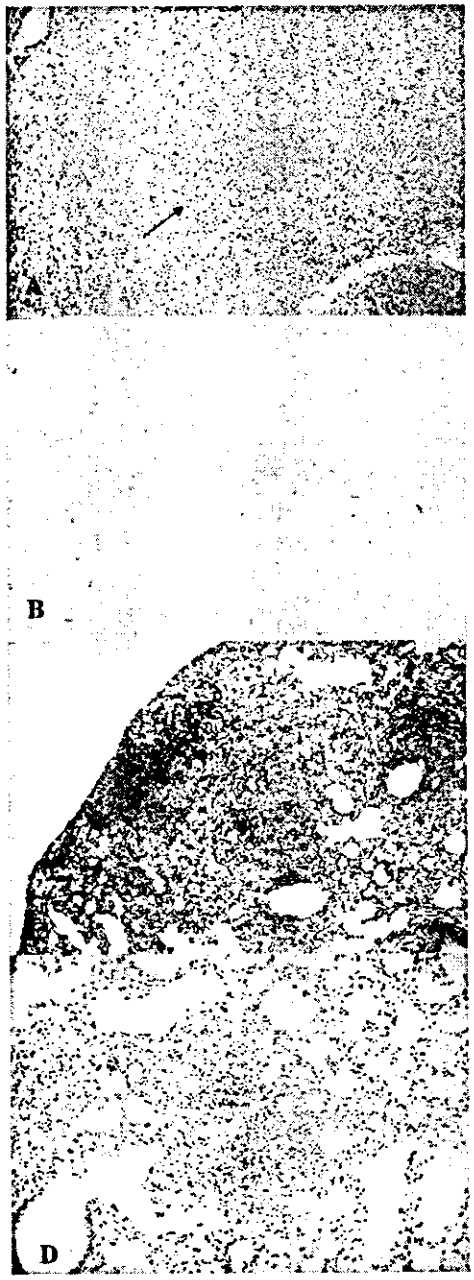

Fig. 3. Histological analysis of lung sections with *M. tuberculosis* Kurono strain. Lungs were removed from STAT1 KO (A) and WT mice (C) 35 days after infection with Kurono strain (10^6 cfu) by an airborne route. Necrosis (→) is recognized in the lung of the STAT1 KO mouse. Hematoxykin and eosin stain; original magnification, ×200. B: Stain for acid-fast bacilli in the lung of STAT1 KO infected with Kurono strain. Original magnification, ×600. D: Pulmonary tissue (5 weeks after infection) from STAT1 KO mice infected with *M. tuberculosis* Kurono strain and treated four times subcutaneously with recombinant IL-12. The granuloma became smaller. Original magnification, ×200.

much higher than that from WT mice at week 1 through 5 after aerosol infection (Fig. 2). At 3 and 5 weeks after infection, there were statistically significant differences in both lung and spleen counts between WT and STAT1 KO mice ($p<0.01$).

Light microscopic observations of infected lungs

The mice were sacrificed 5 weeks after being infected by an airborne route and formalin-fixed sections were stained with hematoxylin and eosin and by the Ziehl-Neelsen method. Macroscopically, there were extensive necroses in the lungs, spleen, and liver of STAT1 KO mice infected with *M. tuberculosis* Kurono strain. On the contrary, pulmonary discrete granulomatous lesions were recognized in WT mice and these granulomas lacked necrosis. Histopathologically, multiple foci of abscesses consisting of neutrophils and epithelioid macrophages were noted in the lungs, spleen, and liver, whereas WT mice developed granulomatous lesions lacking neutrophil infiltration (Fig. 3). Epithelioid macrophages and lymphocytes were not recognized near the necrotic lesions. The necrotic lesions displayed central necrosis, neutrophils and cell debris. Ziehl-Neelsen staining revealed diffuse proliferation of tubercle bacilli in the necrotic lesions (Fig. 3B).

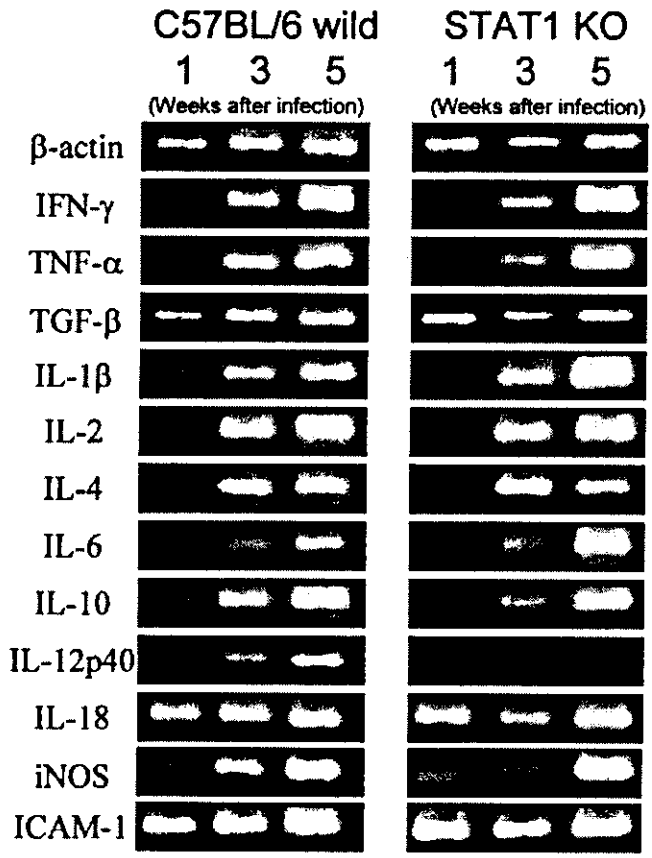

Fig. 4. RT-PCR analysis of cytokine, TGF-β, iNOS and ICAM-1 mRNA expression. Total RNA was isolated from lung tissues of STAT1 KO and WT mice at 1, 3, and 5 weeks after aerosol infection and subjected to RT-PCR using the specific primer sets. IL-12 mRNA expression level is low significantly in the lung tissues of STAT1 KO mice.

TABLE 1. *Size of pulmonary granulomas infected with M. tuberculosis Kurono strain*

Mice	Size of granulomas (μm)
STAT1 KO	5500±105
STAT1 KO treated with IL-12	450± 20
WT	400± 25
WT treated with IL-12	350± 15

Ten pulmonary granulomas per group consisting of 6 mice 5 weeks after aerosol infection were measured with a micrometer. The difference in size of granulomas between STAT1 KO and STAT1 KO mice treated with IL-12 was statistically significant at Student's t-test ($p<0.01$).

RT-PCR analysis

Fig. 4 shows the results of the RT-PCR data from infected lung tissues at 1, 3 and 5 weeks after aerosol infection (three mice each). In STAT1 KO mice, expression of IFN-γ, TNF-α, IL-10, IL-18 and iNOS mRNA was low at weeks 3 after infection compared with that of WT mice. Expression of IL-12 p40 mRNA was very low at weeks 3-5 after infection than that of WT mice and the difference in IL-12 mRNA expression between STAT1 KO and WT mice was statistically significant ($p<0.01$). Expression of IL-1β, IL-2, IL-4, IL-6 and ICAM-1 mRNA was similar in both STAT1 KO and WT mice.

Reconstitution of STAT1 KO mice with exogenous recombinant murine IL-12

As the defect in the STAT1 KO mice was genetically defined, the possibility of recovering immune response function by administering exogenous recombinant IL-12 subcutaneously to the KO mice was investigated. As shown in Table 1, when recombinant IL-12 was given four times subcutaneously, the sizes of the granulomatous pulmonary lesions 5 weeks after aerosol infection with *M. tuberculosis* were reduced significantly (mean diameter, 450±20 μm), and there were virtually no mycobacteria in the pulmonary lesions (Fig. 3D). The sizes of the granulomatous lesions without recombinant IL-12 treatment 5 weeks after aerosol infection were 5, 500±105 μm. There was a statistically significant difference in the sizes of pulmonary granulomas between STAT1 KO and STAT1 KO mice treated with IL-12 WT ($p<0.01$). The sizes of WT and WT mice treated with recombinant IL-12 were 400± 25 and 350±15 μm, respectively. There were no apparent side effects of recombinant murine IL-12 when it was given four times subcutaneously to both STAT1 KO and WT mice.

DISCUSSION

The purpose of this study was to determine the roles of STAT1 protein as a transcription factor in defense against mycobacterial infection. STAT1 KO mice developed multifocal necrotic lesions in the lung, liver and spleen tissues and died of disseminated tuberculosis by the 35 th day of infection. We have shown that STAT1 is essential for the control and survival of the *M. tuberculosis* infection. We made aerosol infection experiments twice and the results were reproducible. We also noted marked reduction of IL-12 mRNA expression in STAT1 KO mice. It is reported that IL-12 is crucial to the development of protective immunity in *M. tuberculosis*-infected mice (Cooper et al. 1997). This marked reduction of IL-12 may be explained as follows. Interferon regulatory factor (IRF)-1 mRNA is significantly reduced in IFN-γ-treated macrophages from STAT1 KO mice (Meraz et al. 1996). Stat 1 is placed upstream from IRF-1 (Darnell 1997). It is known that IL-12 production by macrophages is impaired in IRF-1 KO mice (Lohoff et al. 1997;

Hermann et al. 1998). We have also reported that IRF-1 KO mice developed multifocal necrotic lesions in the lung, liver and spleen tissues and died of disseminated tuberculosis within 43 days of infection (Yamada et al. 2002).

There are seven STAT proteins. We have recently reported the role of STAT4 in mycobacterial infection (Sugawara et al. 2003). STAT4 KO mice infected with *M. tuberculosis* developed large granulomas with massive neutrophil infiltration over time, but did not die of disseminated tuberculosis. Pulmonary iNOS, and IFN-γ mRNA levels were low in STAT4 KO mice, but expression of IL-12 mRNA was not affected in STAT4 KO mice. No significant difference was recognized in severity of mycobacterial infection between WT and STAT6 KO mice. Therefore, STAT1 plays the most essential role for defense against mycobacterial infection among seven STAT proteins.

IFN-γ, TNF-α, and IL-18 mRNA expression was lower in STAT1 KO mice than WT mice. Their reduced expression contributes to the progression of murine tuberculosis. We and other researchers have already reported that IFN-γ, TNF-α and IL-18 play major roles for defense against murine tuberculosis (Cooper et al. 1993; Flynn et al. 1993; Flynn et al. 1995; Sugawara et al. 1998; Kaneko et al. 1999; Sugawara et al. 1999).

It is reported that STAT4 and extracellular regulated kinase 1 activation by IL-12 was intact, whereas the activation of STAT1, -3, and -5 by IL-12 was lost in a patient with atypical mycobacterial infection (Gollob et al. 2000). This imapairment of STAT activation was specific for IL-12 because STAT activation IL-2, IL-15, and IFN-γ was unaffected. In our mycobacterial experiments, STAT1 activation is impaired by IL-12 because IL-12 mRNA expression was severely lost. This indicates that the activation of STAT4 alone is not sufficient for IL-12-induced IFN-γ production. There is another interesting report stating that *M. tuberculosis* inhibits IFN-γ transcriptional responses without inhibiting activation of STAT1 (Ting et al. 1999). One mechanism for *M. tuberculosis* to evade the immune response is to inhibit the IFN-γ signaling pathway and the mechanism of inhibition is distinct from that reported for *Leishmania donovani* or cytomegalovirus (CMV) infection (Nandan and Reiher 1995; Miller et al. 1998). It, however, seems that mice with no STAT1 have no innate response to either vesucular stomatitis virus (VSV) or *Listeria monocytogenes* infection because the first line of defense against potential pathogens requires the IFN response (Durbin et al. 1996; Meraz et al. 1996).

When recombinant murine IL-12 was administered subcutaneously to the infected STAT1 KO mice, the sizes of the pulmonary granulomas were reduced significantly ($p<0.01$). Therefore, changing the therapeutic regimens administered to the experimental model mice in our study may enable mycobacterial infection to be prevented completely (Phillpotts et al. 2003). Treatment with exogenous IL-12 also reduced the bacterial load, indicating that IL-12 plays an important role in the immune response to *M. tuberculosis* (Cooper et al. 1997). However, other factors than IL-12 may be involved in tuberculosis of STAT1 KO mice because IL-12 addition did not cure pulmonary tuberculosis completely.

In summary, STAT1 KO mice were highly susceptible to *M. tuberculosis* infection. Our data demonstrtate clearly that the STAT1-mediated cytokine regulation pathway is critical for the development of protective immunity against tuberculosis.

Acknowledgements

This study was supported in part by an International Collaborative Study Grant awarded to Dr. I. Sugawara from the Ministry of health, Labor and Welfare, Japan.

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INFECTION AND IMMUNITY, Mar. 2004, p. 1804–1806 Vol. 72, No. 3
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.3.1804–1806.2004

Rat Neutrophils Prevent the Development of Tuberculosis

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Received 31 July 2003/Returned for modification 14 September 2003/Accepted 9 October 2003

To understand the role of neutrophils in the development of rat tuberculosis in vivo, we utilized lipopolysaccharide (LPS)-induced neutrophilia in the lungs. LPS (50 μg/ml) was administered intratracheally to male Fischer rats. Rats were then infected with *Mycobacterium tuberculosis* by an airborne route. Intratracheal injection of LPS significantly blocked the development of pulmonary granulomas and significantly reduced pulmonary CFU ($P < 0.01$). LPS treatment with amphotericin B (an LPS inhibitor) or neutralizing anti-rat neutrophil antibody reversed the development of pulmonary lesions. LPS-induced transient neutrophilia prevented early mycobacterial infection. The timing of LPS administration was important. When given intratracheally at least 10 days after aerial infection, LPS did not prevent development of tuberculosis. Neutrophils obtained by bronchoalveolar lavage killed *M. tuberculosis* cells. These results indicate clearly that neutrophils participate actively in defense against early-phase tuberculosis.

When tubercle bacilli are introduced into the lung, neutrophils migrate and accumulate in the infected pulmonary lesions during the early stage of tuberculosis. This rapid neutrophil response controls fast-replicating intracellular bacteria but not slow-replicating *Mycobacterium tuberculosis* (5). However, it has been reported that murine neutrophils play a protective nonphagocytic role in systemic *M. tuberculosis* infection in mice (3). Neutrophils from *M. avium*-infected mice produce tumor necrosis factor alpha, interleukin-12 (IL-12), and IL-1β (4). Thus, little is known about immunologic function in the lungs (although neutrophils have been identified as sources of inflammatory cytokines and chemokines) (2). In circumstances in which neutrophils are not activated with granulocyte colony-stimulating factor, severe mycobacterial infection results (7). However, it remains unclear whether neutrophils play a protective role in defense against mycobacterial infection. In vivo studies of neutrophils in the lungs are challenging, because it is difficult to induce neutrophilia. We therefore modified the lipopolysaccharide (LPS)-induced neutrophilia system in rats so that we could utilize it to study rat tuberculosis (1).

Permission to experiment on animals was given by the Animal Experiment Committee of The Research Institute of Tuberculosis. We injected LPS (*E. coli* 0111:B4 LPS) purchased from Sigma (St. Louis, Mo.) (50 μg/0.5 ml) intratracheally into anesthetized 6-week-old female Fischer rats (three rats/group). On the day after the injection, the rats were infected with the *M. tuberculosis* Kurono strain (ATCC 25618) by an airborne route by placing them in the exposure chamber of an airborne infection apparatus (model 099CA4241; Gals-Col, Inc., Terre Haute, Ind.). The concentration was calculated to result in the uptake of around 200 viable bacilli by the rat lungs after inhalation exposure for 90 min under the experimental conditions of this study (8). For the pulmonary CFU assay, 7 weeks later the lungs were removed, homogenized, diluted with physiological saline, placed on Ogawa slant agar, and incubated at 37°C for 4 weeks. Lung tissue sections from paraffin blocks were stained with hematoxylin and eosin or stained using the Ziehl-Neelsen method for acid-fast bacilli. Similar infection experiments were performed three times. Bronchoalveolar lavage (BAL) was carried out to obtain neutrophils 24 h after the intratracheal introduction of LPS. Under these conditions, $>10^7$ rat neutrophils were obtained by BAL. The cell differential counts showed that more than 99% were neutrophils and less than 1% were alveolar macrophages; no eosinophils were found. This LPS-induced neutrophilia in the rat lung disappeared 4 weeks later. We changed the dosage of LPS administered, and an inoculation of 50 μg of LPS/rat was sufficient to induce optimal neutrophilia. No side effects were recognized with the use of LPS.

We grew cultures of neutrophils with *M. tuberculosis* overnight (multiplicity of infection, 10:1); then, the collected neutrophils were grown on Ogawa slant medium for 4 weeks. No CFU were found on the media. It is suggested that neutrophils possess direct or indirect killing activity for tubercle bacilli. As shown in Fig. 1A, less than 10% of the neutrophils ingested tubercle bacilli. Fig. 1B shows tubercle bacilli in phagosomes of neutrophils (9). Fig. 1C shows alveoli filled with neutrophils. No exudate was found in the alveoli. We performed time course infection experiments to examine the mechanism of rat neutrophil-mediated reduction of mycobacterial growth in vivo. The rats were infected by an airborne route at days 1, 3, 5, 7, and 10 after LPS (50 μg/0.5 ml) was injected intratracheally. The lung homogenates were grown on Ogawa slant culture for 4 weeks, and colonies were counted thereafter. As shown in Fig. 2, there was marked reduction of tubercle bacilli at day 1 after LPS injection but the pulmonary CFU level became gradually higher with the passage of time. These results show that rat neutrophils prevented early mycobacterial infection without ingesting tubercle bacilli. In the lungs treated intratracheally with LPS and infected with *M. tuberculosis* the following day, 200 ± 10 CFU were found; in the lungs infected with *M. tuberculosis* without LPS treatment, $1.3 \times 10^5 \pm 1 \times 10^4$ CFU were found ($P < 0.01$) (Fig. 3). At 7 weeks after aerosol infection, the lung tissues were removed for CFU assays and the lung homogenates were grown on Ogawa slant

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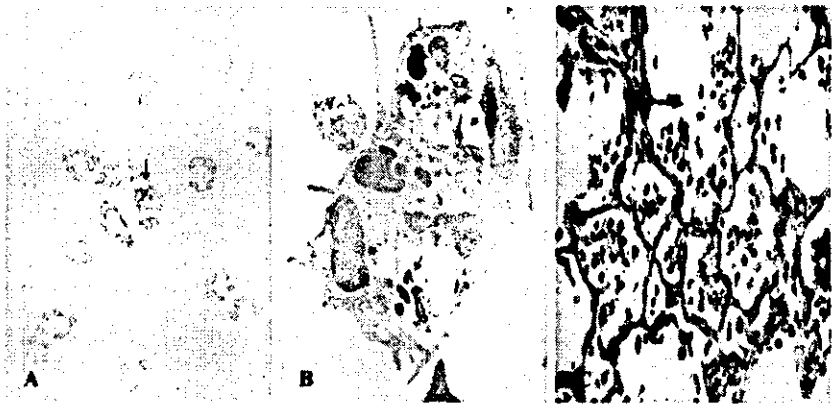

FIG. 1. (A) Neutrophil-ingesting tubercle bacilli. The results of Ziehl-Neelsen staining are shown at a magnification of ×600. Arrow, neutrophil ingesting tubercle bacilli. (B) Electron micrograph of neutrophil-ingesting tubercle bacilli. Several tubercle bacilli (arrow) are evident in the neutrophil phagosomes. The image is shown at a magnification of ×5,000. (C) Alveoli filled with neutrophils. No exudate was found in the alveoli. The results of hematoxylin and eosin staining are shown at a magnification of ×200.

cultures for 4 weeks (Fig. 3). Pulmonary histopathology also showed that the size of granulomas was reduced markedly (Fig. 4B).

Next, amphotericin B (Sigma), an LPS inhibitor, was used to demonstrate the direct effect of LPS on the rapid induction of rat neutrophils. LPS (50 μg/0.5 ml of pyrogen-free saline) and amphotericin B (50 μg) were mixed for 30 min; the mixture was then given to rats intratracheally. The following day, the rats were infected with *M. tuberculosis* aerially. At 7 weeks later, the CFU assay and pulmonary histopathology were performed. The pulmonary CFU level was $1.2 \times 10^5 \pm 1.0 \times 10^4$, and the difference between the results seen with LPS-treated rats and those seen with rats treated with LPS plus amphotericin B was statistically significant ($P < 0.01$). This marked increase of pulmonary CFU was confirmed by lung histopathology, which showed that large pulmonary granulomas were present (Fig. 4D).

To examine the effects of the presence of neutrophils induced by LPS on the growth of *M. tuberculosis* in vivo, neutralizing anti-rat neutrophil antibody (Ab) (catalog no. 22840D; PharMingen International) was used (6). LPS was inoculated intratracheally into the lungs of Fischer rats. The following day, 0.5 mg of Ab/0.5 ml of pyrogen-free saline was given intratracheally. One hour after Ab introduction into the rat lungs, the rats were infected with *M. tuberculosis* aerially. At 7 weeks later, pulmonary CFU were counted and histopathology was undertaken. As shown in Fig. 3 and 4E, pulmonary CFU levels increased and the granulomas became large because the rat neutrophils were killed with Ab and, thus, did not kill the tubercle bacilli.

Lastly, we examined the effect of the timing of the intratracheal inoculation of LPS on the development of tuberculosis. The rats were infected with the *M. tuberculosis* Kurono strain

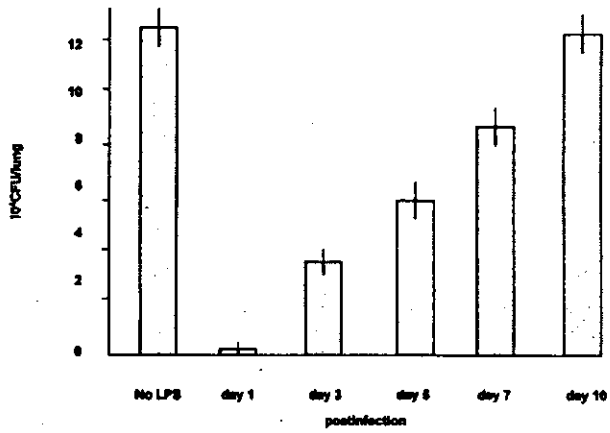

FIG. 2. Bacterial load in a time course study starting at day 1 postinfection. The rats were infected by an airborne route at days 1, 3, 5, 7, and 10 after intratracheal injection of LPS (50 μg/0.5 ml). The lung homogenates were grown on Ogawa slant medium at 37°C for 4 weeks, and CFU were counted thereafter.

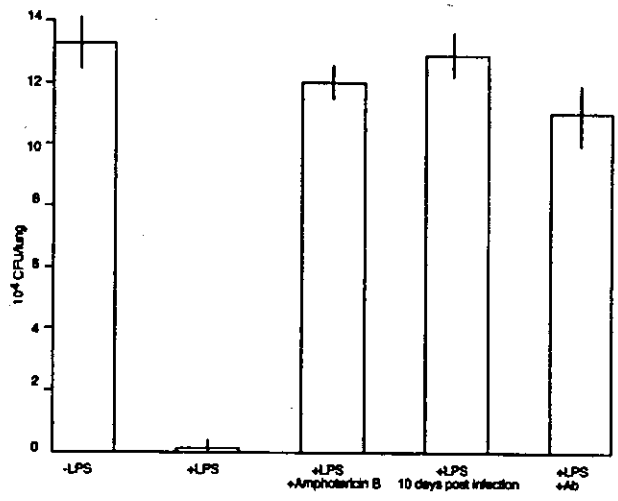

FIG. 3. CFU assay of infected lung tissues left untreated (−LPS), treated with LPS (50 μg/0.5 ml) (+LPS), treated with LPS (50 μg/0.5 ml) and amphotericin B (50 μg/0.5 ml) (+LPS +Amphotericin B), treated with LPS (50 μg/0.5 ml) 10 days after aerial infection (+LPS 10 days post infection), and treated with LPS and anti-rat neutralizing neutrophil Ab (500 μg/rat) (+LPS +Ab).

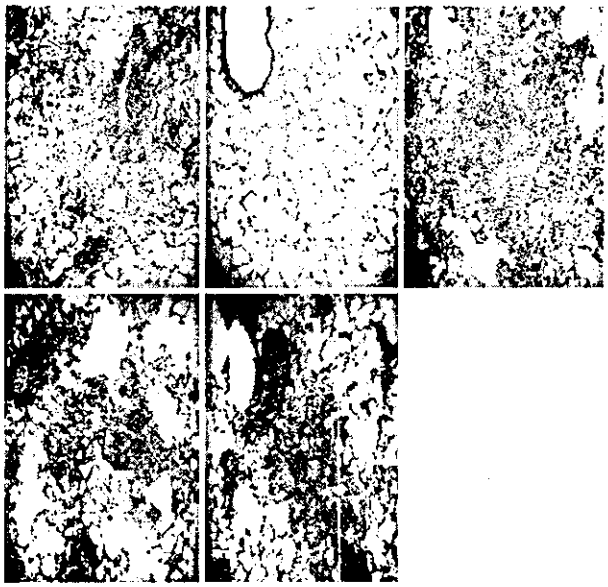

FIG. 4. Representative histopathology of untreated infected lung tissues (A) and infected lung tissues treated with LPS (50 μg/0.5 ml) 1 day before aerial infection (B), treated with LPS 10 days after aerial infection (C), treated with LPS and amphotericin B (50 μg/0.5 ml) 1 day before aerial infection (D), and treated with LPS and anti-rat neutrophil Ab (500 μg/rat) 1 day before aerial infection (E). The results of hematoxylin and eosin staining are shown at a magnification of ×100.

aerially. At 10 days later, LPS was administered intratracheally to the infected rats. We already know that the initial small pulmonary granulomas are formed 10 days after aerial infection. At 7 weeks after aerial infection with *M. tuberculosis*, pulmonary CFU and histopathology were examined again. Pulmonary CFU levels were not reduced significantly (Fig. 3), and the size of granulomas was not reduced substantially (Fig. 4C). Once the pulmonary granulomas were formed, rat neutrophils were ineffective for both the reduction of granuloma size and the elimination of tubercle bacilli.

Editor: J. T. Barbieri

It is concluded that the presence of LPS-induced neutrophilia does not affect the reduction of established pulmonary granulomas. We carried out reverse transcription-PCR using neutrophils obtained by BAL from *M. tuberculosis*-infected rats. We observed significant expression of tumor necrosis factor alpha, IL-1 β, and IL-12 mRNA in *M. tuberculosis*-infected rat neutrophils (data not shown) (4, 10).

In summary, neutrophils play a protective role in the host defense mechanism against *M. tuberculosis* (early-phase tuberculosis). LPS-induced transient neutrophilia prevents early mycobacterial infection, and rat neutrophils possess some phagocytic activity. LPS-induced neutrophilia in the lung is useful for examining neutrophil function in the development of tuberculosis.

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International Immunology, Vol. 17, No. 1, pp. 1–14
doi:10.1093/intimm/dxh186

Review Article

Toll-like receptors in innate immunity

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Keywords: adaptor, innate immunity, signal transduction, TIR domain, Toll-like receptor

Abstract

Functional characterization of Toll-like receptors (TLRs) has established that innate immunity is a skillful system that detects invasion of microbial pathogens. Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct development of antigen-specific acquired immunity. TLR signaling pathways are finely regulated by TIR domain-containing adaptors, such as MyD88, TIRAP/Mal, TRIF and TRAM. Differential utilization of these TIR domain-containing adaptors provides specificity of individual TLR-mediated signaling pathways. Several mechanisms have been elucidated that negatively control TLR signaling pathways, and thereby prevent overactivation of innate immunity leading to fatal immune disorders. The involvement of TLR-mediated pathways in autoimmune and inflammatory diseases has been proposed. Thus, TLR-mediated activation of innate immunity controls not only host defense against pathogens but also immune disorders.

Introduction

Host defense against invading microbial pathogens is elicited by the immune system, which consists of two components: innate immunity and acquired immunity. Both components of immunity recognize invading microorganisms as non-self, which triggers immune responses to eliminate them. To date, both components have been characterized independently, and the main research interest in the immunology field has been confined to acquired immunity. In acquired immunity, B and T lymphocytes utilize antigen receptors such as immunoglobulins and T cell receptors to recognize non-self. The mechanisms by which these antigen receptors recognize foreign antigens have been intensively analyzed, and the major mechanisms, such as diversity, clonality and memory, have been well characterized. However, these receptors are present only in vertebrates, and accordingly we do not fully understand the mechanism for non-self recognition in less evolved organisms. In addition, the innate immune system in mammals has not been well studied. As a result, although mammalian innate immune cells such as macrophages and dendritic cells are known to be activated by microbial components (non-self) such as lipopolysaccharide (LPS) from Gram-negative bacteria, a receptor responsible for the recognition remained unknown.

At the end of the 20th century, Toll was shown to be an essential receptor for host defense against fungal infection in *Drosophila*, which only has innate immunity (1). One year later, a mammalian homolog of the Toll receptor (now termed TLR4) was shown to induce expression of genes involved in inflammatory responses (2). In addition, a point mutation in the *Tlr4* gene has been identified in a mouse strain that is unresponsive to LPS (3). These studies have made innate immunity a very attractive subject of research, and in recent years there has been rapid progress in our understanding that the innate immune system possesses a skillful system that senses invasion of microbial pathogens by Toll-like receptors (TLRs). Furthermore, activation of innate immunity is a critical step to the development of antigen-specific acquired immunity. In this review, we will describe the mechanisms by which innate immunity is activated through TLRs.

Identification of the TLR family

After the characterization of the first mammalian TLR, TLR4, several proteins that are structurally related to TLR4 were identified and named Toll-like receptors (4). Mammalian TLRs comprise a large family consisting of at least 11 members. TLR1–9 are conserved between the human and mouse. However, although TLR10 is presumably functional in the human, the C-terminal half of the mouse *Tlr10* gene is substituted to an unrelated and non-productive sequence,

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indicating that mouse TLR10 is non-functional (our unpublished observation). Similarly, mouse TLR11 is functional, but there is a stop codon in the human *TLR11* gene, which results in a lack of production of human TLR11 (5).

The cytoplasmic portion of TLRs shows high similarity to that of the IL-1 receptor family, and is termed a Toll/IL-1 receptor (TIR) domain. Despite this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-1 receptors possess an immunoglobulin-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain. Functionally, a critical role of TLR4 in the recognition of the microbial component LPS was initially characterized (3). Subsequently, it has been rapidly established that individual TLRs play important roles in recognizing specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses (Fig. 1).

TLR1, TLR2 and TLR6

TLR2 recognizes a variety of microbial components. These include lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis*, zymosan from fungi and glycolipids from *Treponema maltophilum* (6). In addition, TLR2 reportedly recognizes LPS preparations from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pyroli* (7–9). These LPS structurally differ from the typical LPS of Gram-negative bacteria recognized by TLR4 in the number of acyl chains in the lipid A component, which presumably confers differential recognition (10). However, a recent report indicates that LPS preparation from *P. gingivalis* contaminates lipoproteins that activate TLR2, and LPS from *P. gingivalis* only poorly activates TLR4 (11). Therefore, more careful analysis will be required to conclude that some LPS are recognized by TLR2, but not TLR4.

There are two aspects proposed for mechanisms that could explain why TLR2 recognizes a wide spectrum of microbial components. The first explanation is that TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2. Macrophages from TLR6-deficient mice did not show any production of inflammatory cytokines in response to mycoplasma-derived diacyl lipopeptides. However, these cells showed normal production of inflammatory cytokines in response to triacyl lipopeptides derived from Gram-negative bacteria (12). In contrast, macrophages from TLR1-deficient mice showed a normal response to mycoplasma-derived diacyl lipopeptides, but an impaired response to triacyl lipopeptides (13). Thus, TLR1 and TLR6 functionally associate with TLR2 and discriminate between diacyl or triacyl lipopeptides. Moreover, the involvement of TLR1 in the recognition of the outer surface lipoprotein of *Borrelia burgdorferi* has also been shown (14). The second explanation involves recognition of fungal-derived components by TLR2 (15). In this model, TLR2 has been shown to functionally collaborate with distinct types of receptors such as dectin-1, a lectin family receptor for the fungal cell wall component β-glucan. Thus, TLR2 recognizes a wide range of microbial products through functional cooperation with several proteins that are either structurally related or unrelated.

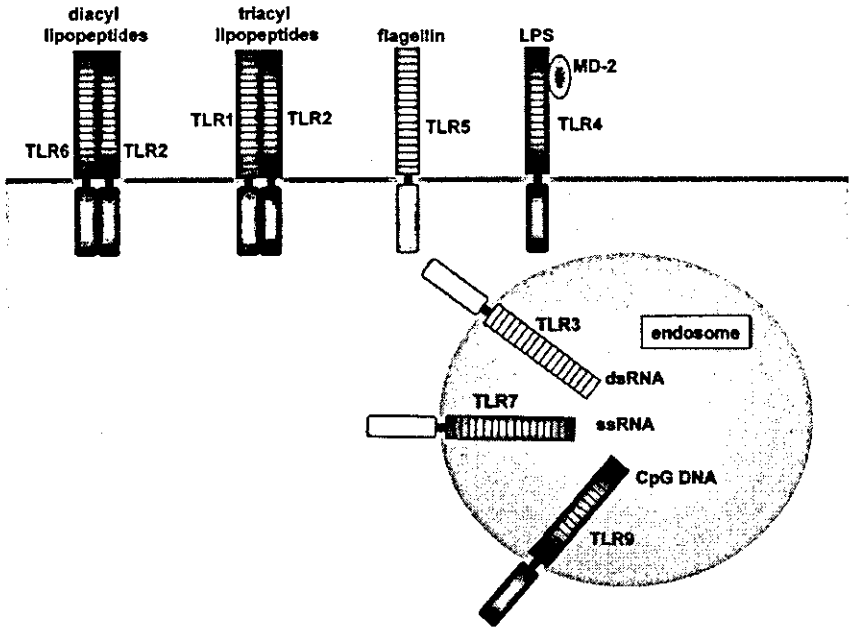

Fig. 1. TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR5 recognizes flagellin. Thus, the TLR family members recognize specific patterns of microbial components.

TLR3

Expression of human TLR3 in the double-stranded RNA (dsRNA)-non-responsive cell line 293 confers enhanced activation of NF-κB in response to dsRNA. In addition, TLR3-deficient mice are impaired in their response to dsRNA (16). dsRNA is produced by most viruses during their replication and induces the synthesis of type I interferons (IFN-α/β), which exert anti-viral and immunostimulatory activities. Thus, TLR3 is implicated in the recognition of dsRNA and viruses. However, TLR3-independent mechanisms of dsRNA recognition exist, as discussed below.

TLR4

As described above, TLR4 is an essential receptor for LPS recognition (3,17). In addition, TLR4 is implicated in the recognition of taxol, a diterpene purified from the bark of the western yew (*Taxus brevifolia*) (18,19). Furthermore, TLR4 has been shown to be involved in the recognition of endogenous ligands, such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen. However, all of these endogenous ligands require very high concentrations to activate TLR4. In addition, it has been shown that contamination of LPS in the HSP70 preparation confers ability to activate TLR4 (20). LPS is a very potent immuno-activator, and accordingly, TLR4 can be activated by a very small amount of LPS, contaminating these endogenous ligand preparations. Therefore, more careful experiments will be required before we can conclude that TLR4 recognizes these endogenous ligands.

TLR5

Enforced expression of human TLR5 in CHO cells confers response to flagellin, a monomeric constituent of bacterial flagella (21). TLR5 has further been shown to recognize an evolutionarily conserved domain of flagellin through close physical interaction between TLR5 and flagellin (22). TLR5 is expressed on the basolateral, but not the apical side of intestinal epithelial cells (23). TLR5 expression is also observed in the intestinal endothelial cells of the subepithelial compartment (24). In addition, flagellin activates lung epithelial cells to induce inflammatory cytokine production (25). These findings indicate the important role of TLR5 in microbial recognition at the mucosal surface. A common stop codon polymorphism in the ligand-binding domain of TLR5 has been shown to be associated with susceptibility to pneumonia caused by the flagellated bacterium *Legionella pneumophila* (25).

TLR7 and TLR8

TLR7 and TLR8 are structurally highly conserved proteins, and recognize the same ligand in some cases. Analysis of TLR7-deficient mice revealed that murine TLR7 recognizes synthetic compounds, imidazoquinolines, which are clinically used for treatment of genital warts associated with viral infection (26). Human TLR7 and TLR8, but not murine TLR8, recognizes imidazoquinoline compounds (27). Murine TLR7 has also been shown to recognize another synthetic compound, loxoribine, which has anti-viral and anti-tumor activities (28,29). Both imidazoquinoline and loxoribine are structurally related to guanosine nucleoside. Therefore, TLR7 and human TLR8 were predicted to recognize a nucleic acid-like structure of the virus. This prediction has recently been shown to be true from the finding that TLR7 and human TLR8 recognize guanosine- or uridine-rich single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus, vesicular stomatitis virus and influenza virus (30–32). ssRNA is abundant in the host, but usually the host-derived ssRNA is not detected by TLR7 or TLR8. This might be due to the fact that TLR7 and TLR8 are expressed in the endosome, and host-derived ssRNA is not delivered to the endosome.

TLR9

Analysis of TLR9-deficient mice revealed that TLR9 is a receptor for CpG DNA (33). Bacterial DNA contains unmethylated CpG motifs, which confer its immunostimulatory activity. In vertebrates, the frequency of CpG motifs is severely reduced and the cysteine residues of CpG motifs are highly methylated, leading to abrogation of the immunostimulatory activity. There are at least two types of CpG DNA, termed A/D-type CpG DNA and B/K-type CpG DNA. B/K-type CpG DNA is conventional, which was identified first, and is a potent inducer of inflammatory cytokines such as IL-12 and TNF-α. A/D-type CpG DNA is structurally different from conventional CpG DNA and has a greater ability to induce IFN-α production from plasmacytoid dendritic cells (PDC), but less ability to induce IL-12 (34,35). TLR9 has been shown to be essential for the recognition of both types of CpG DNA (36). The fact that TLR9 recognition of A/D-type CpG DNA leads to induction of an anti-viral cytokine IFN-α in PDC indicates that TLR9 is involved in viral recognition. Indeed, in addition to bacterial CpG DNA, TLR9 has been shown to recognize viral-derived CpG DNA in PDC (37,38). Furthermore, TLR9-mutant mice have been shown to be susceptible to mouse cytomegalovirus (MCMV) infection (39). TLR9-dependent recognition of MCMV in PDC or other types of DC elicits an anti-MCMV response through activation of NK cells (40). In addition to bacterial and viral CpG DNA, TLR9 is presumably involved in pathogenesis of autoimmune disorders. Sequential engagement of IgG2a–chromatin complex by the B cell receptor and TLR9 mediates effective production of rheumatoid factor by auto-reactive B cells (41). In this model, the IgG2a is bound and internalized by the B cell receptor, and the chromatin, including hypomethylated CpG motifs, is then able to engage TLR9, thereby inducing rheumatoid factor (42). Similarly, internalization by the Fc receptor and subsequent exposure of the chromatin to TLR9 mediates PDC induction of IFN-α by immune complexes containing IgG and chromatin, which are implicated in the pathogenesis of systemic lupus erythematosus (SLE) (43). Thus, TLR9 appears to be involved in the pathogenesis of several autoimmune diseases through recognition of the chromatin structure. Chloroquine is clinically used for treatment of rheumatoid arthritis and SLE, but its mechanisms are unknown. Since chloroquine also blocks TLR9-dependent signaling through inhibition of the pH-dependent maturation of endosomes by acting as a basic substance to neutralize acidification in the vesicle (44), it may act as an anti-inflammatory agent by inhibiting TLR9-dependent immune responses.

TLR11

The most recently identified TLR11 has been shown to be expressed in bladder epithelial cells and mediate resistance to infection by uropathogenic bacteria in mouse (5). TLR11-deficient mice are highly susceptible to uropathogenic bacterial infection. Although the ligand has not yet been identified, these findings indicate that mouse TLR11 mediates anti-uropathogenic bacterial response. As described above, there is no functional TLR11 protein in the human (5). These findings may indicate that the human TLR11 protein was futile in the human environment and became lost through evolution.

Subcellular localization of TLRs

Individual TLRs are differentially distributed within the cell. TLR1, TLR2 and TLR4 are expressed on the cell surface, as demonstrated by positive staining of the cell surface by specific antibodies. In contrast, TLR3, TLR7, TLR8 and TLR9 have been shown to be expressed in intracellular compartments such as endosomes (29,45–47). TLR3-, TLR7- or TLR9-mediated recognition of their ligands has been shown to require endosomal maturation (30–32,44,46,48). The TLR9 ligand CpG DNA is first non-specifically captured into endosomes, where TLR9 is recruited from the endoplasmic reticulum upon non-specific uptake of CpG DNA (44,47,49). Thus, it can be hypothesized that in the case of bacterial infection, macrophages and dendritic cells engulf bacteria by phagocytosis. CpG DNA is then exposed after degradation of bacteria in phagosomes/lysosomes or endosomes/lysosomes, where TLR9 is recruited or expressed. In the case of viral infection, viruses invade cells by receptor-mediated endocytosis, and the viral contents are exposed to the cytoplasm by fusion of the viral membrane and the endosomal membrane. Occasionally, the viral particles are degraded in the endosomal compartment, which results in exposure of TLR ligands such as dsRNA, ssRNA and CpG DNA. Even TLR2, which is expressed on the cell surface, is recruited to the phagosomal compartment of macrophages after exposure to zymosan (50). Thus, phagosomal/lysosomal or endosomal/lysosomal compartments may be the main sites for TLR recognition of microbial components.

TLR-independent recognition of micro-organisms

TLR-independent recognition of viruses and dsRNA

Although TLR3 is involved in the recognition of viral-derived dsRNA, the impairment observed in TLR3-deficient mice is only partial (16,51). In addition, introduction of dsRNA into the cytoplasm of dendritic cells leads to the induction of type I IFNs via a mechanism partially dependent on dsRNA-dependent protein kinase (PKR), but independent of TLR3 (52). These findings indicate that molecules responsible for TLR3-independent recognition of dsRNA and viruses do exist. Although PKR is implicated in dsRNA recognition, it is still controversial whether PKR plays a critical role in dsRNA-induced type I IFN expression (53). Recently, a key molecule was identified, which mediates the TLR3-independent dsRNA recognition (Fig. 2). Retinoic acid-inducible gene I (RIG-I), which encodes a DExD/H box RNA helicase containing a caspase recruitment domain, was identified from the screening of a cDNA library that augments dsRNA-dependent activation of the IRF-3-dependent promoter. Studies with ectopic expression and RNA interference (RNAi)-mediated knockdown of RIG-I clearly demonstrated that RIG-I is critical in dsRNA- and viral infection-induced type I IFN expression (54). It is interesting to analyze the correlation between TLR3 and RIG-I in the recognition of dsRNA and viruses.

NOD1 and NOD2

TLRs are membrane-bound molecules that recognize microbial components on the surface or within extracellular compartments of cells. Accordingly, intracellular recognition of bacteria appears to involve a TLR-independent system. Recent accumulating evidence indicates that the nucleotide-binding oligomerization domain (NOD) family of proteins plays an important role in the recognition of intracellular bacteria (Fig. 2). Peptidoglycan (PGN) has previously been shown to be recognized by TLR2 (55). However, PGN is a thick rigid layer that is composed of an overlapping lattice of two sugars that are crosslinked by amino acid bridges, and the exact structure of PGN that is recognized by TLR2 remains unclear. NOD1 was originally identified as a molecule that is structurally related to the apoptosis regulator, Apaf-1. It contains a caspase-recruitment domain (CARD), a NOD domain and a C-terminal LRR domain. Recent studies have demonstrated that overexpression of NOD1 enables 293 cells to respond to preparations of PGN (56,57). Characterization of the PGN motif detected by NOD1 revealed that γ-D-glutamyl-meso diaminopimelic acid (iE-DAP) is the minimal structure required for NOD1 detection. NOD2 was identified as a molecule that shows structural similarity to NOD1, but which possesses two CARD domains in its N-terminal region. Similar to NOD1, expression of NOD2 confers responsiveness to PGN in 293 cells. Biochemical analyses identified the essential structure recognized by NOD2 as muramyl dipeptide MurNAc-L-Ala-D-isoGln (MDP) derived from PGN (58,59). Thus, NOD1 and NOD2 recognize different structures within PGN. MDP is found in almost all bacteria, whereas iE-DAP is restricted to Gram-negative bacteria. Therefore, NOD1 may play an important role in sensing Gram-negative bacterial infection inside cells. Although TLR2 has been reported to recognize PGN, it is possible that TLR2 recognizes lipoprotein/lipopeptide contaminants that are trapped within the layers of the PGN mesh.

Mutations in the *NOD2* gene have been shown to be associated with Crohn's disease, an inflammatory bowel disease of unknown etiology (60,61). These mutations are found in the LRR domain of *NOD2*, and result in defective NF-κB activation. However, the mechanisms by which *NOD2* mutations result in an increased susceptibility to Crohn's disease are unclear. One of the answers to this question has recently been demonstrated. In the absence of NOD2 or the presence of a Crohn's disease-like *Nod2* mutation, TLR2-mediated activation of NF-κB, especially the cRel subunit, has been shown to be enhanced, which explains enhanced NF-κB activity and Th1 responses in Crohn's disease patients (62). *NOD2* mutations are also associated with Blau syndrome, a disease characterized by granulomatous arthritis, uveitis and skin rash (63). The *NOD2* mutations in Blau syndrome

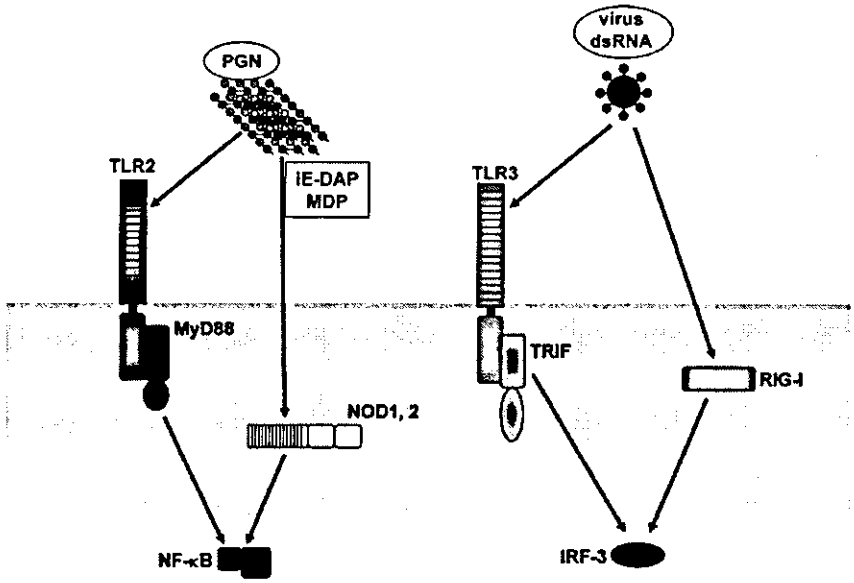

Fig. 2. TLR-dependent and -independent recognition of microbial components. TLR2 has previously been shown to mediate peptidoglycan (PGN) recognition. However, NOD1 and NOD2 have recently been shown to recognize motifs found in the layer of PGN. It is possible that TLR2 recognizes lipoprotein contamination in the PGN layer. Viral recognition is also mediated by TLR-dependent and -independent mechanisms. TLR3-mediated recognition of viruses or dsRNA results in TRIF-dependent activation of IRF-3 and NF-κB. However, viruses or dsRNA are recognized in a TLR3-independent manner, since the impairment of the responsiveness to viruses or dsRNA in TLR3-deficient mice is only partial. RIG-I is identified as a molecule that is responsible for viral recognition and that mediates activation of IRF-3.

patients are located in the NOD domain, leading to an increase in NF-κB activity. Thus, *NOD2* is associated with certain human diseases.

Recognition of PGN motifs by NOD1 and NOD2 results in their oligomerization, which induces the recruitment of Rip2/RICK, a serine/threonine kinase (64). Rip2/RICK has a CARD domain in its C-terminal portion and an N-terminal catalytic domain that shares sequence similarity with Rip, a factor essential for NF-κB activation through the TNF receptor. NODs and Rip2/RICK interact via their respective CARD domains, and induce recruitment of the IKK complex to the central region of Rip2/RICK. This in turn leads to activation of NF-κB. Rip2/RICK-deficient mice have been shown to be highly sensitive to infection with the intracellular pathogen *Listeria monocytogenes* (65). Introduction of NOD1 or NOD2 into Rip2/RICK-deficient embryonic fibroblast cells does not induce NF-κB activation (66). Thus, Rip2/RICK is essential for NOD1- and NOD2-mediated responses, although its involvement in the recognition of PGN motifs needs to be more precisely analyzed in Rip2/RICK-deficient mice.

Phagocytosis and TLRs

Phagocytosis is an important step for host defense against microbial pathogens, since it triggers both degradation of pathogens and subsequent presentation of pathogen-derived peptide antigen. TLR recognition of pathogens leads to expression of genes such as inflammatory cytokines and co-stimulatory molecules. Phagocytosis-mediated antigen presentation together with TLR-dependent gene expression of inflammatory cytokines and co-stimulatory molecules, instruct development of antigen-specific acquired immunity (Fig. 3). Therefore, it is of interest to characterize the relationship between phagocytosis and TLRs. In the absence of TLR2/TLR4 or MyD88, a common adaptor in TLR signaling, phagocytosis of bacteria including *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* has been shown to be impaired due to impaired phagosome maturation (67). Further studies indicate that TLR-mediated MyD88-dependent activation of p38 is required for phagosome maturation (67,68). Thus, TLRs are linked to phagocytosis of bacteria.

TLR signaling pathways

Stimulation of TLRs by microbial components triggers expression of several genes that are involved in immune responses. The molecular mechanisms by which TLRs induce gene expression are now rapidly being elucidated through analyses of TLR-mediated signaling pathways (69). Microbial recognition of TLRs facilitates dimerization of TLRs. TLR2 is shown to form a heterophilic dimer with TLR1 or TLR6, but in other cases TLRs are believed to form homodimers (70). Dimerization of TLRs triggers activation of signaling pathways, which originate from a cytoplasmic TIR domain. In the signaling pathways

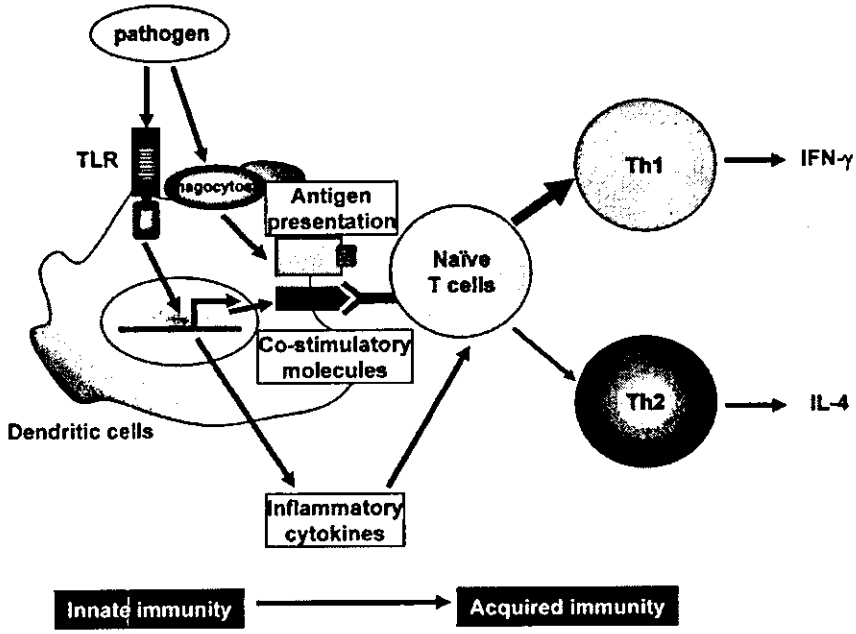

Fig. 3. Innate and adaptive immunity. Innate immune cells, such as dendritic cells and macrophages, engulf pathogens by phagocytosis, and present pathogen-derived peptide antigens to naïve T cells. In addition, TLRs recognize pathogen-derived components and induce expression of genes, such as co-stimulatory molecules and inflammatory cytokines. Phagocytosis-mediated antigen presentation, together with TLR-mediated expression of co-stimulatory molecules and inflammatory cytokines, instruct development of antigen-specific adaptive immunity, especially Th1 cells.

downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, was first shown to be essential for induction of inflammatory cytokines such as TNF-α and IL-12 through all TLRs (21,26,71–74). However, activation of specific TLRs leads to slightly different patterns of gene expression profiles. For example, activation of TLR3 and TLR4 signaling pathways results in induction of type I interferons (IFNs), but activation of TLR2- and TLR5-mediated pathways does not (75–77). TLR7, TLR8 and TLR9 signaling pathways also lead to induction of type I IFNs through mechanisms distinct from TLR3/4-mediated induction (36,78). Thus, individual TLR signaling pathways are divergent, although MyD88 is common to all TLRs. It has also become clear that there are MyD88-dependent and MyD88-independent pathways (Fig. 4).

MyD88-dependent pathway

A MyD88-dependent pathway is analogous to signaling pathways through the IL-1 receptors. MyD88, harboring a C-terminal TIR domain and an N-terminal death domain, associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IRAK-4 to TLRs through interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6, leading to the activation of two distinct signaling pathways. One pathway leads to activation of AP-1 transcription factors through activation of MAP kinases. Another pathway activates the TAK1/TAB complex, which enhances activity of the IκB kinase (IKK) complex. Once activated, the IKK complex induces phosphorylation and subsequent degradation of IκB, which leads to nuclear translocation of transcription factor NF-κB.

As its name suggests, in the MyD88-dependent pathway, MyD88 plays a crucial role. MyD88-deficient mice do not show production of inflammatory cytokines such as TNF-α and IL-12p40 in response to all TLR ligands (21,26,71–74). Thus, MyD88 is essential for inflammatory cytokine production through all TLRs.

A database search for molecules that are structurally related to MyD88 led to identification of the second TIR domain-containing molecule TIRAP (TIR domain-containing adaptor protein)/Mal (MyD88-adaptor-like) (79,80). Similar to MyD88-deficient macrophages, TIRAP/Mal-deficient macrophages show impaired inflammatory cytokine production in response to TLR4 and TLR2 ligands (81,82). However, TIRAP/Mal-deficient mice are not impaired in their response to TLR3, TLR5, TLR7 and TLR9 ligands, Thus, TIRAP/Mal has been shown to be essential for the MyD88-dependent signaling pathway via TLR2 and TLR4.

MyD88-independent/TRIF-dependent pathway

In MyD88-deficient macrophages, TLR4 ligand-induced production of inflammatory cytokines is not observed; however, activation of NF-κB is observed with delayed kinetics (72). This indicates that although TLR4-mediated production of inflammatory cytokines completely depends on the MyD88-dependent pathway, a MyD88-independent component exists

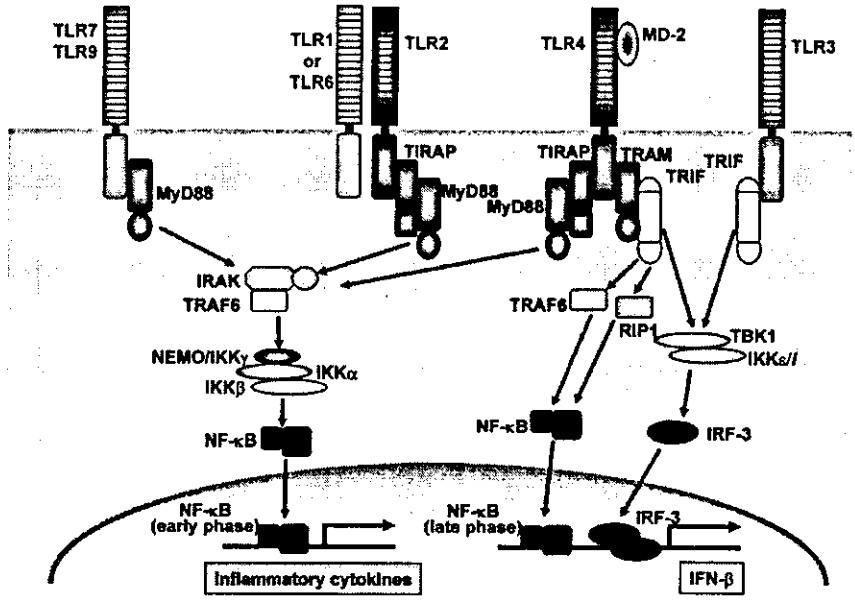

Fig. 4. TLR signaling pathway. TLR signaling pathways originate from the cytoplasmic TIR domain. A TIR domain-containing adaptor, MyD88, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, leading to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. The IKK complex phosphorylates IκB, resulting in nuclear translocation of NF-κB which induces expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is essential for the MyD88-independent pathway. Non-typical IKKs, IKKi/IKKε and TBK1, mediate activation of IRF-3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

in TLR4 signaling. Subsequent studies have demonstrated that TLR4 stimulation leads to activation of the transcription factor IRF-3, as well as the late phase of NF-κB activation in a MyD88-independent manner (83). TLR4-induced activation of IRF-3 leads to production of IFN-β. IFN-β in turn activates Stat1 and induces several IFN-inducible genes (75–77). Viral infection or dsRNA was found to activate IRF-3 (84). Accordingly, the TLR3-mediated pathway also activates IRF-3 and thereby induces IFN-β in a MyD88-independent manner. Hence, TLR3 and TLR4 utilize the MyD88-independent component to induce IFN-β.

Characterization of MyD88 and TIRAP/Mal prompted us to hypothesize that TIR domain-containing molecules regulate the MyD88-independent pathway, and also facilitated the search for such molecules. A database search led to identification of a third TIR domain-containing adaptor, TIR domain-containing adaptor inducing IFN-β (TRIF) (85). This molecule was identified as a TLR3-associated molecule by two-hybrid screening and was named TIR domain-containing adaptor molecule (TICAM-1) (86). The physiological role of TRIF/TICAM-1 was then demonstrated by generation of TRIF-mutant mice. TRIF-deficient mice generated by gene targeting showed no activation of IRF-3 and had impaired expression of IFN-β- and IFN-inducible genes in response to TLR3 and TLR4 ligands (52). Another mouse strain mutated in the *Trif* gene generated by random germline mutagenesis also revealed that they were defective in TLR3- and TLR4-mediated induction of IFN-β- and IFN-inducible genes (87). Thus, TRIF has been demonstrated to be essential for TLR3- and TLR4-mediated MyD88-independent pathways.

Database searches further led to identification of a fourth TIR domain-containing adaptor, TRIF-related adaptor molecules (TRAM)/TICAM-2 (88–91). Studies with TRAM-deficient mice and RNAi-mediated knockdown of TRAM expression showed that TRAM is involved in TLR4-mediated, but not TLR3-mediated, activation of IRF-3 and induction of IFN-β- and IFN-inducible genes (88–90). Thus, TRAM is essential for the TLR4-mediated MyD88-independent/TRIF-dependent pathway.

In TRIF- and TRAM-deficient mice, inflammatory cytokine production induced by TLR2, TLR7 and TLR9 ligands was observed, as well as TLR4 ligand-induced phosphorylation of IRAK-1 (52,89). These findings indicate that the MyD88-dependent pathway is not impaired in these mice. However, TLR4 ligand-induced inflammatory cytokine production was not observed in TRIF- and TRAM-deficient mice. Therefore, activation of both the MyD88-dependent and MyD88-independent/TRIF-dependent components is required for the TLR4-induced inflammatory cytokine production, but the mechanisms are unknown.

Key molecules that mediate IRF-3 activation have been revealed to be non-canonical IKKs, TBK1 and IKKi/IKKε (92). Introduction of TBK1 or IKKi/IKKε, but not IKKβ, resulted in phosphorylation and nuclear translocation of IRF-3. RNAi-mediated inhibition of TBK1 or IKKi/IKKε expression led to impaired induction of IFN-β in response to viruses and dsRNA

(92,93). Embryonic fibroblast cells obtained from TBK1-deficient mice showed impaired activation of IRF-3 and expression of IFN-β and IFN-inducible genes in response to TLR3 and TLR4 ligands (94–96). In contrast, embryonic fibroblast cells from IKKi/IKKε-deficient mice were not defective in their response to TLR3 and TLR4 ligands (95). However, TLR3-mediated activation of IRF-3 and expression of IFN-β and IFN-inducible genes were almost completely abolished in embryonic fibroblast cells lacking both TBK1 and IKKi/IKKε. Thus, TBK1 and IKKi/IKKε are critical regulators of IRF-3 activation in the MyD88-independent pathway.

The mechanisms by which the TRIF-dependent pathway leads to activation of NF-κB and IRF-3 are now under investigation. The TIR domain of TRIF is located in the middle portion of this molecule, flanked by the N-terminal and C-terminal portions. Both N-terminal and C-terminal portions of TRIF mediate activation of the NF-κB-dependent promoter, whereas only the N-terminal portion is involved in IFN-β promoter activation (85). Accordingly, the N-terminal portion of TRIF was shown to associate with IKKi/IKKε and TBK1, which mediate IRF-3-dependent IFN-β induction (93,97). The N-terminal portion of TRIF was also shown to associate with TRAF6 (97,98). Since TRAF6 is critically involved in TLR-mediated NF-κB activation (99), TRAF6 may regulate NF-κB activation derived from the N-terminal portion of TRIF. The C-terminal portion of TRIF was shown to associate with RIP1 (100). Embryonic fibroblast cells from RIP1-deficient mice showed impaired NF-κB activation in response to the TLR3 ligand. Thus, RIP1 is shown to be responsible for NF-κB activation that originates from the C-terminal portion of TRIF.

Negative regulation of TLR signaling

Stimulation of TLRs by microbial components triggers the induction of inflammatory cytokines such as TNF-α, IL-6 and IL-12. When all these cytokines are produced in excess, they induce serious systemic disorders with a high mortality rate in the host. It is therefore not surprising that organisms have evolved mechanisms for modulating their TLR-mediated responses.

Exposure to microbial components such as LPS results in a severely reduced response to a subsequent challenge by LPS. This phenomenon was first described over 50 years ago and is now called endotoxin (or LPS) tolerance, but the precise mechanisms remain unclear (101). The mechanisms are now being analyzed in the context of TLR signaling, and several models are proposed. LPS stimulation of macrophages results in reduced surface expression of the LPS receptor complex composed of TLR4 and MD-2, a co-factor that facilitates LPS binding (102,103). TLR2, TLR7 and TLR4 ligands induce reduced expression of IRAK-1 (104–106). Several other mechanisms are also shown to be involved in LPS tolerance (107).

In addition, molecules that negatively regulate TLR signaling have been identified. IRAK-M, a member of the IRAK family of serine/threonine kinases, is induced by TLR stimulation in monocyte/macrophages, and lacks kinase activity (108). IRAK-M-deficient mice show increased production of inflammatory cytokines in response to TLR ligands and defective induction of LPS tolerance (109). Inhibitory activity of IRAK-M seems to be elicited by IRAK-M prevention of IRAK-1/IRAK-4 dissociation from MyD88, thereby preventing formation of the IRAK-1–TRAF6 complex.

An alternatively spliced variant of MyD88 that lacks the intermediary domain of MyD88 (MyD88s) is induced in monocytes upon LPS stimulation. Overexpression of MyD88s results in impaired LPS-induced NF-κB activation through inhibition of IRAK-4-mediated IRAK-1 phosphorylation (110).

SOCS1 is a member of the SOCS family of proteins that are induced by cytokines and that negatively regulate cytokine signaling pathways (111). In addition to cytokines, TLR ligands such as LPS and CpG DNA induced expression of SOCS1 in macrophages (112,113). SOCS1-deficient mice were hypersensitive to LPS-induced endotoxin shock and showed defective induction of LPS tolerance (114,115). Ectopic expression of SOCS1 resulted in impaired LPS-induced NF-κB activation in macrophages. These findings indicate that SOCS1 directly down-modulates TLR signaling pathways, although the precise mechanism by which SOCS1 inhibits TLR signaling remains unclear.

Membrane-bound proteins harboring the TIR domain, such as SIGIRR (single immunoglobulin IL-1 receptor-related molecule) and T1/ST2, have also been shown to be involved in negative regulation of TLR signaling. In both SIGIRR- and T1/ST2-deficient mice, the LPS-induced inflammatory response was enhanced (116,117).

Ubiquitination-mediated degradation of TLRs is also proposed as a mechanism to inhibit activation of the signaling pathway. A RING finger protein, Triad3A, is shown to act as an E3 ubiquitin ligase and enhance ubiquitination and proteolytic degradation of TLR4 and TLR9 (118). Thus, several molecules are postulated to modulate TLR signaling pathways (Fig. 5). Combination of these negative regulators may finely coordinate the TLR signaling pathway to limit exaggerated innate responses causing harmful disorders.

Involvement of TLRs and immune disorders

Several lines of evidence indicate that TLRs are implicated in inflammatory and immune disorders. For example, constitutive activation of innate immune cells caused by defective IL-10 signaling results in development of chronic enterocolitis (119). Introduction of TLR4 deficiency into these mutant mice results in improvement of intestinal inflammation, indicating that TLR-mediated microbial recognition in the intestine triggers development of chronic enterocolitis (120). The MyD88-dependent pathway is seemingly involved in allograft rejection (121). Development of atherosclerosis observed in apolipoprotein E-deficient mice is rescued by introduction of MyD88 deficiency, indicating that the TLR-mediated pathway is responsible for the development of atherosclerosis (122,123). Involvement of the TLR9–MyD88-dependent pathway in the induction of auto-antibodies in SLE and rheumatoid arthritis was also demonstrated, as described above. In addition to these immune-related disorders, TLR recognition of commensal bacteria has been shown to play a crucial role in the maintenance of intestinal epithelial homeostasis (124). Thus, TLR-mediated pathways are probably involved in many aspects of immune responses, even in the absence of infection.

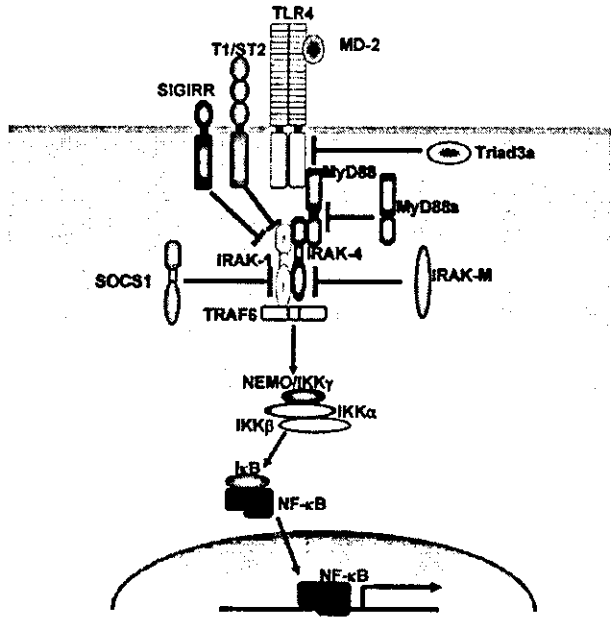

Fig. 5. Negative regulation of TLR signaling pathways. TLR signaling pathways are negatively regulated by several molecules. IRAK-M inhibits dissociation of IRAK-1/IRAK-4 complex from the receptor. MyD88s blocks association of IRAK-4 with MyD88. SOCS1 is likely to associate with IRAK-1 and inhibits its activity. TRIAD3A induces ubiquitination-mediated degradation of TLR4 and TLR9. TIR domain-containing receptors SIGIRR and T1/ST2 are also shown to negatively modulate TLR signaling.

Phylogenetic divergence in the role of Toll

It is well established that mammalian TLRs recognize specific molecular patterns found in microbial components, possibly through a close physical interaction. The Toll receptor in *Drosophila melanogaster* plays an essential role in the host defense against infection by fungi and Gram-positive bacteria. In *Drosophila*, fungal and Gram-positive bacterial infection triggers activation of the Toll-mediated pathway. Toll signaling induces the activation of the Pelle serine/threonine kinase via the adaptor DmMyD88 and degradation of the ankyrin-repeat protein Cactus, and causes activation of the Rel-type transcription factors Dorsal and DIF. Thus, the *Drosophila* Toll signaling pathway is very similar to that of mammalian TLR, especially the participation of the adaptor MyD88, the serine/threonine kinase IRAK, the ankyrin-repeat protein IκB and the Rel-type transcription factor NF-κB (125,126). However, there are some functional differences between the mammalian TLR system and the *Drosophila* Toll system (Fig. 6). First, unlike the mammalian TLR-mediated pathway, the *Drosophila* Toll pathway does not seem to utilize homologs of the mammalian IKKβ and IKKγ/NEMO proteins. These molecules have been shown to be involved in the IMD pathway that senses Gram-negative bacterial infection (125,126). In addition, *Drosophila* Toll is activated by an endogenous ligand, Spätzle (127). Spätzle is initially produced as a pro-Spätzle, and is cleaved into the active signaling form by an as yet unidentified serine protease in response to invasion by fungi or Gram-positive bacteria. This indicates that Toll is not directly involved in the pattern recognition of micro-organisms. In the case of fungal infections, a serine protease that is encoded by the *Persephone* gene has been shown to activate Toll (128). The *Persephone* gene product possesses no obvious pattern recognition motif. Thus, the molecule that is responsible for the recognition of fungi remains unclear. In the case of Gram-positive bacterial infections, Gram-negative binding protein (GNBP) and PGRP-SA, a member of the peptidoglycan recognition protein (PGRP) family, play an essential role in the activation of Toll. This is demonstrated by the finding that mutant flies lacking GNBP or PGRP-SA are defective in the activation of the Toll-mediated pathway in response to Gram-positive bacterial infection (129,130). Subsequently, PGRP-SA has been shown to be responsible for the recognition of Gram-positive lysine-type peptidoglycan, demonstrating that PGRP-SA is a pattern recognition molecule in *Drosophila* (131). In addition, another member of the PGRP family, PGRP-LC, has been shown to play an essential role in the host defense against Gram-negative bacterial infection through the recognition of Gram-negative diaminopimelic acid-type peptidoglycan (132–134). In sharp contrast, mammalian PGRP-S has been shown to play only a minor role in the recognition of pathogens (135). Thus, although the signaling molecules associated with *Drosophila* Toll and mammalian TLR are shared, the actual pattern recognition of pathogens is mediated by quite different mechanisms. In particular, it is of note that only one Toll is actually involved in the *Drosophila* immune response (126). The complete lack of immune functions in other members (18-Wheeler/Toll-2 to Toll-9) of the *Drosophila* Toll family strongly suggests that the ancestral function of the Toll family is not to mediate immune response. In contrast, all members of the mammalian TLR family are specialized for functioning in immune responses, indicating that they have arisen from a common ancestor possessing immune recognition function. Thus, the systems by which pathogens are recognized in vertebrates and insects seem to have evolved separately.

Future prospects

We now know that innate immunity plays an important role in the initiation of an immune response that follows the activation of antigen-specific acquired immunity. Although signaling pathways via TLRs are now being unveiled, there still remain several unanswered questions. For example, activation of TLR7, TLR8 and TLR9 leads to induction of IFN-α/β in a MyD88-dependent manner in PDC. It is possible that there might be a unique pathway downstream of MyD88 that specifies the signaling cascade of these TLRs. The mechanism for regulation of TLR-mediated gene induction is also of interest. A TLR-inducible nuclear factor, IκBζ, has been shown to regulate a subset of TLR-inducible genes (136). In this model, IκBζ, which is immediately induced by TLR stimulation, mediates induction of a certain group of TLR-inducible genes such as IL-6, IL-12p40 and GM-CSF in macrophages. It is of interest to analyze whether this two-step TLR-mediated gene induction model can be applied to other subsets of genes that are induced by TLRs. A complete understanding of the mechanisms of innate immunity will be helpful for the future

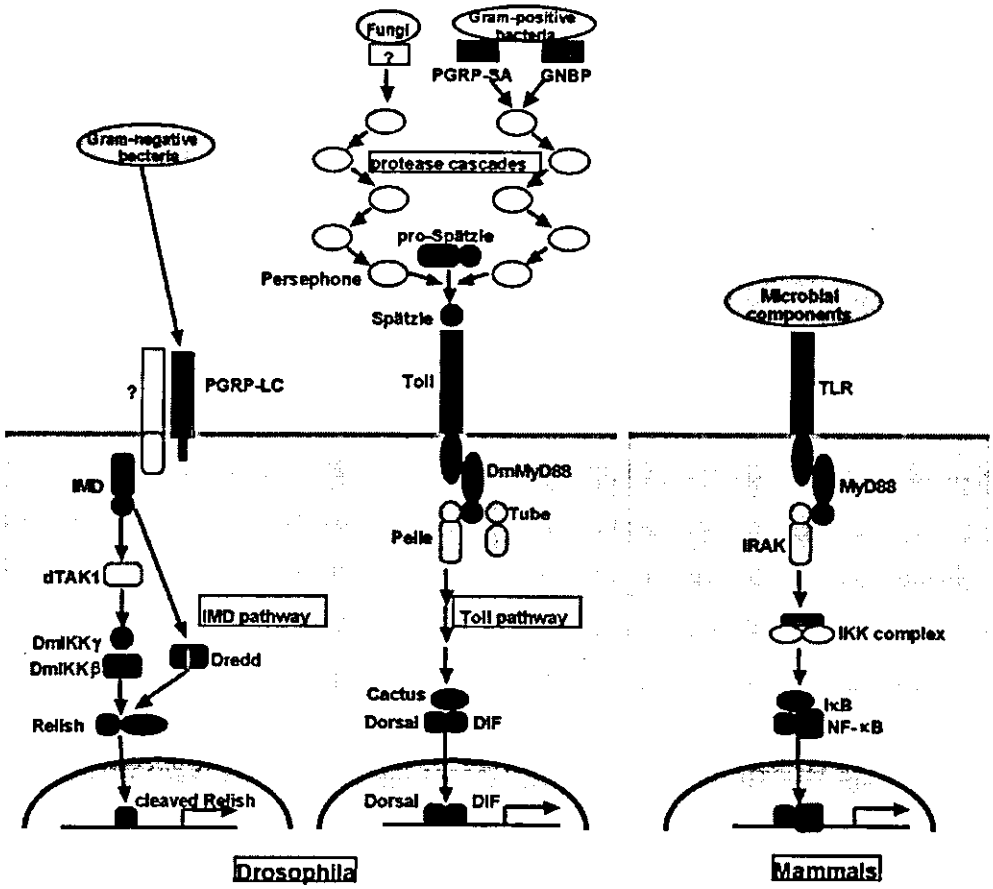

Fig. 6. Toll pathway in *Drosophila* and TLR pathway in mammals. In *Drosophila*, fungal and Gram-positive bacterial infections are sensed by pattern recognition proteins. GNBP1 and PGRP-SA are responsible for the recognition of Gram-positive bacteria. Recognition of micro-organisms is followed by activation of proteolytic cascades, leading to the cleavage of Spätzle. Spätzle activates Toll, which leads to degradation of Cactus and nuclear translocation of the Rel-type transcription factor DIF. In the case of Gram-negative bacterial infection, the IMD pathway is activated in *Drosophila*. In mammals, microbial infections are sensed by TLRs, which leads to activation of the Rel-type transcription factor NF-κB.

development of innovative therapies for manipulation of infectious diseases, cancer and allergies.

Acknowledgements

We thank M. Hashimoto and M. Kurata for secretarial assistance. This work was supported by grants from the Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology, the Uehara Memorial Foundation and the Naito Foundation.

Abbreviations

CARD	caspase-recruitment domain
dsRNA	double-stranded RNA
HSP	heat shock proteins
iE-DAP	γ-D-glutamyl-meso diaminopimelic acid
IKK	IκB kinase
LRRs	leucine-rich repeats
Mal	MyD88-adaptor-like
MCMV	mouse cytomegalovirus
MDP	MurNAc-L-Ala-D-isoGln
NOD	nucleotide-binding oligomerization domain
PDC	plasmacytoid dendritic cells
PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PKR	dsRNA-dependent protein kinase
RNAi	RNA interference
SIGIRR	single immunoglobulin IL-1 receptor-related molecule
ssRNA	single-stranded RNA
TICAM	TIR domain-containing adaptor molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLRs	Toll-like receptors
TRIF	TIR domain-containing adaptor inducing IFN-β

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