9 (TLR7 and 8 are sensors of single-stranded RNA, and TLR9 is a sensor of CpG DNA). Many TLR are able to recognize endogenous ligands such as fibronectin domain A synthesized in response to damage, or heat shock proteins in normal conditions localized in the cytoplasm and accessible for TLR only after cell damage or death [15]. At the present time over 12 types of human TLR are described.

In vivo different agents including LPS, polynucleotides, pathogens of viral diseases, malaria, tuberculosis, corynebacteria, *Listeria*, *Staphylococcus aureus*, some

lactobacteria, and acidic polymers induce secretion of IFN-γ [16], thus providing for the first priming signal. Priming stimulates the influx of Mph cells into the focus, thus sharply increasing CCR2 expression in monocyte-macrophages and their chemotactic abilities [17]. Besides, in response to IFN-γ Mph cells produce IL-12/IL-18 that recruit into the inflammation zone NK and Th1 cells and stimulate IFN-γ production in them. Priming is followed by trigger signal.

The succession of events *priming by interferon-γ → TLR-mediated activation* is well documented in numerous works. However, according to some observations TLR agonists can activate Mph without IFN-γ, or on addition to Mph of IFN-γ and TLR agonists in sequence opposite to the classical one [18]. It is assumed that this is due to auto- and paracrine effect of Mph-produced cytokines and synergism of IFN-γ and TLR signal transduction pathways [18]. An example of autocrine induction of classical activation is the effect of TNF-α that in cooperation with CD40 and adaptor protein TRAF-6 (TNRF-associated factor-6) itself induces in Mph high antimicrobial activity even independently of IFN-γ [11]. The presence of TNF-α in an inflammation focus as triggering signal can bring monocyte-macrophages migrating into the infiltrate region to activated condition and cause a self-maintained pathological process even in the absence of products of infection agents. In our opinion, the possibility of IFN-γ-independent sensitization and activation of Mph cells means that the term "primed Mph" includes cell phenotypes that are not identical.

Criteria of classical activation. Since TNF-α is a good inducer of inducible NO synthase (iNOS), activated mouse Mph cells can be easily identified by NO production [19, 20]. The priming of Mph cells by IFN-γ results in NO production only when IFN-γ is contaminated with LPS, which often occurs in commercial preparations. Human monocyte-macrophages synthesize almost no NO in response to classical activating stimuli. Therefore, in addition to TNF-α, not NO generation but rather the ability of Mph cells to express MHCII and CD86 molecules, to present antigens, to eliminate intracellular pathogens, and to produce IL-12, IL-1β, IL-6, and IL-15 is used as criteria of Mph activation quality. The use of the microarray technique made the situation more complicated, because it became clear that Mph activation changes expression of at least 25% of genes, which makes necessary the selection of new real and reliable activation criteria from several hundreds of genes [21]. Moreover, Mph activation involves epigenetic factors that may be such criteria, for example, in response to LPS many histone deacetylases are transiently repressed and then are induced [22].

Molecular mechanisms of classical activation. Priming. The IFN-γ-induced monocyte-macrophage priming is an extremely complicated and poorly understood cross- and autoregulated process. It is launched via IFN-γ receptors R1 and R2 (Fig. 1) activating receptor-associated Jak tyrosinases, which results in phosphorylation of latent cytoplasmic STAT (signal transducer and activator of transcription) proteins, their dimerization, and translocation into the nucleus where they bind to appropriate promoter sequences and activate transcription. Phosphorylation of transcription-activating domains at serine residues potentiates the STAT transcription activity. Different kinases can be involved in this potentiation, including MAPK (mitogen-activated protein kinases), protein kinase C, and calmodulin-dependent protein kinase II. IFN-γ activates mainly STAT1 [23].

In addition to STAT1 homodimer binding to responsive elements of promoters of IFN-γ-responding genes (GAS elements), including genes of IFN regulatory factors (IRF1, 8, etc.), response to IFN-γ involves the IFN-stimulated response elements (ISRE) induced by type I IFN, octamer element, etc. IRF functions are controlled by DNA methylation and other mechanisms [24]. The interaction pattern is complicated because STAT1 is able to induce expression of different transcription factors such as CIITA (class II transactivator) that, in turn, stimulate work of their own target genes. In particular, CIITA induces expression of major histocompatibility complex (MHC) class II genes. Priming does not activate Mph cells, but it dramatically increases intracellular STAT1 content, thus making cells hyperreactive towards re-stimulation by lowest doses of IFN-γ (0.1-1 unit/ml). Simultaneously IFN-γ induces the following priming mechanisms: increase in TLR expression and activity; synergistic activation of promoters by transcription factors NF-κB and STAT1 binding to their own promoter elements; potentiation of NF-κB activation; additive functions of NF-κB/MAPK- and STAT1-induced target genes [23, 25]. The latter include, in particular, the STAT1-induced CD40 production blocked by inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMGR), a key enzyme of the mevalonate metabolic pathway.

It is known that the prenylating isoprenoids farnesyl and geranylgeranyl that posttranslationally modify numerous regulatory proteins including large and small G-proteins are generated in this pathway. Our data show [26, 27] that mevalonate and other factors increasing the intracellular pool of prenylating isoprenoids exhibit the Mph-priming effect. At the same time, reduction of this pool in response to HMGR inhibitors blocks the classical Mph activation. This correlates with data on the selective activation of HMGR and inhibition of squalene synthase by proinflammatory stimulation [28] and the IFN-γ-stimulated increase in the prenylating isoprenoid pool in Mph [29, 30]. Evidently, prenylation of G-proteins, including those of Ras family like Cdc42 [25], Rac1, RhoA, etc., and of the Rab family, is extremely important for Mph priming because these proteins mediate intracellular signal transduction cascades from IFN-γ receptors

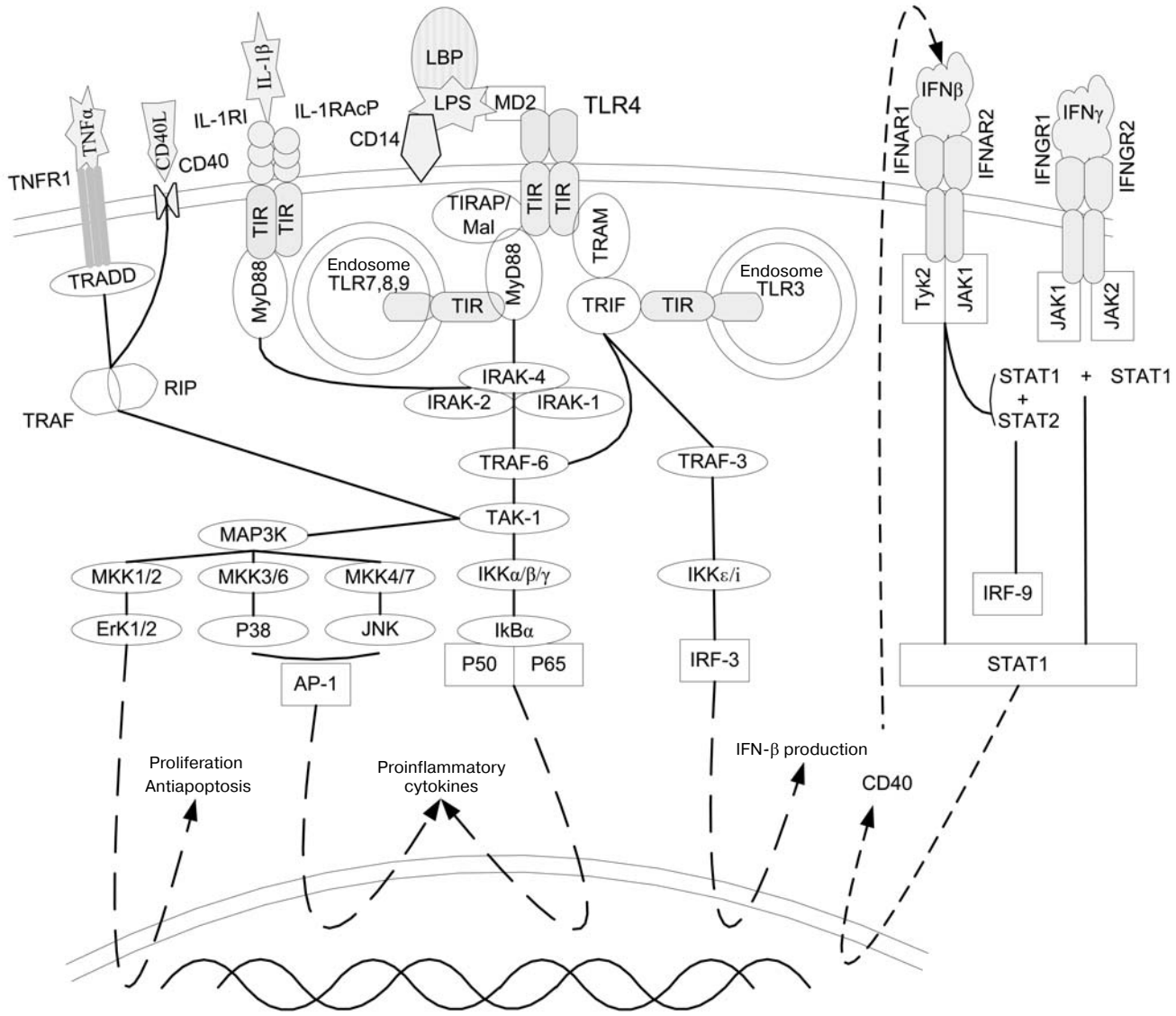

Fig. 1. Signal transduction mechanisms upon classical activation of Mph cells. Receptors TLR3, TLR7/8, and TLR9 are located mainly in endosomes, while the rest, including TLR2, TLR4, TLR5, and TLR11, are located on the plasma membrane. After binding to appropriate ligands and recruiting adaptor molecules MyD88 (all TLR except TLR3) and TRIF (TLR3 and TLR4), protein kinases IRAK4, TRAF6, and IKKε/TBKI undergo activation, which results in activation of transcription factors NF-κB, AP1, IRF3, and STAT1 with following production of type I IFN and proinflammatory cytokines.

[31], costimulatory molecules such as CD40 [32], proinflammatory cytokines [33], and TLR. The same proteins mediate NADPH-oxidase activation upon respiratory burst and actin cytoskeleton rearrangement upon formation of filopodia, lamellipodia, and invaginations [34] during locomotion, adhesion, phagocytosis, endosome traffic, etc. Although molecular co-stimulation mechanisms necessary for complete Mph activation are not quite clear, it is evident that cooperative action of several transcription factors is necessary for optimal induction of at least some mediators and cytokines. As mentioned above, both direct interactions between IFN-γ and TLR signal pathways and autocrine mechanisms using TNF-α, IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), and also other factors can be involved in Mph activation [17, 35, 36].

Triggering signal. Bacterial LPS are the best-studied triggering signal. Great part of LPS circulate in the bloodstream in complexes with high affinity proteins, first of all with LPS-binding protein (LBP). The LPS–LBP complex binds membrane receptor CD14 of monocyte-macrophages (Fig. 1) anchored at glycosyl-phosphatidylinositol [37], then CD14 presents this complex to TLR4 [38], thus launching TL4 dimerization and extracellular dimer fragment association with MD2 protein (myeloid differentiation protein-2) carrying the leucine-

rich repeat (LRR) domain [39]. The incorporation of TLR4–MD2 complex into cholesterol-rich membrane microdomains (lipid rafts) is necessary for LPS recognition [40]. Like in other TLR and in IL-1 receptor (IL-1R), the cytoplasmic terminus of TLR4 contains highly conservative TIR residue (Toll-IL-1R) [41], while the ectodomain of all TLR contains LRR responsible for ligand recognition. In the case of TIR-mediated TLR4 dimerization, TIR domain recruits adaptor molecules MyD88 and TIRAP (Toll receptor IL-1R domain-containing adaptor protein), thus launching an intracellular signal cascade resulting in activation of transcription factors NF-κB, JNK, p38-kinase, etc. [42]. This cascade includes recruiting of IL-1R-associated serine-threonine kinase-1 (IRAK1) to MyD88, recruiting of IRAK4 phosphorylating/activating IRAK1, and formation of IRAK4/2/1–TRAF6 (tumor necrosis factor receptor-activated factor 6) complex. This results in TRAF6 oligomerization and acquiring ligase activity, the IRAK–TRAF6 complex undergoes polyubiquitination and degrades, thus activating the TAK1-binding protein (TAB1) and the TGF-β-activated kinase (TAK1) necessary for classical activation of NF-κB and mitogen-activated protein kinases.

There is a different, MyD88-independent pathway of signal transduction from LPS using TRIF (TIR-domain-containing adapter-inducing IFN-β) and TRAM (TRIF-related adaptor molecule) (Fig. 1). For membrane colocalization with TLR4, TRAM undergoes myristoylation [43]. Like the first pathway, this TRIF-dependent pathway activates NF-κB via TRAF6, but unlike the MyD88-dependent one it results in activation of IRF3, IRF3-mediated expression of IFN-β (type I IFN), and IFN-inducible chemokines such as CXCL10, CCL5, and CCL2 [44]. Transcription/expression of IFN-β is caused by IRF3 dimerization, dimer entry into the nucleus, its interaction with coactivator p300/CBP, and binding within the enhanceosome to ISRE in the IFN-β gene promotor. It is important that, in turn, IFN-β is capable of autocrine binding to its own membrane receptor and activation of signal pathway Jak/STAT1 launching iNOS transcription and NO generation [41]. Along with STAT1, IFN-β activates STAT2, which results in STAT1 heterodimerization with STAT2, dimer translocation into the cell nucleus and association with IRF9, and formation of a heterocomplex of IFN-stimulated gene factor 3 (ISGF3) interacting with ISRE in numerous gene promoters. Thus, LPS recruits various STAT-dependent mechanisms, and in this way it itself is able to provide for Mph priming and further activation, though with significantly lower dynamics.

Nonclassical mechanisms of "classical" Mph activation. Recognition of pathogens by Mph is carried out both via TLR and different membrane and cytosol pattern recognition receptors (PRR) interacting with pathogen-associated molecular patterns (PAMP). Along with TLR, they include C type membrane lectin receptors (CLR), cytosol NOD-like receptors (NLR), and RIG (encoding retinoic acid-inducible gene-I)-like helicase receptors (RLRs). As shown below, the proinflammatory activation of Mph cells via these receptors differs significantly from "classical".

NLR. Over 20 NOD-like receptors have been described in humans and over 30 in mice. All of them have the three-domain structure (shown schematically for the example of NLRP in Fig. 2): central nucleotide-binding domain (NBD or NOD), C-terminal variable leucine-rich repeat LRR (similarly to PAMP-recognizing LRR motives of TLR ectodomains), and N-terminal effector domain. Depending on the effector domain type, NLR are subdivided to not less than five subfamilies: NLRA, i.e. containing acidic transactivating domain; NLRB (NLR apoptosis inhibitory protein (NAIP)) containing the BIR domain (baculovirus inhibitor of apoptosis repeat); NLRC (NOD) containing CARD domain (caspase activating and recruitment domain); NLRP (NALP) containing the pyrin domain (PYR), and NLRX containing an unknown domain. NLRs recognize both PAMP of infectious agents such as LPS, flagellin, bacterial and viral nucleic acids, components of yeast cell walls, and different noninfectious danger signals ("alarmins" or danger-associated molecular patterns – DAMP) such as molecular markers of cell destruction including non-histone chromosomal protein HMGB1 and heat-shock proteins, calcium-binding S100 proteins (S100A8, S100A9, S100A12), ATP generated by infectious pathogens or entering the environment from damaged necrotic cells or TLR-stimulated monocytes, altered proteins of extracellular matrix, β-amyloid, hyaluronate and heparan sulfate, as well as crystals including those of uric acid, asbestos, cholesterol, silicon dioxide, calcium pyrophosphate, and aluminum salts [45]. Despite the sensory role of NLRs for PAMP and DAMP, no specific PAMP or DAMP ligands interacting with NLRs have been identified yet [46]. Nevertheless, it is known that the interaction of PAMP or DAMP with NLRs can result in formation of large (700 kDa) cytoplasmic multimolecular complexes ("inflammasomes") activating inflammation caspases, first of all, caspase-1 (formerly IL-1β converting enzyme (ICE)) necessary for proteolytic activation of IL-1β and IL-18, and for proteolysis of IL-33. Such complexes are similar to the Apaf-1-containing "apoptosomes", multiprotein structures complexing for caspase-9 activation, and to caspase-8 activating apoptogenic signal complex Fas/CD95-DISC. An apoptogenic process with involvement of caspase-1 acquired the name of "pyroptosis" – it lacks some essential features of classical apoptosis and has some symptoms of cell necrotic death [47, 48]. Four types of inflammasomes are best studied: pyrin-containing NLRP/NALP1, 2, and 3 and CARD-containing NLRC4/IPAF (IL-1β-converting enzyme protease-activating factor).

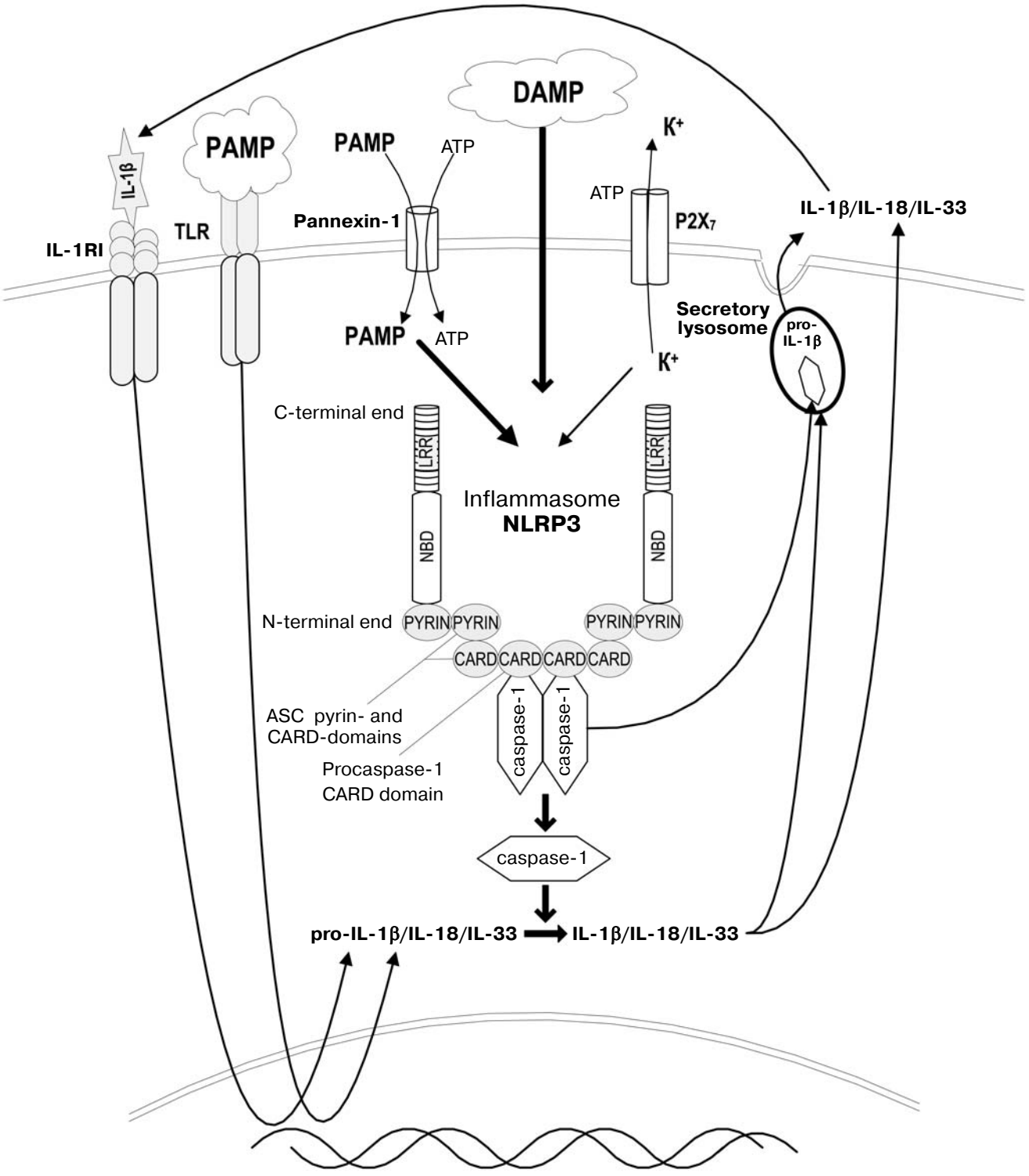

Fig. 2. Mechanisms of caspase-1 activation by NLR3 inflammasomes and secretion of the IL-1 cytokine family in Mph cells. PAMP molecules affect membrane TLRs or *via* a pannexin-1-mediated way enter the cytoplasm where they interact with NLR3. DAMP molecules interact mainly with NLR3, entering the cytoplasm through variable receptor-mediated interiorization mechanisms. The TLR or IL-1 receptor activation induces transcription and translation of IL-1-cytokine precursors. Activation of P2X7 receptors by extracellular ATP, potassium release from Mph cells, and decrease in its intracellular concentration launch NLRP3 complex formation with procaspase-1 molecules and formation of active NLRP3 inflammasomes. The processing of procaspase-1 by inflammasomes in the cytosol or secretory lysosomes results in formation of active caspase that transforms IL-1 precursors into active molecules secreted by macrophages. In turn, the secreted IL-1 can influence in an autocrine way the receptor for IL-1, maintaining production of pro-IL-1 and different proinflammatory cytokines via the cytosol TIR domain similar to those in TLR.

NLRP3/NALP-3/Cryopyrin-inflammasomes. Unlike different type inflammasomes activated by a limited number of PAMP/conservative microbial ligands, these inflammasomes are activated by many various PAMP and DAMP, including LPS, muramyl dipeptide, double-stranded RNA, lipoteichoic acid, peptidoglycan, hyaluronate, etc. The organization of NLRP3 inflammasomes and NLRP3-mediated signaling are shown schematically in Fig. 2. Note that adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), carrying pyrin and CARD domains, is involved in NLRP3 formation/activation. ASC recruits procaspase-1 to NLR at the expense of pyrin-mediated CARD–CARD interactions, which results in caspase-1 activation. Caspase-1, in turn, transforms inactive IL-1β and -18 forms into active secreting forms. It is known that caspase-1 activation is most pronounced when PAMP and DAMP affect Mph cells in the presence of ATP [49]. Therefore, it is logical to assume that PAMP and DAMP, influencing NLRP3, prime Mph cells for ATP trigger effect on production of IL-1 family cytokines. It seems that it is an absolutely special form of priming: it does not directly concern transcription mechanisms and relates only to a narrow spectrum of proinflammatory cytokines, to the IL-1 family.

The mechanism of the ATP priming effect is associated with activation of membrane purinergic receptors/channels P2X7, releasing from cells potassium ions important for NLRP3 (and NLRP1) activation. Therefore, similarly to the ATP effect, NLRP3 activation is caused by microbial toxins with K^+-ionophore effect such as nigericin, aerolysin, maitotoxin, gramicidin, and α-toxin. Activated P2X7 receptors recruit protein pannexin-1 forming its channels on the cell membrane. Pannexin is supposed to provide for penetration of PAMP, activating inflammasomes, into the cytoplasm (Fig. 2). In addition, the ATP-induced activation of P2X7 stimulated generation of reactive oxygen metabolites, able to activate inflammasomes, whereas blocking their generation cancels the NLRP3 activation [49]. In monocytes, inflammasomes are activated constitutively and due to this TLR ligands or proinflammatory cytokines, including IL-1β itself, will induce processing of IL-1β precursor and its secretion, thus making monocytes very good producers of this cytokine. However, there are no inflammasomes within Mph cells in constitutive activation, and due to this their activation/effective production and IL-1β secretion require co-stimulation of Mph cells by an proinflammatory agent and by an agent inducing potassium efflux from the cells [50].

NLRP1 and NLRC4. Different pyrin-containing inflammasomes NLRP1, reacting to muramyl dipeptide, lethal toxin of *Bacillus anthracis* and others, and CARD-containing inflammasomes NLRC4/IPAF, responding to flagellin, structurally differ from NLRP3 but they activate caspase-1 with the same efficiency. They exhibit lower than NLRP3 dependence on ASC, but IL-1β processing in the presence of ASC is sharply enhanced. In fact, ASC plays a major role in inflammasome formation and in caspase-1 activation. Therefore, mice with knockout of ASC, similarly to caspase-1-deficient mice, are highly resistant to endotoxic shock. In ASC-deficient mice infected by intracellular bacteria or stimulated by bacterial ligands and ATP, Mph cells are not able to produce IL-1β and IL-18. It is important that the potential ability of Mph cells to produce such proinflammatory cytokines as TNF-α and IL-6 is retained in these mice [51].

Thus, the inflammasome-dependent Mph activation is highly specific, only a part of the spectrum of proinflammatory cytokines, characteristic of "classical" Mph activation, is selectively induced. It may be that features of complete proinflammatory activation can appear later, when IL-1β and IL-18 will in an autocrine way influence Mph cells together with different co-stimulator molecules. At the same time, IL-33 production can potentiate IL-13 effects and induce in Mph cells expression of so-called alternative activation markers including arginase-I, mannose receptors, CCL24, CCL17, IL-4Rα, and Ym1 [52]. So, NLR ligands can stimulate special, nonclassical forms of Mph priming and activation, seemingly forming a "noncanonical" phenotype of Mph activation.

There are individual reports on the ability of some DAMP ligands to interact with both NLR and with TLR [53], but these data need significantly broader experimental support. There are significantly more data showing that simultaneous activation of various PRR, including NLR and TLR, takes place upon interaction with Mph cells of complex agents carrying different ligands [54-57]. Besides, mutual potentiation of signal pathways originating from different PRR is possible. Evidently, *in vivo* inflammasome-dependent "nonclassical" Mph activation takes place under mainly DAMP ligand effects, while PAMP ligands exhibit synergistic effects on TLR, CLR, NLR, and RLR. In particular, TLR stimulation, providing for pro-IL-1β synthesis, "primes" Mph for caspase-1-mediated production of IL-1β, while IL-1β, in turn, is capable of autocrine maintenance of Mph activation by influencing IL-1 receptors. Recent reports suggest the possible involvement of ASC in inflammasomes-independent signaling [58-60]. Therefore, it might be that ASC can be involved in formation of both NLR-mediated "nonclassical" and classical Mph activation.

RLR. A large group of PAMP ligands consists of nucleic acids of infectious agents including recognized by TLR3 and NLR double-stranded RNA (dsRNA) formed as a part of viral genome RNA, as an intermediate product of viral replication or RNA transcripts of DNA viruses, as well as endogenous dsRNA from apoptotic and dying cells [61]. In addition to NLR- and TLR-recognition of viral and microbial nucleic acids, their penetration/replication within a cell is detected by three highly

Main sensors of foreign nucleic acids mediating proinflammatory response of macrophages (considering data of [77])

Receptor type	Localization	Ligands	Receptors	Adaptors/mediators
TLR	endosomes	CpG DNA, damaged DNA	TLR9	MyD88
		ssRNA	TLR7, TLR8	–"–
		dsRNA	TLR3	TRIF
NLR	cytosol	bacterial and viral RNA	NLRP3	ASC
RLR	–"–	ssRNA 5′-triphosphate	RIGI	IPS1
		dsRNA	MDA5	–"–
		–"–	LGP2	–"–
RNA polymerase III	cytosol and, possibly, the nucleus	dsDNA	RNA polymerase III	RIGI via dsRNA 5′-PPP
DAI	cytosol	–"–	DAI	STING?
HIN200/IFI200	cytosol, nucleus	–"–	IFI16/P204	STING
			P204	STING?
			AIM2	ASC
STING	cytosol	cyclic dinucleotides	STING	unknown
KU70	cytosol and, possibly, the nucleus	CpG DNA, damaged DNA	KU70	–"–

homologous cytosol receptors of the RLR family (table). The first is the RIGI-like helicase (RIG-I-like helicase), the second is MDA5 protein (melanoma differentiation associated gene 5), and the third is LGP2 helicase (encoded by the gene of laboratory of genetics and physiology-2). RIGI and MDA5 recognize 5′-triphosphorylated and uncapped single-stranded RNA (ssRNA) and dsRNA, and in this case RIGI recognizes short and MDA5 detects long dsRNA. RIGI detects 5′-triphosphorylated dsRNA transcribed by DNA-dependent RNA polymerase III from AT-rich dsDNA or bacterial and viral DNA, whereas MDA5 can be activated by dsRNA having no 5′ triphosphate. Significantly, unlike viral RNA, 5′-triphosphorylated regions in mammalian RNA are removed or capped, which makes possible to differentiate pathogen RNA and host RNA [62, 63].

RIGI. The RIGI molecule contains two CARD-like sites at the N-terminus, a central DExD/H helicase domain containing an ATP-binding motif, and a C-terminal RNA-binding repressor domain (RD) (Fig. 3). In the case of viral infection, RNA binding to RIGI monomers results in their complex formation accompanied by interaction of CARD with signal molecules and production of IFN-β and proinflammatory cytokines.

MDA5. This protein contains at its N-terminus tandem CARD-like sites and DExD/H helicase domain (Fig. 3), but RNA-binding RD activity of the MDA5 C-terminus has not been found.

LGP2. This protein contains DExD/H helicase domain and RD but has no CARD-like site necessary for homotypic interaction forming a complex with RLR-adaptor IPS1 (IFN-β promoter stimulator 1) and different signaling molecules. There is contradictory information concerning the significance of LGP2 in antiviral response. Some works show that the LGP2 domain RD inhibits response to viral RNA, because it competes with RIGI and MDA5 for dsRNA and ssRNA by binding them with highest affinity, and simultaneously it inhibits signaling from RIGI by binding components of the IFN-β signal pathway [49, 64, 65]. It is shown in other works that the helicase domain of LPG2 enhances RIGI- and MDA5-mediated response [66].

Signaling launched in Mph from RIGI- and MDA5-receptors is presented schematically in Fig. 3. After the

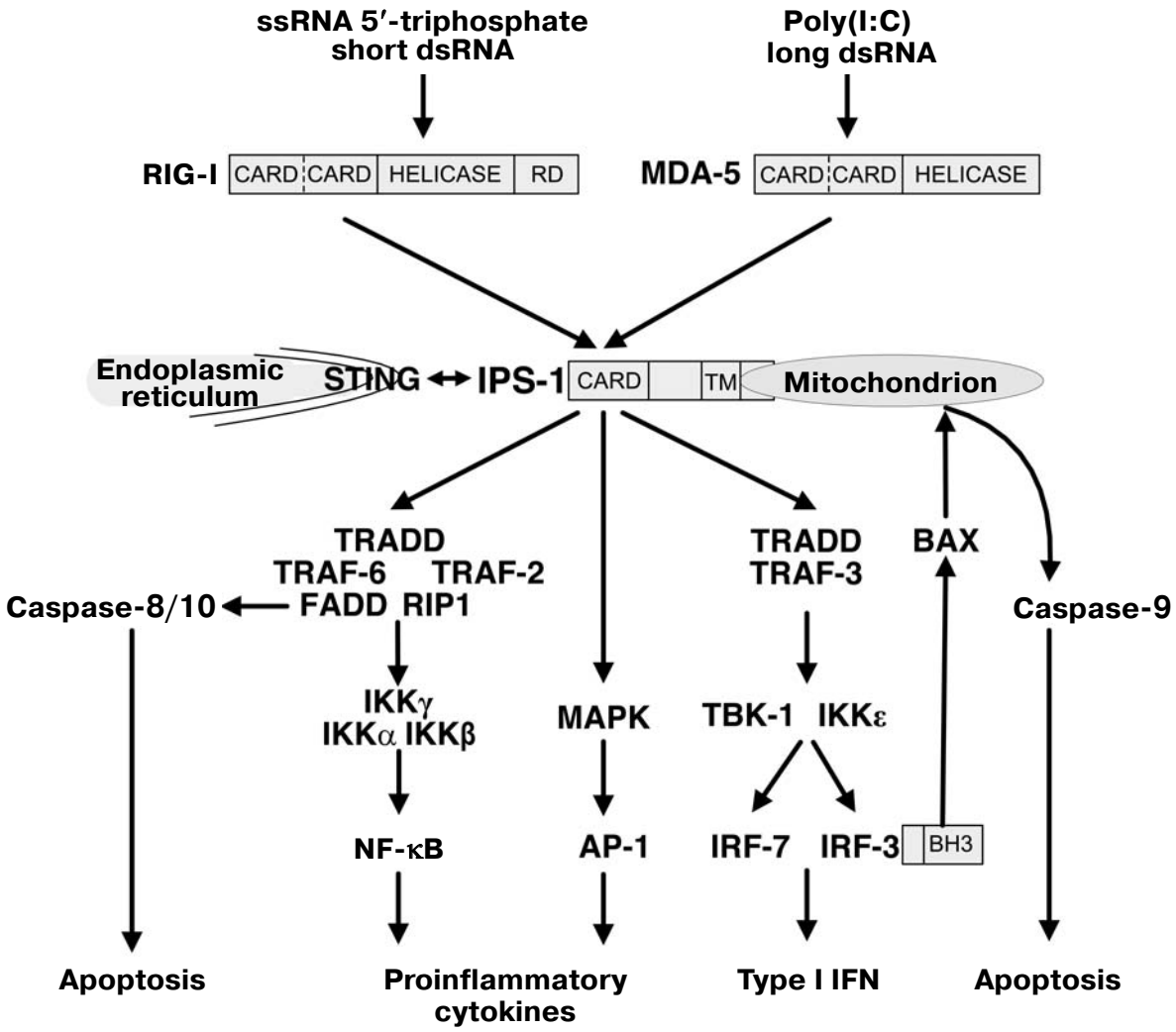

Fig. 3. Mechanisms of RIGI and MDA5-mediated Mph activation. The cytosol RNA helicases RIGI and MDA5 recognize foreign RNA, and as a result they undergo conformational alterations resulting in homotypic CARD-mediated interactions between RIGI/MDA5 and adaptor IPS1 localized on mitochondria. In turn, IPS1 activates TBK- and IKKε-kinases phosphorylating transcription factors IRF3 and IRF7, as well as IKKαβγ and MAP kinases inducing, correspondingly, NF-κB and AP1. Adaptors RIP1 and FADD, which cause apoptosis via caspases-8/10, are involved in IPS1-induced activation of NF-κB. IRF3 and IRF7 induce expression of type I IFN. Simultaneously, IRF3 induces mitochondrial apoptosis.

CARD–CARD interaction, RIGI/MDA5 activate adaptor IPS1 (IFN-β promoter stimulator 1) bound to the outer mitochondrial membrane by its transmembrane domain TM. The activation of IPS1 is significantly enhanced upon complex formation with another adaptor STING (stimulator of interferon genes), a transmembrane protein located on endoplasmic reticulum. The activation of IPS1 results in TRADD (TNFR1-associated death domain) recruiting, and then TRAF3 (TNFR-associating factor 3) and following TRAF3-mediated activation of noncanonical serine-threonine kinases IKKε (IκB kinase ε/ι) and TBK1 (TANK-binding kinase-1) phosphorylating/activating transcription factors IRF-3 and IRF-7. Besides, the interaction of RIG-I/MDA5 with IPS1 activates protein kinase IKK complex consisting of IKKα, IKKβ, and IKKγ (NEMO). This complex catalyzes phosphorylation and following degradation of IκB, releasing the p50/p65 form of NF-κB for translocation into the nucleus and launching transcription of proinflammatory cytokines. It is also known that IPS1 activates AP1 via MAP kinases, although the mechanism of this activation is unknown.

So, RIGI and MDA5 use mitochondrial membrane-bound protein IPS1 and recruit several TRAF family members activating the same protein kinases and transcription factors as TLR3 membrane receptor recognizing extracellular dsRNA entering endosomes. Transcription factor activation by TLR3 and RLR results

in transcription of IFN and numerous IFN-stimulated antiviral protection genes. Keeping in mind convergence of RLR and TLR signaling to the same molecular intermediates, one can suppose RLR agonists induce Mph phenotype characteristic of "classical" TLR activation. However, antiviral mechanisms of TLR3 and RLR activation, not directly associated with IFN-induced transcription, differ significantly. The most important difference between them is different ability to cause apoptosis of infected Mph (limiting viral replication and dissemination). The interaction of dsRNA with TLR3 does not always cause apoptosis in Mph cells because TLR3 activation induces expression of both pro- and antiapoptotic molecules. On one side, TLR3 activates NF-κB, inducing production of proinflammatory cytokines and several antiapoptotic and growth-stimulating proteins, while on the other side it results in apoptogenesis launched by TRIF–RIP1–FADD–caspase-8 cascade, independent of NF-κB and not associated with mitochondrial mechanisms of apoptosis [61]. Apoptosis induced by dsRNA is much more often mediated by RIGI than by TLR3 [67]. According to work [67], apoptosis caused by RIG-I ligands is due to activation of IRF3 (but not of IRF7) and leads to apoptosis in two ways. The first way is associated with IRF3-initiated transcription of antiviral protection genes including proapoptotic genes *TRAIL*, *Noxa*, etc., while the second, "non-transcriptional", is caused by the BH3 domain of IRF3. The non-transcriptional pathway is realized due to the binding of the BH3 domain to proapoptotic protein BAX of the Bc12 family. This results in activation of BAX and co-translocation of both proteins to a mitochondrion, which serves as an extremely efficient trigger of mitochondrial apoptogenesis. The binding of IRF3 to BAX is a necessary and sufficient condition for dsRNA-induced apoptosis. Therefore, it is independent of interferonogenesis and NF-κB activation, but it is critically dependent on functioning of RIGI, IPS1 bound to mitochondrial membrane, TRAF3, and TBK1 [67-69].

Thus, the RLR-mediated activation of Mph cells is accompanied by increased production of proinflammatory cytokines and type I IFN, i.e. it has features of "classical" activation with auto-priming. However, the pathways of signal transduction initiated by TLR3 and RIGI/MDA5, preceding TRAF, do not cross, i.e. the RLR-mediated activation has dynamics different from TLR-mediated activation. In this case activation of RIGI causes very effective apoptogenesis that is not characteristic of "classical" Mph activation. In other words, the RLR-mediated stimulation forms a noncanonical type of "classical" Mph activation.

Different sensors of nucleic acids. Along with TLR, NLR, and RLR, different sensors of foreign nucleic acids have been recently found in Mph cells, but they are still poorly characterized (table). The role of these intracellular molecules in formation of Mph phenotype is still almost unstudied. Among such molecules there is the dsDNA-dependent activator of IFN-regulatory factors (DAI) of cytosol inducing production of type I IFN using still unknown adaptors via the TBK1–IRF3 pathway [70]. Another such molecule is the RLR signaling adaptor STING. The latter not only serves as an adaptor of the RLR cascade, but itself it is a sensor of unique small bacterial RNA – cyclic dinucleotides consisting of two cAMP molecules bound by 3′,5′-phosphodiester bond or of two cGMP molecules. Detecting these dinucleotides, STING directly, i.e. independently from IPS1, induces NF-κB activation, type I IFN production, TBK1 kinase, and IRF3 [71]. Another example of an intracellular DNA-detecting molecule is DNA-repairing protein KU70 inducing type III IFN production [72, 73]. Among IFN-inducing molecules involved in intracellular detection of viral and bacterial DNA, there is RNA polymerase III able to transcribe AT-rich dsDNA to 5′-triphosphate dsRNA and, as has been recently shown, to stimulate RIG-I [74]. Another group of intracellular DNA-sensors consists of IFN-inducible/inducing proteins containing one or two long conservative 200-amino-acid-residue repeats mediating their action via STING or ASC adaptors (HIN200 human family and IFI200 mouse family) [75]. Most of these molecular sensors stimulate the apoptotic death of Mph cells. In response to IFN, members of the HIN200/IFI200 family translocate into the nucleus, where some of them are involved in regulation of cell growth and differentiation, playing the role of DNA binding transcription regulators or of activity modulators of different transcription factors. The HIN200/IFI200 family includes pyrin-domain-containing dsDNA sensor IFI16 (interferon γ-inducible protein 16), its mouse ortholog P204, AIM2 (absent in melanoma 2), and other proteins. Upon interaction with microbial DNA, AIM2 stimulates formation of caspase-1-activating inflammasomes, while IFI16 stimulates transcription of type I IFN (possibly AIM2, like IFI16, induces via ASC formation of Mph inflammasomes, but it is still shown only in endothelial cell nuclei [76]). STING- and ASC-dependent interferonogenesis, NF-κB activation, inflammasome formation, and IL-1 production suggest that, on the whole, intracellular DNA sensors induce proinflammatory phenotypes in Mph cells. However, these phenotypes evidently differ significantly from the "classically activated" one, because in most cases they are associated with induction of pyrin- and/or IRF3-dependent apoptosis and include a limited spectrum of proinflammatory cytokines.

CLR. C-Type lectin receptors comprise a family of transmembrane and soluble receptors recognizing carbohydrate residues of a pathogen or host molecules in a Ca^{2+}-dependent pathway using the highly conservative CRD (carbohydrate recognition domain). During the last decade many receptors similar to CRD or containing at their C-terminus a conservative motif characteristic of C-

type lectins and consisting of three cysteine residues have been discovered. These receptors were called C-type lectin-like (CTLL) receptors, because most of them are devoid of carbohydrate-binding activity or bind carbohydrates in a cation-independent way [78]. In all, there are 60-80 members of the superfamily; in accordance with their structure they are separated into 17 groups [79]. Some CTLL are transmembrane polypeptides carrying numerous CRD. They include mannose receptors (MR), receptors to phospholipase A2, DEC205, Endo180, etc. CTLL with a single CRD include DC-SIGN (dendritic cell-specific ICAM3-grabbing nonintegrin), langerin, MGL (macrophage galactose-type C-type lectin), collectins, dectin-1 and -2 (dendritic cell-associated C-type lectin-1 and -2), etc. In our opinion, the effect of CLR on Mph phenotype is ambiguous.

Mannose receptors. These receptors bind in a Ca^{2+}-dependent way mannose, fucose, N-acetylglucosamine, and glucose residues of Gram positive and Gram negative bacteria, yeasts, parasites, and viruses, as well as of carbohydrate-modified host molecules via their internalization and following processing necessary for presentation to MHC molecules [80, 81]. In this case the MR-mediated capture of mannosylated antigen may cause a weak antigen-specific response or even immunological tolerance, thus probably providing for inhibition of the autoimmune reaction [82]. Enhanced MR expression is observed in alternatively activated Mph cells. However, it is not known whether this expression is the result or a factor responsible for acquiring this phenotype. A short cytoplasmic MR terminus does not contain the classical signal domains and, according to different data, it can mediate production in Mph cells of both pro- and antiinflammatory cytokines [83, 84]. So, it seems that MR agonists induce some "mixed" Mph phenotype. Perhaps the character of MR-mediated macrophage response depends on the extent of MR oligomerization on plasma membrane varying in density of lectin ligands on the surface of the pathogen [85].

DC-SIGN receptors. These receptors are able to multimerize on the cell surface and bind in a Ca^{2+}-dependent way mannosylated residues, thus recognizing viruses, bacteria (including mycobacteria), fungi, and Protozoa, as well as host antigens by providing for their capture, processing, and presentation. Although the expression of DC-SIGN is increased in alternatively activated Mph cells, there are controversial data about the role in Mph cells of signal transduction downstream DC-SIGN. Some are indicative of anti-, while the other suggests proinflammatory effects. Thus, according to the authors of works [86, 87], DC-SIGN signaling downregulates proinflammatory TLR effects by acetylation of the NF-κB subunit p65 via the Raf-kinase pathway, and in this way it causes enhanced expression of IL-10 and immunosuppression. According to work [88], ligand binding to DC-SIGN disturbs STAT1 phosphorylation in Mph cells and TLR3 expression in them. It was shown in [89] that mycobacterial lipoarabinomannan interacts with MR and DC-SIGN of macrophages, and due to this it inhibits production of proinflammatory cytokines IL-12 and TNF-α and induces production of IL-10. However, according to other authors [90] neither increased expression of the mouse homolog of DC-SIGN – SIGNR1 – nor its deficiency influence the activity of lipoarabinomannan towards LPS-stimulated production of NO and proinflammatory cytokines. Moreover, binding of the LPS polysaccharide part to SIGNR1 enhances LPS-stimulated oligomerization of TLR4, degradation of IκB-α, and production of proinflammatory cytokines [90]. SIGNR3, the mouse ortholog of DC-SIGN, activates Mph cells via a signal cascade involving tyrosine kinase Syk and induces NF-κB- and Raf1-ERK-dependent signaling with production of proinflammatory cytokines [91]. Thus, it is evident that DC-SIGN agonists induce complex Mph phenotypes different from the canonical ones.

MGL receptors. MGL is distinguished among CLR due to its ability to recognize galactose and N-acetylgalactosamine residues on glycoproteins, glycolipids, and bacterial LPS. These residues are characteristic of many infectious pathogens and tumor-associated glycosylated antigens carrying, in particular, Tn (α-GalNAc-*O*-Ser/Thr), one of most specific structures of human tumors. After binding to ligands, MGL enhances phagocytosis and presentation of MHC class I and II antigens [92, 93] even in the presence of CpG [94]. The interaction of MGL with Tn stimulates immunosuppressive phenotype of tumor-associated Mph cells [92, 95, 96]. Similarly to MR and DC-SIGN, MGL receptors are increasingly expressed on alternatively activated Mph cells and can be considered as a marker of this phenotype [97, 98]. In mice there are two MGL isoforms encoded by two homologous genes – *MGL1* and *MGL2*. It was shown in [99] that bacterial activation of MGL1 induces in Mph cells production of IL-10 thus exhibiting antiinflammatory effect. However, it was shown in [100] that MGL is able to stimulate classical pathway of NF-κB and ERK1/2 MAP kinase. It is reported in [101] that MGL activation causes Th17 response, while in [102] production of proinflammatory cytokines was reported to occur in MGL(+) cells. Thus, the role of MGL in formation of mononuclear phagocyte phenotype requires further elucidation.

Dectin-1 and dectin-2 (dendritic cell-associated C-type lectin-1 or -2) are lectin receptors with moderate homology. Dectin-1 is calcium-independent main receptor recognizing β-glucans (components of fungal, yeast, mycobacterial, and other pathogens). Dectin-2 is a calcium-dependent low-affinity receptor for fucose and highly mannosylated structures. Splice-variants of dectin-1 and dectin-2 are found, and different ability of splice-variants to bind their own agonists has been shown [103, 104].

Conservative tandem amino acid sequence ITAM (immunoreceptor tyrosine-based activation motif), characteristic of immunoreceptor subunits FcRγ and DAP12, is present in the cytoplasmic domain of dectin-1. Binding of such ligands as zymosan (β-glucan-rich cell wall of *Saccharomyces cerevisiae*) to dectin-1 stimulates ITAM phosphorylation on tyrosine, which results, in particular, in activation of cytoplasmic tyrosine kinase Syk (spleen tyrosine kinase) phosphorylating in Mph cells numerous substrates, including adaptor protein SLP-76 (SH2 domain-containing 76-kDa leukocyte protein). SLP-76, in turn, activates phospholipase Cγ and dependent production of inositol-3-phosphate and diacylglycerol [105]. Syk also mediates activation of phosphatidylinositol-3-kinase (PI3 kinase). PI3-kinase along with diacylglycerol activates proteinase C, which, in turn, activates CARD9, and the latter via a number of intermediates activates TRAF-6 complex, which causes degradation of IκB with canonical activation of NF-κB and activation of MAP kinases. Activation of these transcription factors results in production of proinflammatory cytokines and in expression of classical activation markers [56, 106]. Thus, the dectin-1-induced pathway of signal transduction and classical TLR-induced pathway converge to the same molecular intermediates and effectors. Some data suggest that the convergence can be carried out already at the level of CARD9 [107].

In addition to distinctions in a series preceding TRAF-6, an essential distinction between the dectin-1-induced cascade and that induced by TLR is calcium release from intracellular depots and resulting Ca-dependent signaling mediated by ITAM-phospholipase-Cγ. The TLR-induced cascade causes only indirect and weak Ca-signaling [56]. As follows from [108], zymosan activates in a Ca-dependent way transcription factor NFAT, which is involved in Mph activation. It was shown in [56] that zymosan launches Ca-dependent calmodulin-kinase–tyrosine-kinase Pyk2 (proline-rich tyrosine kinase-2)–ERK cascade, which causes generation of respiratory burst, CREB (cAMP response element-binding protein) activation, and IL-10 production. Evidently, dectin-1-induced antiinflammatory cytokine IL-10 production in Mph cells combined with production of proinflammatory cytokines make it impossible to consider such Mph cells as "classically" activated. Increased expression of dectin-1 is observed on alternatively activated Mph cells [109].

Probably, in actual pathologies cooperation of ITAM- and TLR/TIR-mediated cascades is possible, and in this case, depending on phosphorylation extent, ITAM-containing receptors can either enhance or inhibit TLR signaling and formation of classically activated Mph cells [23]. In this case such intermediaries as osteopontin-1 [110], Rac1, and CDC42 [106] potentiate costimulation of dectin-1- and TLR-signaling. Similarly to ITAM–TLR interactions, ITAM-dependent cascades and IFN-γ/Jak-STAT-signaling may interact, and analogously, depending on intensity of ITAM signaling, either enhancement or inhibition of classical activation can occur [23]. Interaction of dectin-1–Syk- and NLR-signaling has been recently shown. It has been shown [111] that β-glucans stimulate formation of NLRP3 inflammasomes and the NLRP3-dependent production of IL-1β. In this case, the dectin-1–Syk pathway is involved in NLRP3 activation either via NF-κB or generation of reactive oxygen species, but also directly via Syk-phosphorylated targets. It is logical to conclude that ligand binding to dectin-1 and variable inter-cascade interactions form a special, extremely complex Mph phenotype essentially different from "classically activated".

Unlike dectin-1, dectin-2 has no signal sites in its cytoplasmic domain and induces signal transduction after association with intracellular site FcRγ of immunoreceptors and ITAM activation. The signal cascade launched from dectin-2 is still unstudied, but considering ITAM-dependent mechanisms one can suppose that activation of Syk kinase and its substrates is very probable. Nevertheless, dectin-1 and dectin-2 induce production of different sets of cytokines, in particular the first induces IL-12 and IL-10, while the second induces TNF-α and antagonist of receptor to IL-1β [112, 113]. Significant increase in dectin-2 expression on monocyte-macrophages in response to proinflammatory stimuli as well as NF-κB activation and production of proinflammatory cytokines in response to dectin-2 [114] are at first glance manifestations of "classically activated" phenotype, but high production of antagonist to IL-1β receptor and potential ability of ITAM activation to downregulation of Mph proinflammatory response is indicative of formation of mixed "nonclassical" phenotype.

So, contemporary ideas of Mph proinflammatory response were formed mainly during investigation of TLR ligand effects, first of all of bacterial LPS. As a result, now the notions that are most widespread are of some typical proinflammatory reaction of these cells called "classical" activation. Analysis shows that in reality there is no typical reaction, and the character of Mph proinflammatory response/phenotype is extremely variable and critically depends on concrete PAMP and DAMP. Investigations of interaction of PRR-dependent signal cascades undoubtedly will soon explosively complicate concepts of proinflammatory phenotypes. In this connection, it would be evidently most correct to use the term "classical activation" for concrete macrophage phenotype formed under TLR-induced MyD88- and/or TRIF-mediated signaling. In our opinion, it is a peculiar case of an endless set of variable macrophage phenotypes of proinflammatory, mainly biocidal-destructive direction. Even in this peculiar case different priming variants are possible.

One cannot be not surprised at numerous and evident at first glance "excessiveness" of mechanisms/variants of Mph proinflammatory reactions (remember, for

example, the variability of sensors/responses to nucleic acids). The following questions arise: why was such excess necessary for evolution? To what extent do different activation mechanisms overlap each other, and what phenotypes evolve in actual inflammation? How are different activation pathways dependent on different pattern recognition receptors – coordinated with each other? What are the limitations/inclination of Mph cells of different histogenesis, localization, and differentiation stage to different variants of proinflammatory response? There are still no answers to these questions.

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