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REVIEWS

Septic Shock: Innate Molecular Genetic Mechanisms of the Development of Generalized Inflammation

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Abstract—The capacity for immune surveillance and protection against genetically alien agents is a basic property of multicellular organisms, and increasing significance in realizing this capacity is assigned to mechanisms of innate immunity. The data accumulated to date show that many components of these mechanisms have a very wide spectrum of biological functions and play essential roles at different stages of ontogeny. An illustrative example is the signal system activated by tumor necrosis factor alpha (TNFα), which is responsible for the inflammation process. Analysis of its structural organization has shown that signaling mechanisms initiating inflammation largely overlap with mechanisms of programmed cell death. This is why hypersecretion of TNFα may lead to systemic inflammatory reaction, or septic shock, and, hence, have a fatal outcome. Although studies on the TNFα-dependent mechanism have long history, many aspects of its regulation remain obscure. In particular, this concerns the nature of interspecific differences in the sensitivity of mammals to TNFα action and the ability of TNFα to activate oppositely directed cell programs depending on cell type or ambient conditions. The numerous data obtained in studies on different experimental systems need generalization and critical analysis. This review is an attempt at such an analysis. Its scope is concentrated on modern views on the divergence of TNFα-induced signal at the level of intracellular receptor-associated proteins. A description is given to potential "molecular triggers" responsible for switching between the main TNFα-dependent signaling pathways: inflammation, apoptosis, and necroptosis. The contribution of necroptosis (genetically programmed necrotic cell death) to the development of systemic inflammation and the lethal effect of TNFα are described. Consideration is also given to various lines of mice possessing natural resistance or sensitivity to TNFα, which hold much promise as models for deciphering the molecular genetic bases of the regulation of innate immune reactions and other TNFα-dependent processes.

Keywords: inflammation, tumor necrosis factor alpha, septic shock, necroptosis, mouse models, forward genetics

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INTRODUCTION

The inflammatory response is an innate (genetically determined), evolutionarily conserved property that plays a major role in the ontogeny of a multicellular organism by maintaining its molecular genetic integrity and biochemical and cellular homeostasis. The development of inflammation involves various mechanisms of cell activation related to the induction of certain sets of genes. However, recent studies have shown that many inducers and mediators of cell activation in the course of inflammatory response are at the same time central regulators of programmed cell death, with their functioning being also important for normal morphogenesis and immunogenesis during embryonic development. The leading role in initiating the inflammatory response is played by tumor necrosis factor-alpha (TNFα) and its receptor, which, depending on conditions, can activate opposite genetic programs of cell activation ("inflammation–survival pathway") or cell death. There also are counter-mechanisms that control the magnitude and spread of the inflammatory response, but they fail in certain situations, with consequent development of systemic inflammation and eventual septic shock.

Despite considerable advances in the control of infectious diseases, sepsis and septic shock still present a major problem for clinical medicine and fundamental science (Marshall, 2014). The development of septic shock may be caused not only by the direct entry of bacterial infection into the circulation but, for example, also by damage to the intestinal wall and consequent exposure of endosymbiotic bacteria to peritoneal macrophages. The burst-type development of systemic inflammation generally proves fatal within a short time, and statistical data on annual mortality from the consequences of septic shock confirm the urgency of the problem at issue (King et al., 2014).

Uncontrolled TNFα hyperproduction by macrophages resulting from their interaction with Gram-negative bacteria or bacterial cell wall components is the triggering factor of systemic inflammation (King et al., 2014). Different species and laboratory lines of

mammals are known to differ in sensitivity to septic shock (Warren, 2009), with humans falling within the highly sensitive group. Such phenotypic differences may be accounted for by various factors. In particular, insensitivity to septic shock may be due either to resistance against the effect of TNFα or to a defect in recognition of bacterial infection and insufficient TNFα production. Experimental systems have been designed to model the development of septic shock and analyze the mechanisms of TNFα induction and action on other tissues and organs. The data obtained in these experiments suggest that phenotypic differences in the response to TNFα are accounted for by specific molecular genetic features of the organism. Many components of TNFα-activated reactions are already known, but it is still unclear why the human organism is supersensitive to this factor. The current concept of the TNFα-mediated signaling pathway cannot adequately explain the complexity of the corresponding process and, hence, does not allow prediction of its outcome. Studies on model experiments systems (see below) have shown that signaling mechanisms triggered by TNFα or responsible for its hyperproduction are much more complex than it has been considered until recently and that they involve additional, previously unknown components acting as triggers or modulators. Therefore, further analysis of mechanisms activating TNFα production and the action of this factor is a highly relevant task for specialists in immunology and immunogenetics. Identification of new factors determining the rate, intensity, and variants of development of septic shock reactions is a means to reveal novel targets for therapy aimed at reducing their lethal effect. Knowledge of hereditary (genetic) factors accounting of individual sensitivity to septic shock provides the possibility of developing diagnostic and prognostic test systems, evaluating individual risks, and taking appropriate preventive measures. Studies on the mechanisms of inflammatory reactions with the involvement of TNFα are important not only for solving the problem of septic shock: TNFα is a pleiotropic cytokine, and disturbances in the regulation of TNFα-dependent reactions lead to the development of many other human pathologies (in particular, chronic inflammatory, autoimmune, and oncological diseases).

Laboratory mouse strains are widely used to study molecular genetic mechanisms of innate immune response to infection, including factors determining their resistance (sensitivity) to septic shock (Conner et al., 2009; Poltorak, 2012). The availability of inbred mouse strains phenotypically differing in immunity against infections and of a well-annotated mouse genome sequence makes it possible to use classical genetic analysis for revealing genetic factors accounting for susceptibility to bacterial infection in general and TNFα in particular. The high homology between the mouse and human genomes and similarity in their organization allow the data obtained in experimental systems to be extrapolated to clinical studies (Mestas et al., 2004). Apparently, predisposition to the development of septic shock in the course of immune response to infection is a complex, polygenic quantitative trait, and its phenotypic expression depends not only on the individual activities of corresponding genes but also on interactions between them and many other conditions. In this context, the use of inbred mouse strains as experimental objects is especially expedient (Poltorak, 2012).

The development of systemic inflammatory response is a multistage process, and the phenotypic resistance of a species (subspecies) may be accounted for by absolutely different genetic alterations (mutations) affecting certain stages of this process. Of special interest is the variant where a resistant organism is characterized by an intact, fully functional TNFα receptor and produces a normal level of TNFα in response to infection. In this variant, genetic alterations appear to be associated with certain trigger mechanisms due to which the signal from the TNFα receptor is redirected from the cytotoxic to the "survival" pathway.

With regard to the above, the purpose of this review was to analyze published data on the mechanisms of septic shock development obtained in various in vitro and in vivo model systems in order to reveal possible genetic and biochemical factors accounting for resistance or sensitivity to septic shock in mouse models. Special attention was given to current views on the divergence of TNFα-induced signal at the level of intracellular receptor-associated adaptor proteins and enzymes as a possible formation mechanism of resistance to septic shock.

INDUCTION OF TNFα SECRETION AS A RESULT OF ACTIVATION OF INNATE IMMUNITY

Septic shock is a complex pathophysiological state characterized by a generalized (systemic) inflammatory process caused by bacteremia or massive tissue damage (Kumar, 2014). The main role in the initial recognition of pathogens during the development of inflammation is played by components of innate immunity, primarily macrophages. These cells contain on their surface invariant pattern-recognition receptors (PRRs) that specifically bind highly conserved chemical structures common to most groups of microorganisms, which are referred to as pathogen-associated molecular patterns (PAMPs). According to functional classification, some PRRs act as signaling receptors, since their interaction with PAPMs induces the expression of specific genes responsible for the production of proinflammatory signaling molecules in the focus of infection (Schenten et al., 2011).

One of PAMP components is bacterial lipopolysaccharide (LPS, or endotoxin), which plays

the key role in functional activation of macrophages in the course of immune response to Gram-negative bacteria (Cho et al., 2014). Macrophages specifically recognize LPS through the TLR4 receptor, which belongs to the family of evolutionarily conserved Toll-like receptors (TLRs). TLR4 was the first representative of this family revealed in mammals in experiments on identifying the gene of resistance to septic shock in different mutant mouse strains (Poltorak, 2012). The structure of TLR4 corresponds to its function as a signaling PRR: its cytoplasmic domain contains Toll-interleukin-1-receptor (TIR) sequences capable of interacting with a range of cytoplasmic adaptor proteins. Spontaneous or induced mutations leading to amino acid substitutions in the TIR domain in some mouse strains account for their high susceptibility to bacterial infection combined with resistance to endotoxin injection.

It is generally accepted that the signal from the TLR4 receptor may be transduced via two pathways, MyD88-dependent and MyD88-independent (TRIF-mediated), with the former leading to activation of genes for proinflammatory cytokines, and the latter, for type I interferons. Many components of these signaling cascades have already been characterized, but it remains unclear what is the mechanism of "switching" between them, which results in production of two different sets of cytokines. MyD88-dependent signal transduction occurs with the involvement of IRAK protein kinases, TRAF6 ubiquitin ligase, and TAK1 kinase, which activate transcription factor NF-κB or MAP kinases ("inflammatory, pro-survival" pathway). In the TRIF-mediated pathway, the signal is transmitted through interferon regulatory factors (IRFs) interacting with the interferon-stimulated response elements (ISREs) of target genes (Brikos et al., 2008). The signal from some TLRs (including TLR4) can also be switched to the programmed cell death pathway.

In terms of the problem of septic shock ("cytokine storm"), TNFα is the key product of TLR4-mediated MyD88-dependent activation of macrophages (Shuh et al., 2013). Together with interleukins IL-1 and IL-6, TNFα forms the triad of main proinflammatory cytokines, but it should be especially emphasized that the action of TNFα is highly pleiotropic. Depending on the type of target cells and their current state and microenvironment, TNFα may produce either an activating or a cytotoxic effect. When the dose of pathogen is relatively low, macrophage-mediated immune reactions are usually local, with TNFα functioning as an auto- or paracrine regulator. In systemic inflammation, mass activation of macrophages takes place, and TNFα concentration in the circulation may increase to a critical level. The systemic physiological effect of TNFα manifests itself in symptoms such as hypotension, hypothermia, hypoproteinemia, disseminated intravascular coagulation, and various metabolic disturbances. Fatal outcomes result from necrosis of internal organs, acute polyorganic insufficiency, internal bleeding, etc. (King et al., 2014). Thus, if LPS is the inducer (trigger) of systemic inflammation, then TNFα is the key mediator of this process. The effect of TNFα at the cell level depends on the functional features of the TNFα–receptor complex.

THE FUNCTIONING OF TNFα RECEPTOR

The structure of receptor complex and components of TNFα-dependent signaling cascade. The TNFα receptor (TNFR) belongs to the large superfamily of cell death receptors, which currently includes more than 20 members. Two types of this receptor, TNFR1 and TNFR2, have been identified in mammalian cells. TNFR1 (p55), the main signaling receptor for TNFα, is found on almost all human cell types, while the expression of TNFR2 (p75) is generally limited to cells of the immune system, and its biological role has not yet been determined definitely (Naudé et al., 2011).

TNFR1 activation may induce diverse biological responses manifested in the survival and proliferation of target cells and activation of proinflammatory genes (the NF-κB pathway) or, conversely, in cell death via the apoptotic or necrotic pathway (Fig. 1). According to the current model of TNFα-induced activation, the result depends on the spectrum of secondary mediators (adaptor proteins and protein kinases) that receive the signal from TNFR1, the relative levels of their expression, and localization of the receptor complex in a certain membrane compartment of the cell.

TNFα-induced trimerization of TNFR1 provides for convergence of cytoplasmic death domains (DDs) of the latter, which allows their interaction with adaptor proteins containing homologous DDs such as TRADD (TNFR-Associated Death Domain) and FADD (Fas-Associated Death Domain) (Fig. 1). The current model of signal transduction from activated TNFR1 implies the possibility of formation of supramolecular signaling complexes of two types: Complex I (proximal) directs the signal along the "survival"–immune inflammation pathway, and Complex II, along the pathway of genetically programmed cell death. It should be noted that the mechanism of signaling cascade switching between these two complexes has not yet been elucidated (Vanlangenakker et al., 2012; Moriwaki et al., 2013).

Complex I associated with "survival" and inflammation comprises the TRADD adaptor, apoptosis inhibitor proteins cIAPs, factor TRAF2 (the family of E3 ubiquitin ligases), and receptor-associated signaling kinase RIP1 (Vanlangenakker et al., 2011) (Fig. 2). The choice of pathway for further signal transduction supposedly depends on the activity of RIP1 kinase, which, in turn, depends on the level of its ubiquitination. TRAF2 and cIAP1/2 within Complex I catalyze linear polymerization of ubiquitin and Lys63-linked polyubiquitination of RIP1 kinase. This modification

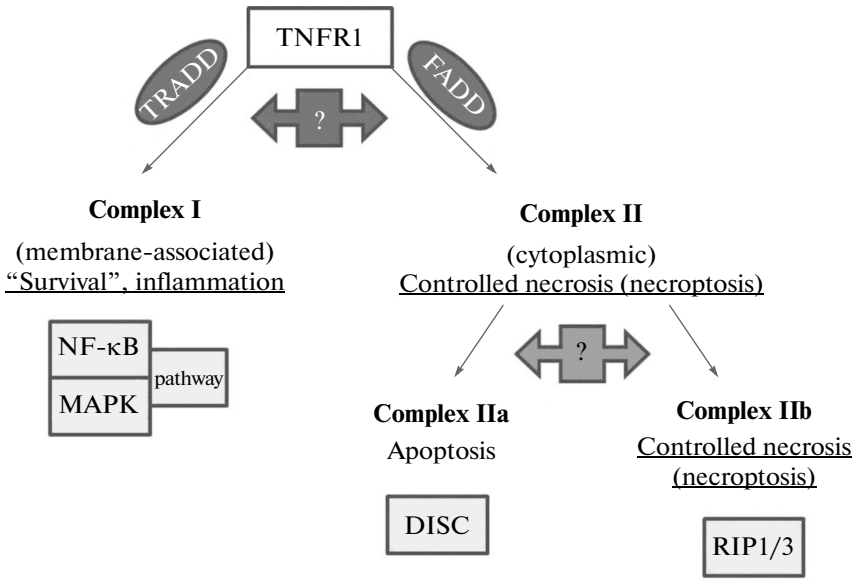

Fig. 1. Scheme of signal divergence during TNFR1 activation.

is not related to proteasome-dependent degradation but plays a regulatory role. It is considered that polyubiquitination inhibits the catalytic (kinase) activity of RIP1 and prevents signal switching to the cell death pathway and, on the other hand, that the polyubiquitin chains play a role of molecular scaffold for the assembly and activation of specific protein kinases TAK1 and IKK, which phosphorylate NF-κB inhibitor (IκB), thereby involving it in proteasome-dependent degradation (Fig. 2). Activated NF-κB (p65:p50) moves from the cytoplasm to the nucleus and interacts with regulatory sequences of various target genes responsible for cell activation, proliferation, inhibition of apoptosis, and inflammatory response.

Additional ubiquitin-dependent protein components have been revealed that facilitate activation of TAK1 and IKK complexes and thereby potentiating the NF-κB pathway. Thus, the RIP1 polyubiquitin scaffold is necessary for binding the NEMO regulatory subunit of the IKK complex (Fig. 2) and, at the same time, accounts for the recruitment and activation of the linear ubiquitination assembly complex (LUBAC). LUBAC builds up ubiquitin chains linked to NEMO, which provide for general stabilization of Complex I and serve as an attachment site for the IKK complex (Emmerich et al., 2011). The TAB2 and TAB3 proteins contain ubiquitin-binding domains and function as adaptors that facilitate the interaction of the TAK1 kinase complex (TAK1:TAB1) with RIP1 kinase (Broglie et al., 2010). The TNFα signal can also be transduced via TRAF2 and TAK1 to terminal MAP kinases (p38 MAPK, JNK, and ERK1/2) and eventually to transcription factor AP-1, whose targets are various genes responsible for cell proliferation and survival under exposure to stress factors (Shuh et al., 2013).

Thus, the formation of RIP1–polyubiquitin molecular scaffold is a key prerequisite for the assembly of Complex I. However, recent experiments with RIP1-knockout cells have cast doubt on the absolute necessity of RIP1 for activating the NF-κB pathway, indicating that some other ubiquitinated proteins within Complex I are probably involved in the recruitment and activation of TAB:TAK1 and IKKα:IKKβ:NEMO complexes (Vanlangenakker et al., 2011). Thus, deubiquitinases A20 and CYLD (cylindromatosis) negatively regulate the stability of receptor-associated Complex I by correcting the length of polyubiquitin chains linked to receptor-proximal signaling proteins. Supposedly, the concerted action of different ubiquitin ligases and ubiquitinases accounts for various modifications and consequent distinct conformations of these chains, which, in combination, form a code that determines the probability to signal transduction by either the NF-κB pathway or the cell death pathway (Hymowitz et al., 2010). However, this hypothesis needs further experimental verification.

The "external" (receptor-dependent) apoptosis pathway and formation of Complex II (IIa). Apoptosis is a genetically controlled mechanism of cell death involving activation of specific groups of genes and accompanied by characteristic morphological and biochemical changes in the cell (Rossi et al., 2003). There are many extra- and intracellular factors that can initiate the apoptosis program, which is usually implemented with the involvement of intracellular cysteine proteases, or caspases (EC 3.4.22). The interaction of cell death ligands and receptors (members of

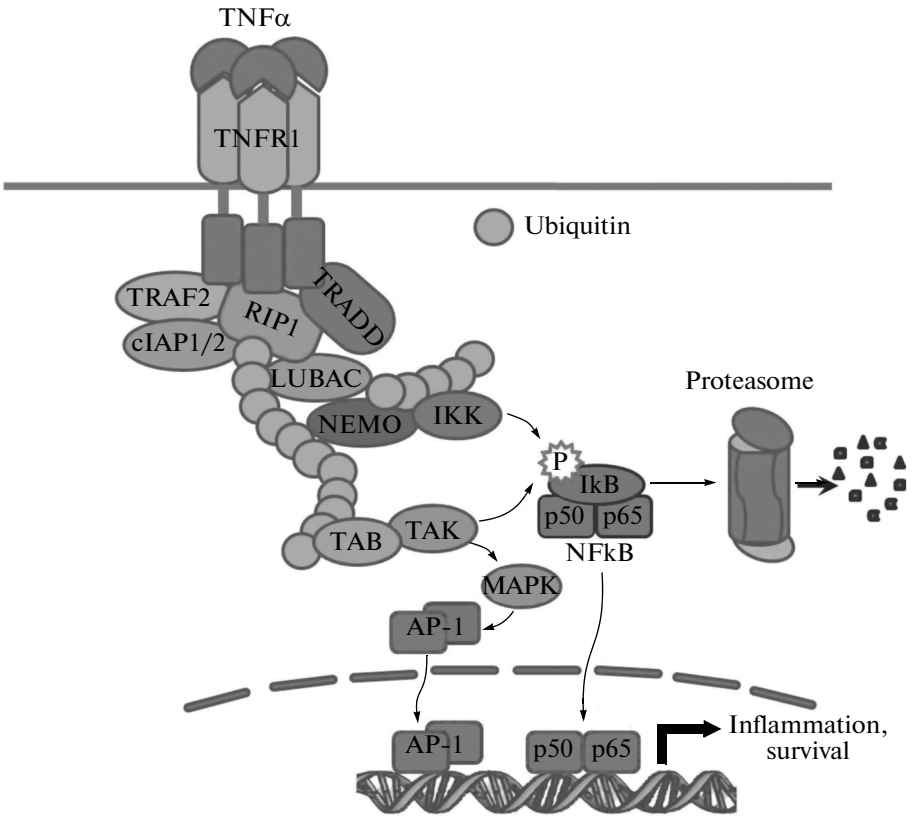

Fig. 2. TNFα signal transduction through Complex I.

the TNF superfamily) is the basic mechanism for inducing the external apoptosis pathway, with FADD performing the function of adaptor protein interacting with DDs of the receptor. In addition to the DD sequence, FADD also contains the death effector domain (DED) that is necessary for the formation of death-inducing signaling complex (DISC), which serves as a platform for multimerization and autocatalytic activation of initiator caspase-8. This enzyme is responsible for the processing of effector caspases that lack the DED sequence and are therefore incapable of signal perception immediately from the death receptor. The formation of active DISC complex may be suppressed by endogenous competitive inhibitor cFLIP, a structural analog of caspase-8 that is devoid of protease activity (Lavrik et al., 2012).

As a result of numerous studies, this "classical" scheme of receptor-mediated activation of the apoptotic cascade has become significantly more complex. It has been shown that molecular processes occurring in the cell under the effect of TNFα have certain specific features, compared to those caused by CD95L or TRAIL. TNFα-dependent signal transduction begins with the formation of receptor-proximal Complex I, which is destabilized in case of RIP1 deubiquitination and dissociates from TNFR1 into the cytoplasm, forming Complex II (Fig. 3). Ubiquitin monomers are cleaved off by CYLD and A20. Polyubiquitin chains may also be attenuated when cIAPs are inhibited, e.g., upon activation of mitochondrial factor Smac/DIABLO or under the effect of Smac mimetics (Moquin et al., 2013). RIP1 kinase devoid of the ubiquitin tail can associate with the FADD adaptor, which binds procaspase-8.

Thus, proapoptotic Complex II is composed of proteins FADD, RIP1, procaspase-8, and cFLIP. It is within this complex that caspase-8 is activated to cleave RIP kinases and the CYLD protein, thereby preventing the development of necroptosis (RIP-dependent cell death) and directing the cell to the apoptosis pathway (Fig. 3). However, the level of caspase-8 activation in Complex II and, hence, the choice between the apoptosis, necroptosis, and survival pathways shows a complex dependence on the expression of cFLIP and apparently on the balance between its "short" and "long" isoforms, which inhibit caspase-8 activity to different extents. As noted above, cFLIP competes with procaspase-8 for binding with FADD. When the level of cFLIP is low, procaspase-8 forms homodimers and undergoes autocatalytic activation, inducing apoptosis; when its level is high, cFLIP forms heterodimers with procaspase-8,

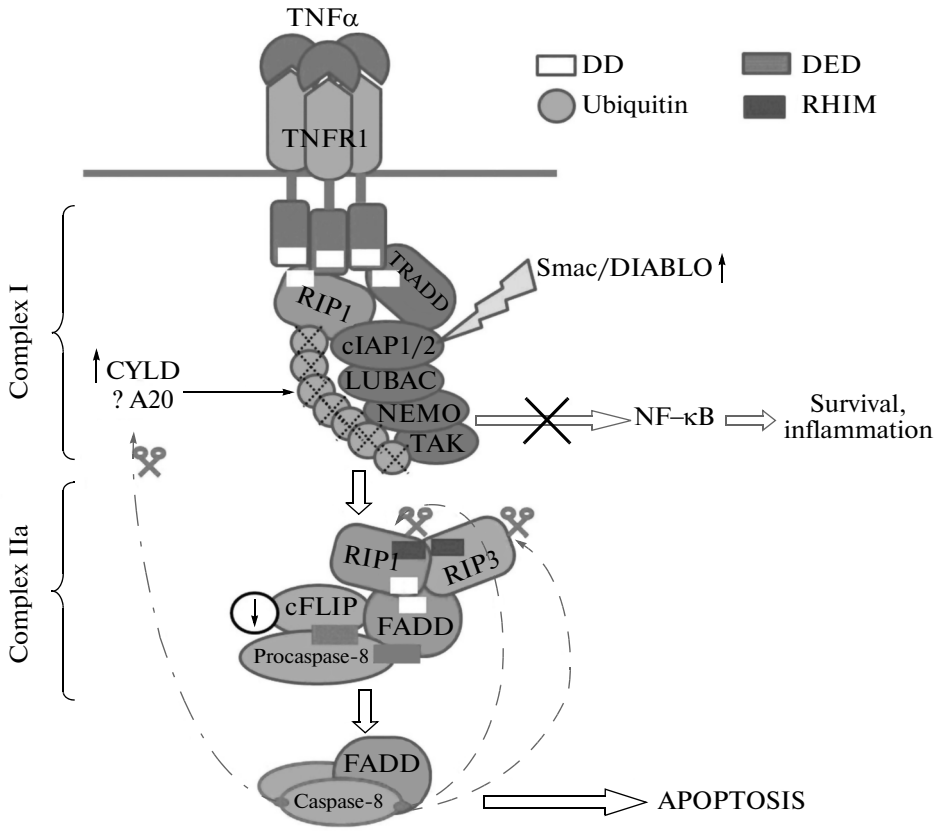

Fig. 3. Scheme of Complex II (IIa) assembly. Rectangles indicate functional domains mediating protein–protein interactions.

thereby blocking its activation. Moreover, cFLIP in the same way inhibits not only caspase-dependent apoptosis but also caspase-independent RIP-mediated necrosis, probably by destabilizing the FADD:RIP1:RIP3 complex. Thus, cFLIP acts as a trigger between cell survival and death (either apoptotic or necroptotic) (Giampietri et al., 2014).

Despite a considerable advance in understanding the mechanisms of induction of receptor-mediated apoptosis, it is still unclear what exactly are the signals that play the role of switches between Complexes I and II in vivo (Christofferson et al., 2010; Vanlangenakker et al., 2011). Apparently, the choice of cell fate (survival or death) largely depends on the balance between (a) ubiquitin ligase and deubiquitinase activities and (b) relative expression levels of caspases and their endogenous inhibitors or activators. The role of RIP1 kinase in Complex IIa formation is not yet fully elucidated, since the inhibition of its expression in certain cases stimulates the formation of the proapoptotic complex instead of suppressing it. Every cell type appears to have its own profile of relative expression of these components, which accounts for the high tissue specificity of response to TNFα. Active consideration is also given to the role of TNFα–receptor complex compartmentalization and microenvironment (incorporation into specific membrane microdomains, or rafts) in determining its protein composition and functional state (Schütze et al., 2008).

NECROPTOSIS AS AN ALTERNATIVE PATHWAY OF TNFα-INDUCED CELL DEATH

Traditionally, two types of cell death are distinguished: necrosis and apoptosis. Necrosis is understood as a passive, accidental form of cell death accompanied by cell membrane rupture and the release of cytoplasm into the intercellular space, in the absence of internucleosomal DNA fragmentation (Wu et al., 2012). It has been found, however, that necrotic cell death in some cases may be associated with activation of certain enzymes and be regulated at the genetic level (Linkermann et al., 2014). Moreover, the results obtained in different experimental systems show that when the activities of caspases are completely blocked (by synthetic peptide analogs or viral inhibitors), treatment with death ligands may induce cell demise with the characteristic cytological symptoms of necrosis (Vercammen et al., 1998). Combined treatment of cells with both inhibitors of proteins activated during necrosis and caspase inhibitors results in the development of resistance to cell death factors such as cytokines of the TNFα family. Thus, a caspase-independent

but genetically programmed form of necrotic cell death has been discovered and termed necroptosis.

Similar to apoptosis, programmed necrosis can be stimulated by different factors (genotoxic stress, hypoenergetic states, disturbances of Ca^{2+} homeostasis, loss of transmembrane potential, depolarization of mitochondria, hyperproduction of reactive oxygen species, etc.). Activation of some cell surface receptors (TNFR1, TNFR2, TRAILR1, TRAILR2, TLR3,4) can also induce necrotic cell (Linkermann et al., 2014), with TNFR-mediated necrosis being especially relevant to the problem of septic shock. It should be noted that TNFα was identified primarily as a factor causing rapid hemorrhagic necrosis of tumors in mice (Carswell et al., 1975). A widespread in vitro model of TNFR-dependent necroptosis is that of murine fibrosarcoma L929 cells treated with TNFα. Necroptosis underlies pathogenesis of many chronic and acute inflammatory diseases, including ischemic heart disease, retinal detachment, atopic dermatitis, Crohn's disease, and acute pancreatitis (Linkermann et al., 2014). In this review, attention is focused on the role of necroptosis in the development of TNFα-induced systemic inflammatory response (septic shock) and on experimental models for its study.

The formation of necroptosis-associated Complex IIb. In the course of studies on molecular mechanisms of TNFα-dependent necroptosis, researchers have arrived at the conclusion that RIP3 kinase is the most probable factor triggering this process; hence, necroptosis is also referred to as RIP3-dependent cell death (in contrast to caspase-dependent cell death). Nevertheless, RIP1 is also an essential component and regulator of the cascade of events associated with necroptosis (He et al., 2009). Research on the mechanism of necroptosis induction was contributed to by the discovery of necrostatin-1 (Nec-1), an allosteric inhibitor of RIP1 kinase that specifically suppresses its kinase activity without affecting its functioning as a molecular polyubiquitin platform. In experiments on different in vitro and in vivo systems, preliminary cell treatment with Nec-1 proved to prevent TNFα-induced cell death without affecting the ability of TNFα to activate the NF-κB pathway. Therefore, it is the kinase activity of RIP1 (which depends on the degree of its polyubiquitination) that is important for switching the TNFα signal to the cell death pathway; in the absence of active caspase-8, this signal is transduced to RIP3, initiating necroptosis. The composition of the necroptosis signaling pathway and functions of its individual components are still largely unknown, and studies are underway to identify and develop new specific inhibitors allowing in-depth analysis of this pathway (e.g., necrosulfonamide was found to be an inhibitor of human MLKL pseudokinase, a necrosome component); much hope is also pinned on potential applications of such inhibitors as medical drugs (Linkermann et al., 2014).

TNFR-induced necroptosis begins with the formation of necrosome, i.e., amyloid-like cytoplasmic complex [RIP1K:RIP3K:MLKL], or Complex IIb (Fig. 4). Supposedly, deubiquitination of RIP1 makes its RHIM domain accessible for interaction with the homologous domain of RIP3; in the resulting complex, RIP1 is autocatalytically activated to phosphorylate RIP3, which then activates proteins involved in the effector phase of necroptosis (Moriwaki et al., 2013). As noted above, the spectrum of these proteins is largely unknown, but there is evidence that the composition of necrosome may include MLKL pseudokinase and mitochondrial phosphoglyceromutase and dynamin-like Drp1 protein, whose activation is associated with increased production of reactive oxygen species. RNAi silencing of MLKL inhibits necroptosis in model systems, as does RIP1 and RIP3 knockdown, which is evidence for its central role in the cascade of necroptosis-associated reactions (Remijsen et al., 2014). RHIM domains have been found in other two proteins, DAI and TRIF; the former has been shown to integrate signals from different intracellular sensors of viral infection, and the latter functions as a cytoplasmic adaptor for TLR3 and TLR4 receptors. Therefore, cell recognition of viral or bacterial infection may be accompanied by necrosome formation and activation of RIP-dependent necroptosis (Linkermann et al., 2014).

The main question as to the mechanism of switching between apoptosis and necroptosis (i.e., between Complexes IIa and IIb) remain open. Necrosome formation depends not only on the relative activities of deubiquitinases and ubiquitin ligases but also on the level of caspase-8 activity. The assembly of the necrosomal complex becomes possible if the process of caspase-8 activation is somehow blocked (e.g., upon cell treatment with the pancaspase inhibitor zVAD) or caspase-8 expression is below the appropriate level. It is in such a case that RIP kinases and CYLD deubiquitinase remain intact, while RIP1 retains its kinase activity.

It should be noted that caspase-8 performs a protective function against necroptosis by blocking necrosome assembly. This important conclusion is based on the results of experiments with knockout mice. It has been found that deletion of caspase-8, FADD, or cFLIP is lethal (mice die at early stages of embryonic development), while mice with double knockout of one of these genes and the RIP3 gene are quite viable. Therefore, the cause of embryonic mortality is RIP3-induced cell death, and the complex [caspase-8:FADD:cFLIP] is its antagonist (Kaiser et al., 2011; Zhang et al., 2011). Thus, inhibition of necrosis is the key nonapoptotic function of caspase-8. In contrast to this enzyme, CYLD deubiquitinase stimulates necroptosis, and its role as a probable trigger of this process is confirmed by the results of experiments on knockdown of the gene *cyld* that inhibits necrotic cell death. In laboratory mice, tissue-specific

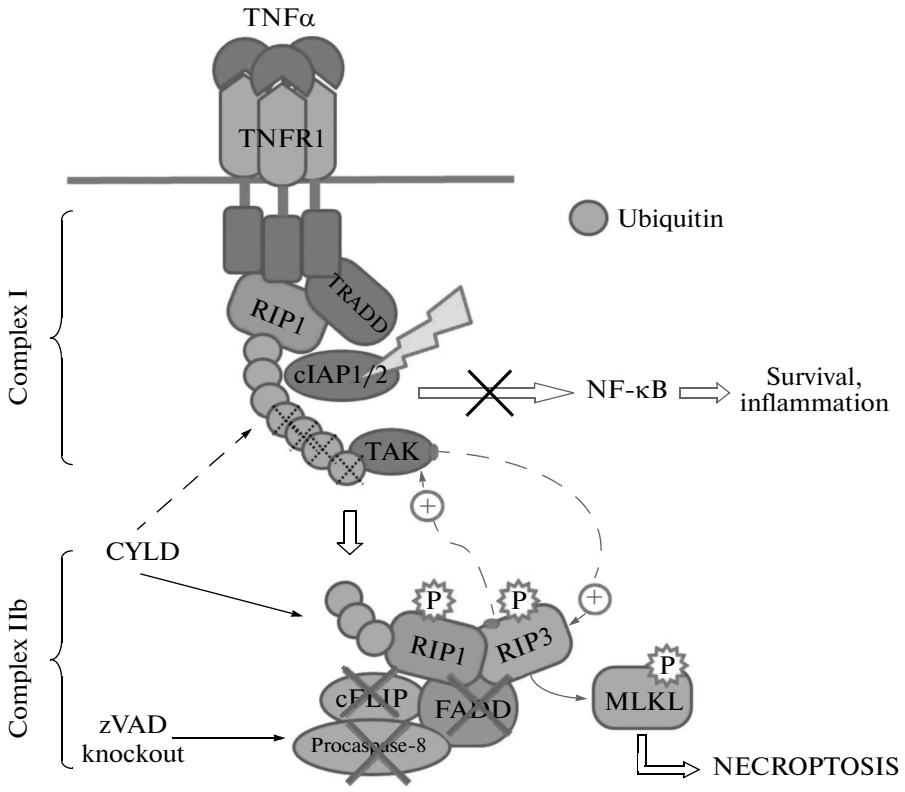

Fig. 4. Scheme of Complex IIb (necrosome) assembly.

knockdown of FADD in epidermal cells leads to severe necrotic skin lesions and inflammation, but their progeny from crosses with mice mutant for CYLD deubiquitination activity recover the normal phenotype (Moquin et al., 2013). Nevertheless, this role of CYLD needs additional experimental verification, because its inhibition does not necessarily results in switching from necroptosis to apoptosis. It may well be that CYLD regulates the dynamics of RIP1–RIP3 complex assembly rather than determines the very possibility of its formation (Moquin et al., 2013).

The results of studies by Morioka (2014) and Dondelinger et al. (2013) suggest a possible role for TAK1 kinase as a molecular switch between TNFα-driven apoptosis and necroptosis. These authors have found that TAK1 kinase, a component of the TNFR-proximal complex (Complex I) can directly activate RIP3 kinase, thereby initiating cell death by necrosis, while inhibition of TAK1 activity leads to caspase-dependent apoptosis, irrespective of the presence or absence of RIP3.

Another question addressed in the literature concerns the significance of other posttranslational modifications of RIP kinases (apart from their ubiquitination and phosphorylation) for the choice of TNFα signal transduction pathway. It has been found, for example, that RIP1 Lys530 deacetylation by SIRT2 deacetylase stabilizes the RIP1–RIP3 complex and enhances TNFα-dependent necrosis in an in vitro model (Narayan et al., 2012), but additional studies are necessary to confirm the role of SIRT 2 in the regulation of necroptosis in vivo (Moriwaki et al., 2013; Newton et al., 2014).

Expectedly, novel protein regulators of the TNFα-dependent signaling pathway will be revealed, or new functions of its already known components will be discovered. In the majority of experimental systems, the role of a certain component in TNFα signaling is assessed using a certain method of its inactivation, but this by no means always allows a deeper insight into how TNF-dependent regulation occurs during the development of a pathological process in natural living systems, where all components of the signal pathway remain functionally active (at least potentially). How is the "decision making" at the cell level done in the absence of any inhibitors? The results of recent studies provide evidence for a remarkable flexibility of this regulatory mechanism and its high tissue specificity. The functions of many its components overlap considerably and are redundant; depending on cell type and conditions, different components may substitute for each other. Therefore, it should be taken into account

that the schemes considered above may pertain to particular cases and only roughly reflect the real regulatory potential of the TNFα-dependent mechanism. Attempts to generalize data obtained by different research groups show that they are often inconsistent with each other, which is usually explained by differences in cell types used as model systems and in experimental conditions.

For example, it has been reported that RIP1 kinase is not an obligate component of the TNFα-dependent cascade leading to cell death by necroptosis or apoptosis (Moriwaki et al., 2013; Moujalled et al., 2013). Some experimental results suggest that, even though RIP1 and RIP3 are key factors in the mechanism of necroptosis, they do not function as triggers between TNFα-driven apoptosis and necroptosis (Kaczmarek et al., 2013; Remijsen et al., 2014). Probably, the ratio between expression levels of RIP3 and RIP1 has decisive significance in this case.

The role of necroptosis in the development of systemic inflammatory response. The question concerning the contribution of RIP1–RIP3 necroptosis to the development and lethal effect of septic shock is still debatable, despite important pieces of experimental evidence obtained during recent years (Linkermann et al., 2012). It is noteworthy that cell death by necroptosis, unlike in apoptosis, involves plasmalemma rupture and is accompanied by the release in the intercellular space of highly immunogenic cytoplasmic proteins referred to as damage-associated molecular patterns (DAMPs) (Kaczmarek et al., 2013). DAMPs can be recognized by the PRR receptors of macrophages, which results in amplification of the innate immunity reactions initiated by bacterial infection (LPS, TNFα), with consequent development of septic shock, or cytokine storm (Linkermann et al., 2014).

The data obtained by Duprez et al. (2011) on the mouse model of "sterile" sepsis provide evidence that the fatal outcome of septic shock results primarily from TNFα-induced necroptosis of hepatocytes. Using a gene knockout technique, these authors revealed the role of RIP3 kinase in promoting systemic inflammation in vivo and the protective effect of caspase-8: RIP3(–/–) mice proved to be absolutely insensitive to TNFα injection, whereas injection of the pancaspase inhibitor zVAD to wild-type mice enhanced the lethal effect of TNFα. Histological analysis revealed no necrotic changes in the liver of RIP3-knockout mice, compared to wild-type animals. The level of circulating DAMPs in RIP3(–/–) mice did not significantly increase after TNFα injection, which confirmed the absence of necrotic damage to internal organs. A noteworthy fact is that knockout of effector caspases 3 and 7 and inflammatory caspase-1 had no effect on the mortality rate of mice with induced septic shock. Therefore, the lethal effect of TNFα and the sensitizing effect of zVAD on hepatocytes are determined exactly at the level of caspase-8, i.e., at the level of signal switching between apoptosis and necroptosis. RIP3 knockout also eliminated the lethal effect of TNFα in the model of sepsis caused by cecal ligation and puncture, which confirmed the universality of the proposed concept (Duprez et al., 2011). Analyzing the time course of changes in the concentrations of circulating proinflammatory cytokines, these authors found that the level of IL-1 and IL-6 in RIP3(–/–) mice increased to the same extent as in wild-type mice during the first 2 h after TNFα injection, but it proved to be markedly lower than in the latter after 6 h. This observation provides a basis for the following conclusions: (1) the proinflammatory NF-κB pathway in RIP3(–/–) mice remains intact, since RIP3 kinase is not involved in it (Fig. 2), and (2) the consistently high level of inflammatory cytokines in wild-type mice is most probably accounted for by the "secondary" response to increase in the concentration of DAMPs resulting from necrotic cell death rather than by primary activation of macrophages under the effect of TNFα. These data confirm the physiological significance of the type of cell death: necroptosis is exactly the type characterized by the formation of a positive feedback loop that amplifies inflammatory reactions. However, Duprez et al. (2011) provide arguments for the protective effect of Nec-1 in the in vivo model of septic shock, but these results are questioned by other researchers (Linkermann et al., 2012).

Damage to the intestinal mucosa is another characteristic process accompanying TNFα-induced septic shock, but epithelial cells in this case die by apoptosis. This is confirmed both by cytomorphological analysis and by experiments on gene knockdown: it is deletion of caspase-3, but not of RIP3 kinase, that prevents intestinal epithelium damage upon TNFα injection. Taking into account that RIP3(–/–) mice survive after septic shock induction, it appears that damage to the intestinal epithelium is not the main cause of mortality (Duprez et al., 2011). It is noteworthy that conditional knockout mice lacking the expression of FADD or caspase-8 in enterocytes are characterized by a high frequency of spontaneous necrosis of these cells and an increased level of RIP3 expression. TNFα injection in such animals also induces necrosis of intestinal epithelial cells, while in wild-type mice the TNFα signal initiates apoptosis (Galluzzi et al., 2011; Welz et al., 2011). Thus, different tissues prove to be highly selective in "choosing" the pathway of cell death in response to the same stimulus (TNFα) and in the absence of any exogenous inhibitors or interventions at the level of genes, which is apparently determined by the natural intracellular "molecular context."

USING MOUSE MODELS TO SEARCH FOR NEW COMPONENTS OF LPS-/TNFα-INDUCED SEPTIC SHOCK

The resistance or sensitivity to septic shock is a complex, polygenic quantitative trait reflecting the multistage and multicomponent process of the development of inflammatory response. To be capable of controlling this process (for therapeutic purposes, drug development, etc.), it is necessary to identify the maximum possible number of genes involved in it and reveal their functions. The existence of natural subspecies or inbred strains of conspecific animals differing in the level of resistance to septic shock makes it possible to study the proteins and mechanisms involved in its development using methods of "forward" genetics. This approach is based on crosses between organisms with contrasting expression of a trait and analysis of corresponding phenotype in a series of filial generations. Attention in this course is focused on recombination events, since their analysis allows mapping of the trait (i.e., determination of chromosome region containing the corresponding gene). A statistically significant genotype–phenotype correlation confirms the involvement of the identified genetic locus in the expression of the trait under study (Poltorak, 2012).

Classical inbred (pure) strains of laboratory mice are a convenient model for analyzing molecular genetic mechanisms controlling the development of immune response. Their advantage is that the phenotypes of such strains are well studied, and some strains have proved to be close to the human in certain aspects. Panels of microsatellite markers developed for genetic analysis on mouse models allow full-genome genotyping of individuals. However, specialists who use classical genetic analysis with standard inbred lines to reveal new genes have problems in finding strains with contrasting (opposite) phenotypes of the trait studied. This is due to the limited genetic diversity of such strains, since most of them have been derived from the same subspecies of the house mouse, *Mus musculus domesticus*. In this respect, especially valuable are the strains derived from wild subspecies or species that diverged from each other more than million years ago (Conner et al., 2009). Based on the results of analysis of different genetic and biochemical markers, the species *Mus musculus* was divided into three subspecies: *M. m. domesticus*, *M. m. castaneus*, and *M. m. musculus* (Moriwaki et al., 2009). *Mus m. domesticus* is the founder species for most of laboratory lines, including the widely used C57BL/6 strain. The inbred strain MSM/Ms has been derived from another subspecies living in Japan, *M. m. molossinus* (Moriwaki et al., 2009), as also was the strain MOLF/Ei. Subspecies *M. m. domesticus* and *M. m. molossinus* diverged about million years ago and have accumulated a considerable number of genetic differences during this long period of independent evolution: on average, these subspecies and derivative lines contain a nucleotide substitution per 100–200 nucleotides (Conner et al., 2009). The high frequency of polymorphisms manifests itself in a considerable number of phenotypic differences. In particular, it has been found that "wild" strains differ from classical strains in many parameters of immune response to pathogenic microorganisms and, hence, can be used to search for the corresponding genes using the forward genetic approach.

Below are given several examples of studies on the molecular genetic mechanisms of septic shock with such wild strains as well as with classical inbred strains of mice with spontaneous mutations in the genes of immune response.

In 1968, two sublines of the classical inbred strain C3H were revealed: LPS-resistant C3H/HeJ and LPS-sensitive C3H/HeN (Sultzer, 1968). Subsequent studies showed that the divergence into these sublines occurred as a result of spontaneous mutation in the single gene coding for the LPS receptor. As a result of cloning and sequencing, this receptor was identified as TLR4, and the mutation responsible for its insensitivity to LPS was revealed: Pro714His substitution in the cytoplasmic domain of the receptor proved to interfere with its correct dimerization, activation, and signal transduction (Poltorak et al., 1998; Xu et al., 2000). Coutinho et al. (1978) characterized another subline of LPS-resistant mice, named C57BL10/ScCR. These mice are also devoid of functional TLR4, but this is due not to a point mutation but to a deletion in the LPS locus (Poltorak et al., 2000). It should be noted that mouse lines resistant to LPS- or TNFα-induced septic shock are usually highly sensitive to bacterial infection, which is evidence for the important role of LPS-/TNFα-dependent reactions in pathogen elimination (O'Brien et al., 1980).

The study by Staelens et al. (2002) is an example of genetic analysis performed with the wild mouse strain SPRET/Ei (derived from the species *Mus spretus*, which diverged from *Mus musculus* about 3×10^6 years ago) to study the mechanism of the lethal action of TNFα during the development of septic shock. Mice of this strain are highly resistant to TNFα, compared to C57BL/6 mice (the difference is no less that 1 log unit). In particular, TNFα injection to SPRET/Ei does not result in hypothermia, increased level of circulating IL-6 or serum transaminase activity, or delamination of intestinal epithelium. Using C57BL/6 × SPRET/Ei intercrossing and a series of reciprocal crosses with one of parental strains, genes involved in the formation of TNFα resistance have been localized to chromosomes 2, 6, and 11.

The "wild" mouse strain MOLF/Ei derived from *M. m. molossinus* has been successfully used to study the temporal regulation of MyD88-/NF-κB-dependent signaling pathway upon TLR activation (Conner et al., 2009). Macrophages of these mice respond to TLR stimulation by increasing the expression of proinflammatory cytokine IL-6 and the IKK kinase

and p38 activities to a higher level, compared to macrophages of C57BL/6 mice. The genetic mapping of this trait resulted in identification of isoforms of MyD88-associated kinase IRAK-2 and the gene encoding its inhibitor as novel components of the MyD88 cascade (Conner et al., 2009). It has also been found that macrophages of MOLF/Ei mice, unlike those of other strains (C57BL/6, MSM, CZECH, SPRETUS, and CAST), show only a slight immune response to TLR9 activation by oligonucleotides containing CpG motifs that mimic bacterial DNA (Moseman et al., 2013). The molecular genetic basis of this phenotype has not been revealed in detail: most components of the signaling cascade are functionally active in all the above mouse strains, and phenotypic differences between them probably reflect specific features in the regulation of the TLR9 pathway.

The MSM/Ms strain is also characterized by high resistance to TNFα-/LPS-induced septic shock. The dose of LPS that is lethal to C57BL/6 mice does not cause any symptoms of systemic inflammation in MSM mice. The survival rate of the latter does not decrease when LPS/TNFα is injected together with D-galactosamine, which blocks the synthetic function of the liver. The resistance to LPS-induced septic shock in MSM mice is not due to disturbances in the TLR4-dependent pathway, since their macrophages produce a large amount of TNFα upon stimulation and, therefore, appears to be determined at the level of TNFα signal perception. This conclusion is confirmed by the fact that MSM mice easily tolerate injection of recombinant TNFα at a dose that is 100% lethal to C57BL/6 mice. According to preliminary data obtained at the Laboratory of Molecular Genetics of Innate Immunity, Petrozavodsk State University (headed by Prof. A.N. Poltorak), the resistance of MSM mice to TNFα is not due to disturbances in TNFα reception; specific features of their phenotype appear to be related to the regulation of TNFα signal switching between necroptosis, apoptosis, and survival–inflammation. This hypothesis is indirectly confirmed by the data that 4-day ingestion of dextran sulfate with drinking water proved to cause severe inflammatory damage of the intestine in C57BL/6 mice, while no signs of necrosis of enterocytes were revealed in MSM mice (Nakanishi et al., 2007).

CONCLUSIONS

Mechanisms regulating the functioning of TNFα in vivo in the norm and under pathological conditions remain largely obscure, despite large-scale research in this field, and the question concerning the molecular genetic bases of high resistance or sensitivity to this factor in different cell types is still open. Most studies on the mechanism of switching between apoptosis, necrosis, and cell survival upon activation of TNFα receptor have been performed on the models of laboratory cell lines (e.g., murine embryonic fibroblasts, MEFs, or murine fibrosarcoma L929 cells) and by the methods of "reverse" genetics (gene knockout, knockdown, etc.). However, the potential of these experimental systems is limited, because they by no means always can provide an insight into the functioning of the regulatory mechanism under natural conditions and at the level of organism as a whole. In this context, in vivo models of septic shock that differ in phenotypic parameters of its development appear especially valuable.

Difficulties in explaining in detail the trigger mechanism of TNFα-dependent signal transduction are apparently due to the fact that not all components of the signaling cascade have been identified and not all functions of already known components have been revealed to date. As noted above, many molecules involved in the TNFα-signal pathway perform not only their basic (catalytic) function by also play a role of molecular platforms, thereby creating an appropriate microenvironment. Mouse strains with natural, genetically conditioned resistance or sensitivity to septic shock appear to be most promising for solving problems in this field.

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