Involvement of Toso in activation of monocytes, macrophages, and granulocytes

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Rapid activation of immune responses is necessary for antibacterial defense, but excessive immune activation can result in life-threatening septic shock. Understanding how these processes are balanced may provide novel therapeutic potential in treating inflammatory disease. Fc receptors are crucial for innate immune activation. However, the role of the putative Fc receptor for IgM, known as Toso/Faim3, has to this point been unclear. In this study, we generated Toso-deficient mice and used them to uncover a critical regulatory function of Toso in innate immune activation. Development of innate immune cells was intact in the absence of Toso, but Toso-deficient neutrophils exhibited more reactive oxygen species production and reduced phagocytosis of pathogens compared with controls. Cytokine production was also decreased in *Toso*$^{-/-}$ mice compared with WT animals, rendering them resistant to septic shock induced by lipopolysaccharide. However, *Toso*$^{-/-}$ mice also displayed limited cytokine production after infection with the bacterium *Listeria monocytogenes* that was correlated with elevated presence of *Listeria* throughout the body. Accordingly, *Toso*$^{-/-}$ mice succumbed to infections of *L. monocytogenes*, whereas WT mice successfully eliminated the infection. Taken together, our data reveal Toso to be a unique regulator of innate immune responses during bacterial infection and septic shock.

sepsis | ROS | LPS

During systemic bacterial infection, bacteria have to be controlled within the first few hours to days to guarantee survival. Macrophages, monocytes, and granulocytes are major contributors to antibacterial immune defense. Each of these cell types contributes to control of infection through phagocytosis of bacteria, production of antibacterial substances, and generation of cytokines, which recruit additional immune cells. Antibacterial immune responses can cause substantial collateral damage to tissue, and therefore immune effector functions have to be well controlled. How the innate immune system manages to restrict activation of effector cells largely to the sites of bacterial infection is still under intensive investigation.

Toso is a plasma membrane protein that contains an extracellular region with homology to the Ig variable (IgV) domains and a cytoplasmic region with partial homology to Fas-activated serine/threonine kinase (FAST kinase) (1). Toso was first identified as a molecule expressed on activated T cells capable of protecting T cells from programmed cell death (1). Toso is additionally expressed on B cells, overexpressed in B-cell lymphomas (2, 3), and is important for macrophage homeostasis (4). Mechanistically, Toso was originally described to influence Fas signaling (1, 5). We previously reported that TNF-induced apoptosis is enhanced in the absence of Toso (6, 7). Other work has shown that Toso can act as an Fc receptor for IgM (8, 9) and is important for the phagocytosis of IgM-coated targets (10). However, the physiological relevance of the diverse functions attributed to Toso remains to be verified.

In this study, we analyzed how Toso influences innate immune activation during bacterial infection in vitro and in vivo. We generated gene-targeted Toso-null (*Toso*$^{-/-}$) mutant mice and found that phagocytosis of pathogenic bacteria into both granulocytes and monocytes is influenced by IgM and Toso. In vivo, the lack of Toso reduced antibacterial cytokines, resulting in enhanced tolerance toward LPS-induced septic shock. However, *Toso*$^{-/-}$ mice were found to succumb to infection with *Listeria monocytogenes* more readily than WT controls.

Results

Toso Is Expressed on Granulocytes and Monocytes. To study the physiological role of Toso, we first generated Toso-deficient mice as described in *Experimental Procedures* and Fig. S1. We then analyzed Toso expression on bone marrow–derived granulocytes from WT and *Toso*$^{-/-}$ animals using a Toso-specific antibody (7). Immature (Ly6G^{med}) and mature (Ly6G^{hi}) granulocytes from WT bone marrow exhibited expression of Toso (Fig. 1*A*). Next we analyzed expression of spleen-derived monocytes. Monocytes showed clear expression of Toso (Fig. 1*B*). These findings indicate that Toso is present on innate immune cells such as monocytes and granulocytes.

Toso Does Not Influence Differentiation of Granulocytes and Monocytes. To determine whether Toso is important for the generation of myeloid cells, differentiation of granulocytes and monocytes from myeloid precursors was analyzed in WT and *Toso*$^{-/-}$ mice (Fig. 2*A*). Pre-GM and GMP cells were comparable between WT and

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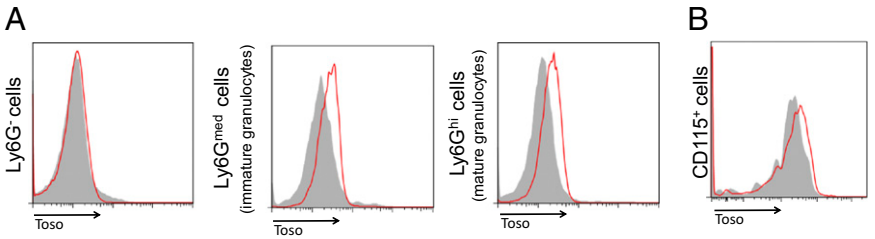

Fig. 1. Toso is expressed on granulocytes and monocytes. (*A*) Bone marrow cells from WT and *Toso*$^{-/-}$ mice were immunostained with anti-Ly6G (high expressing cells = mature granulocytes, medium expressing cells = immature granulocytes) plus rat anti-mouse Toso antibody. Red shows staining for WT cells; gray area shows staining of *Toso*$^{-/-}$ cells. One of two experiments is shown. (*B*) Splenocytes from WT and *Toso*$^{-/-}$ mice were stained with anti-CD115 (monocytes) plus rat anti-mouse Toso antibody (red). Red shows staining for WT cells; gray area shows staining of *Toso*$^{-/-}$ cells. One of two experiments is shown.

Toso$^{-/-}$ mice (Fig. 2 *B* and *C*). Monocytes and immature and mature granulocytes were also present in similar numbers in WT and *Toso*$^{-/-}$ mice (Fig. 2 *D* and *E*), suggesting that differentiation of granulocytes and monocytes was not dramatically impaired in the absence of Toso.

Lack of Toso Enhances Reactive Oxygen Species Production in Granulocytes. Next, the function of Toso in mature granulocytes was investigated by stimulation with *N*-formyl-methionine-leucine-phenylalanine (fMLP). fMLP engages the formyl peptide receptor (encoded by the *Fpr1 gene*) on granulocytes, mimicking pathogen contact (11, 12). About 10% of WT granulocytes produced reactive oxygen species (ROS) and degranulated following fMLP treatment (Fig. 3 *A* and *B*). In contrast, treatment with fMLP activated 50% of *Toso*$^{-/-}$ granulocytes, suggesting that the activation threshold to produce ROS is reduced in Toso-deficient granulocytes (Fig. 3 *A* and *B*). Even without fMLP treatment *Toso*$^{-/-}$ granulocytes also showed enhanced release of ROS compared with WT granulocytes (3.5 ± 0.6% vs. 1.4 ± 0.3%; $P = 0.003$). This phenotype could be reproduced using an independent strain of Toso KO mice (*Toso*$^{del/del}$; Fig. S2). Treatment with either LPS or GM-CSF, two stimuli known to prime granulocytes (13, 14), in combination with fMLP showed similarly enhanced ROS production in Toso-deficient granulocytes. Cellular stress (such as heat or cold shock) can also activate primed granulocytes (15) and represents a source of stimulation thought to contribute significantly to (auto)inflammatory diseases (16–18). To determine whether *Toso*$^{-/-}$ granulocytes were unusually prone to such activation, we incubated WT and *Toso*$^{-/-}$ granulocytes for 30 min at a range of temperatures and measured numbers of degranulating neutrophils. Temperature reduction from 37 °C to 14 °C induced degranulation in about 30% of *Toso*$^{-/-}$ granulocytes but in only a negligible percentage of WT granulocytes (Fig. 3*D*).

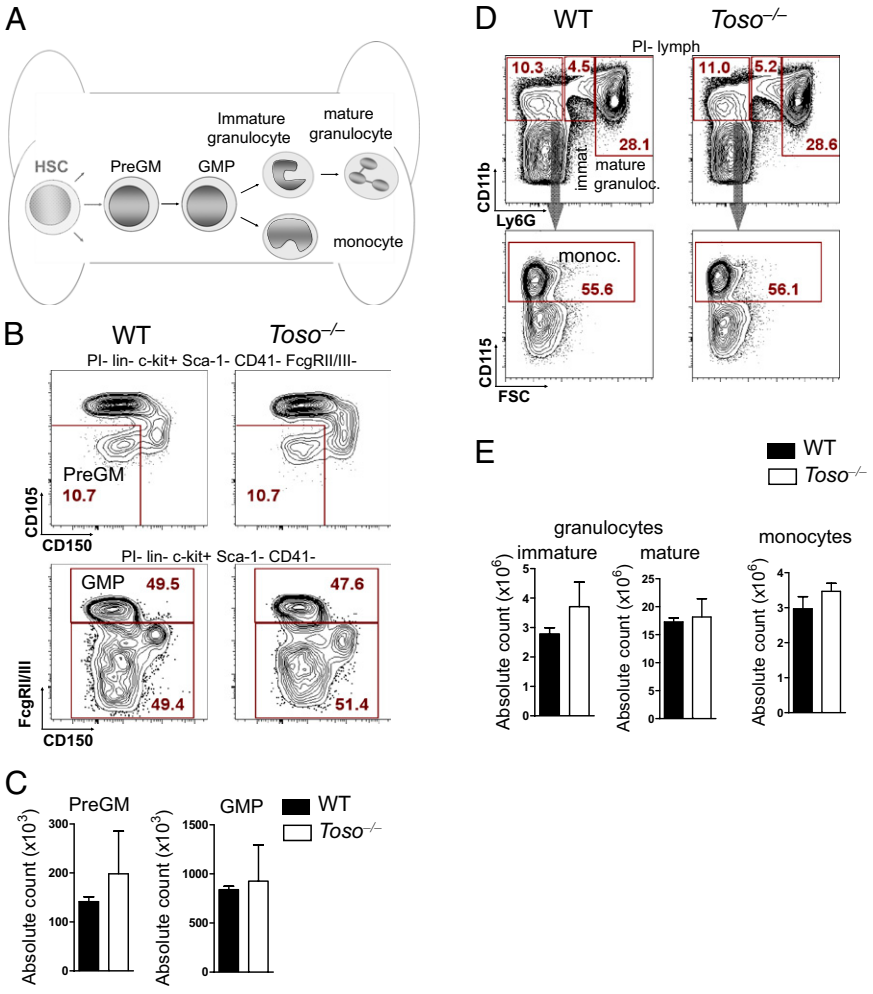

Fig. 2. Toso does not influence granulomonopoiesis in the bone marrow. (*A*) Scheme of granulocytes and monocyte development in bone marrow. (*B* and *C*) Flow cytometric analysis of early granulomonocytic progenitor compartments (PreGM and GMP) of WT mice and *Toso*$^{-/-}$ mice by staining for CD150, CD105, FcgRII/III, lineage markers, c-kit, Sca-1, and CD41. Original FACS contour plots (*B*) and quantification of progenitor compartments (*C*, $n = 3$) are shown. One of two experiments is shown. (*D* and *E*) Flow cytometric analysis of WT mice and *Toso*$^{-/-}$ mice stained for CD11b, Ly6G, CD115, and lymphoid markers (lymph). Original FACS contour plots (*D*) and quantification of different granulo- and monocytic subsets (*E*, $n = 3$) are shown. One of two experiments is shown.

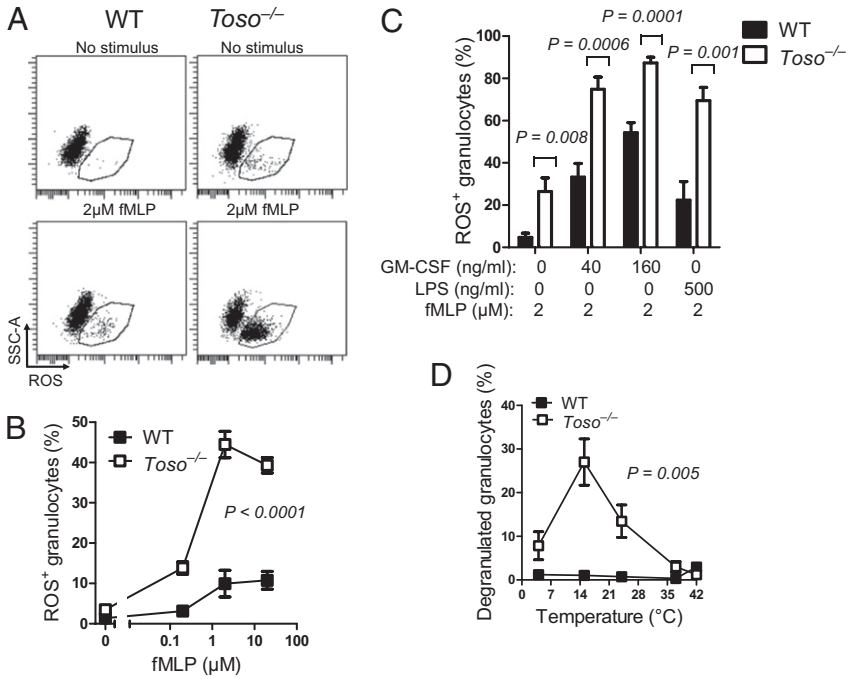

Fig. 3. Lack of Toso enhances ROS production in granulocytes. (*A* and *B*) Blood cells from WT or *Toso*$^{-/-}$ mice were incubated with or without the indicated concentrations of fMLP for 30 min at 37 °C, and granulocyte activation was assessed by measuring ROS production (dihydro-rhodamine staining) and degranulation (side-scatter) as described in *Experimental Procedures*. (*Left*) Representative dot plot of cells gated on total granulocytes (*A*). The mean percentage ± SEM of total granulocytes that was activated at each fMLP concentration (*B*, $n = 6$). (*C*) Blood cells from WT and *Toso*$^{-/-}$ mice ($n = 6$/group) were primed by incubation for 30 min with 40 ng/mL GM-CSF supernatant or 500 ng/mL LPS, and activated by stimulation with 2 μM fMLP for 15 min. The percentage of activated granulocytes was determined as for *A*. (*D*) Blood cells from WT and *Toso*$^{-/-}$ mice ($n = 6$/group) were incubated at the indicated temperatures for 30 min. The percentage of granulocytes that had degranulated was measured using flow cytometric side scatter as described in *Experimental Procedures*. Results shown are the mean percentage of degranulated cells ± SEM.

These findings indicated that the absence of Toso triggered enhanced ROS production in granulocytes.

Toso Influences Phagocytosis of Granulocytes and Monocytes. Next we analyzed whether Toso is involved in the phagocytosis of bacteria. Toso is described as an Fc receptor for IgM, which is internalized after IgM binding (8–10). To determine whether Toso and/or IgM are important for triggering granulocyte-mediated phagocytosis of *Listeria*, we incubated samples of peripheral blood from WT, *Toso*$^{-/-}$, or sIgM$^{-/-}$ mice with carboxyfluorescein succinimidyl ester (CFSE)-labeled *Listeria monocytogenes* and used flow cytometry to evaluate *Listeria* binding by Ly6G-positive granulocytes. WT granulocytes showed greater binding of *Listeria* than either *Toso*$^{-/-}$ or sIgM$^{-/-}$ granulocytes (Fig. 4*A*). Although a significant proportion of *Listeria* could still bind to granulocytes in the absence of Toso, confocal microscopy confirmed that reduced binding of CFSE-positive *Listeria* was also correlated with reduced *Listeria* uptake in Toso deficiency (Fig. S3). Next, we further investigated the mechanism of reduced *Listeria* binding to *Toso*$^{-/-}$ granulocytes by incubating CFSE-labeled *Listeria* with granulocytes isolated from WT or *Toso*$^{-/-}$ mice in the presence of serum obtained on day 3 postinfection from *Listeria*-infected WT or sIgM$^{-/-}$ mice. Binding of *Listeria* by *Toso*$^{-/-}$ granulocytes was reduced compared with WT controls under normal serum conditions, but not in the absence of serum IgM (Fig. 4*B*). Similar to granulocytes, Toso-deficient monocytes also showed reduced binding of *Listeria* (Fig. 4*C*). These data suggest that Toso is integrally involved in the IgM-mediated attachment of *Listeria*. Absence of Toso led to reduced phagocytosis of *Listeria* in *Toso*$^{-/-}$ granulocytes.

Toso Deficiency Leads to Reduced NF-κB Phosphorylation and Cytokine Production. We previously demonstrated that Toso limits signaling induced by the proinflammatory cytokine TNF-α (7). Limited signaling was mainly linked to defects in NF-κB activation. Because Toso-deficient granulocytes showed enhanced ROS production and granulocytes and monocytes exhibited defective phagocytosis, we wondered whether toll like receptor (TLR)-induced cytokine production might also be impaired. Indeed, LPS-stimulated *Toso*$^{-/-}$ macrophages exhibited delayed NF-κB activation after LPS stimulation compared with WT macrophages (Fig. 5).

Lack of Toso Reduces the LPS Response In Vivo. Next we analyzed cytokine production in WT and *Toso*$^{-/-}$ mice during treatment with LPS. We found that systemic injection of LPS led to reduced IL-6 and TNF-α production in *Toso*$^{-/-}$ mice relative to controls (Fig. 6 *A* and *B*). In line with this, *Toso*$^{-/-}$ mice survived LPS treatment, whereas WT mice died (Fig. 6*C*). Those data show that, although ROS production was enhanced in *Toso*$^{-/-}$ mice, the cytokine response during LPS challenge was functionally limited.

Impaired Control of *Listeria* in Toso-Deficient Mice. Our data showed that lack of Toso correlated with enhanced ROS production but

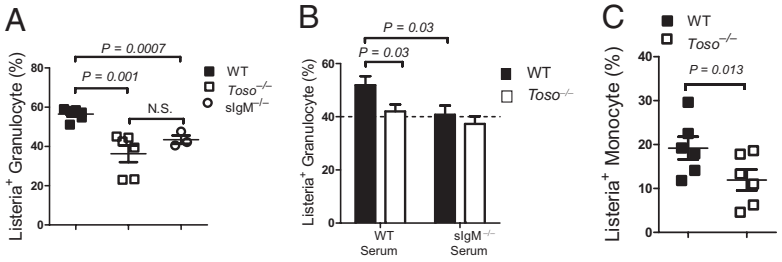

Fig. 4. Toso influences phagocytosis of granulocytes and monocytes. (*A*) Peripheral blood was incubated for 60 min with 10^6 CFU of CFSE-labeled *Listeria* and CSFE-positive granulocytes were analyzed by flow cytometry. Data points are values for individual mice ($n = 3$–6/group). (*B*) Blood leukocytes from WT or *Toso*$^{-/-}$ mice were washed three times to remove IgM. Granulocytes were then incubated for 60 min with CFSE-labeled *Listeria* plus serum from WT or sIgM$^{-/-}$ mice that had been infected with *Listeria* 3 d prior. *Listeria* uptake by granulocytes was analyzed. Horizontal line, mean for IgM independent *Listeria* uptake. Results are the mean ± SEM ($n = 10$). (*C*) Peripheral blood was incubated for 60 min with 10^6 CFU of CFSE-labeled *Listeria*, and CSFE-positive monocytes were analyzed by flow cytometry. Data points are values for individual mice ($n = 6$/group).

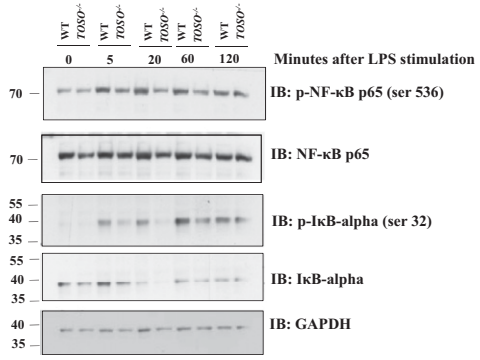

Fig. 5. Toso deficiency leads to reduced NF-κB phosphorylation, Bone marrow–derived macrophages were generated from WT and *Toso*$^{-/-}$ bone marrow. Cells were challenged with LPS (100 ng/mL). NF-κB activation was analyzed after different time points. One of three similar experiments is shown.

reduced cytokine production and phagocytosis. To determine the impact of those deficiencies on the control of bacterial infection, we infected WT and *Toso*$^{-/-}$ mice with *L. monocytogenes*. Consistent with our in vitro results, peripheral blood granulocytes obtained from *Toso*$^{-/-}$ mice on day 2 after systemic *Listeria* inoculation (1×10^4 CFUs) showed elevated ROS production compared with granulocytes from infected WT mice (Fig. 7*A*). This observation correlated with the increased release of myeloperoxidase (MPO) protein into the serum of infected *Toso*$^{-/-}$ mice compared with infected WT animals (Fig. 7*B*). There were no obvious differences in the number of granulocytes in infected organs (Fig. 7*C*). However, the number of bacteria was enhanced within the granulomas compared with WT mice (Fig. 7*C*). Reduced bacterial control in the presence of granulocytes suggested that infiltration of granulocytes into target tissues was not defective in the absence of Toso, but likely that these granulocytes displayed reduced effector function within the tissues. Consistently, cytokine production was reduced in *Toso*$^{-/-}$ mice compared with WT mice after *L. monocytogenes* infection compared with WT animals (Fig. 7*D*). Three days after infection, higher *Listeria* titers were found in *Toso*$^{-/-}$ spleen, liver, and brain tissue compared with the WT tissue (Fig. 7*E*). As a result, *Toso*$^{-/-}$ mice rapidly succumbed to a dose of *Listeria* that was generally lethal to only 20% of WT mice (Fig. 7*F*), indicating that Toso is essential for effective control of *Listeria* in vivo. Taken together, the absence of Toso resulted in limited cytokine production after *L. monocytogenes* infection and death of infected *Toso*$^{-/-}$ mice.

Discussion

In this study, we demonstrated that Toso regulates ROS production, cytokine production, and phagocytosis. A previous report established that the presence of Toso on T cells rendered them less sensitive to Fas-induced cell death (1), and we have recently shown that Toso overexpression reduces apoptotic signaling induced by TNF-α treatment (6, 7). In addition, we found here that Toso is involved in IgM-dependent phagocytosis and that the lack of Toso reduces cytokine responses after LPS treatment. The latter effect correlated with reduced NF-κB phosphorylation. Taking those effects together, we suggest that Toso is probably involved in several signaling pathways. From our findings, we conclude two functions of Toso: (*i*) Toso can act as an FcR that can bind to IgM-coated pathogens, triggering phagocytosis; and (*ii*) Toso regulates surface signals that drive antipathogenic signaling. Such dual functions have been previously described for other granulocyte receptors, including α_M-integrin (CD11b). CD11b not only acts as receptor for several ligands (ICAM-1, iC3b, collagen, fibrinogen) (19) but also negatively regulates TLR signaling (20–22). The mechanism of phagocytosis probably involves small GTPases and actin polymerization (23); however, how these are linked to Toso remains to be studied.

We found that the susceptibility of Toso-deficient mice to bacterial infection correlated with reduced phagocytic capacity and enhanced ROS production. Even without any additional stimulation, ROS were detectable in Toso-deficient granulocytes. Together those data suggest that fMLP signaling was probably not affected specifically by Toso. More likely, Toso expression generally enhances the activation threshold of granulocytes. From our data, we would interpret that the absence of this mechanism in Toso deficiency would result in activation of granulocytes without any additional stimulus. Recently, Cole et al. showed that ROS production enhanced the spread of *Listeria* between cells (24), hastening mouse death. In line with that, depletion of monocytes rather than depletion of granulocytes enhanced susceptibility to *Listeria* infection in mice (25). These studies, and the data described here, underscore that enhanced ROS production of granulocytes likely has an overall negative impact in the control of *Listeria*. How early and enhanced ROS production can negatively impact *Listeria* infection remains largely unexplained. We found that deficiency of Toso led to enhanced bacterial titers in the spleen, liver, and brain. Using histology, we also found enhanced *Listeria* numbers within granulomas. As the granulomas did not show an obvious difference in size between WT and *Toso*$^{-/-}$ mice, we suggest that homing to the site of infection was not altered between WT and *Toso*$^{-/-}$ mice. Instead, it was likely the effector function within the infected tissue, which was defective in the absence of Toso.

We found that, *Toso*$^{-/-}$ granulocytes were still able to uptake *Listeria*, albeit at a reduced level. Because *Toso*$^{-/-}$ was nevertheless more susceptible to *Listeria* infection, it is unclear whether additional mechanisms besides *Listeria* uptake are also defective in the absence of Toso. As activation of innate immune cells can partially depend on the phagocytosis of pathogenic pattern molecules, we postulate that Toso is not only directly affecting innate immune cell effector functions, but perhaps more importantly is regulating the immune activation, which strongly impacts pathogen control. Indeed, using LPS treatment, we found that Toso influences cytokine induction during this model of sterile immune activation.

Enhanced ROS production and deficient cytokine response in parallel to reduced phagocytosis are likely the reason for the rapid death of Toso-deficient mice during *Listeria* infection (26, 27). Theoretically, enhanced ROS levels could also lead to death of mice due to collateral damage to tissue (28). However, because we saw significantly enhanced bacterial titers in the brains of

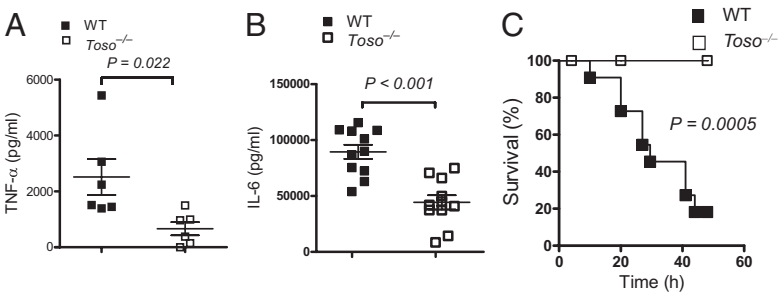

Fig. 6. Lack of Toso reduces the LPS response in vivo. WT and *Toso*$^{-/-}$ mice were injected intraperitoneally at a dose of 25 mg/kg. (*A*) TNF-α was analyzed after 4 h ($n = 6$). (*B*) IL-6 was analyzed after 2 h ($n = 11$). (*C*) Survival was monitored ($n = 11$).

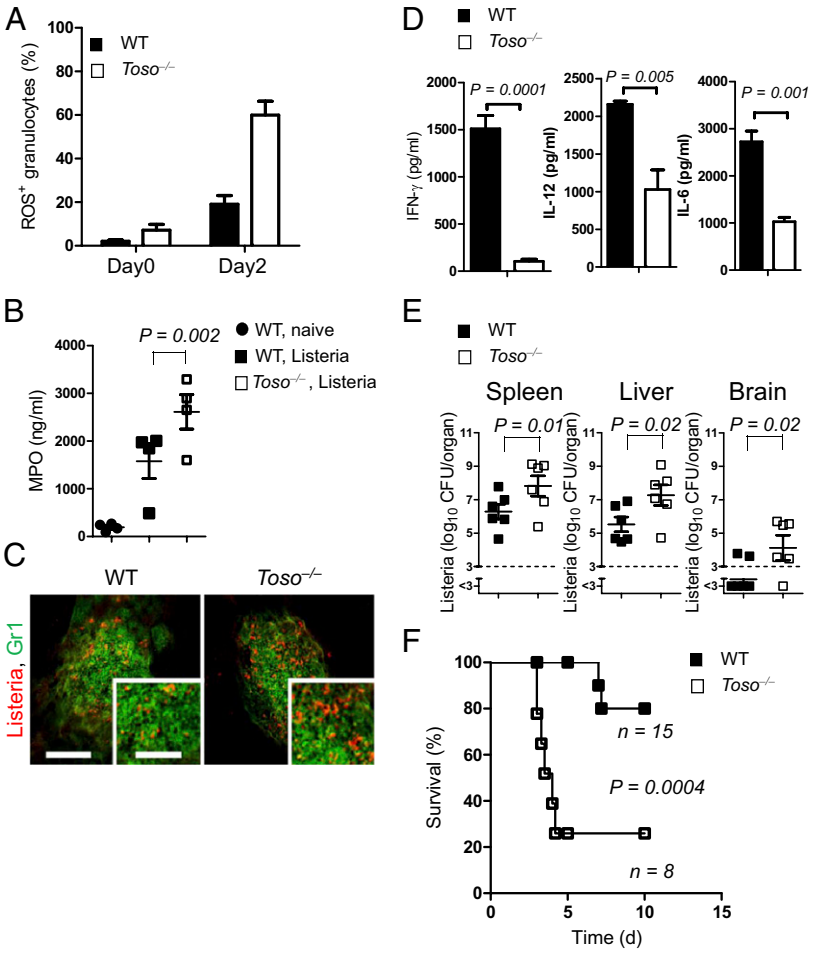

Fig. 7. Impaired control of *Listeria* in Toso-deficient mice. WT and $Toso^{-/-}$ mice were infected with 1×10^4 CFU *Listeria* or 1×10^5 CFU *Listeria* for *B*. (*A*) ROS production on the indicated days postinfection by granulocytes from peripheral blood of WT and $Toso^{-/-}$ mice ($n = 4$–5). (*B*) Levels of MPO protein in the plasma of uninfected WT mice and at 6 h postinfection in the plasma ($n = 4$/group). (*C*) *Listeria* and granulocyte infiltration was analyzed in the liver on day 3. One of three representative slides is shown. (Scale bar = 50 μm; Scale bar inset, 25 μm.) (*D*) IFN-γ, IL-12, and IL-6 concentrations were analyzed in the serum at different time points ($n = 4$). (*E*) *Listeria* titers on day 3–4 postinfection in spleen, liver, and brain of WT and $Toso^{-/-}$ mice ($n = 6$/group). (*F*) Kaplan-Meier survival curves for WT ($n = 15$) and $Toso^{-/-}$ ($n = 8$) mice.

Toso-deficient mice and because we could rescue *Listeria* susceptibility of Toso-deficient mice with transfer of WT granulocytes (Fig. S4), we would rather speculate that bacterial burden was the reason for the hastened death of Toso-deficient mice.

Although the main function of granulocytes is the early control of pathogens, hyperactivation of these cells is a hallmark of autoinflammatory disorders such as periodic fever syndromes and autoimmune diseases such as rheumatoid arthritis, vasculitis, and autoinflammatory lung injury (18, 29–31). The reasons for these instances of inappropriate granulocyte activation are not known (16, 17). In particular, vasculitis is associated with enhanced granulocyte activation and/or autoantibodies directed against granulocytic components (31, 32). Consistent with this disease, Toso-deficient animals exhibit autoantibody formation (33). Future studies will examine the exciting possibility that Toso also influences granulocyte activation in human inflammatory diseases.

In conclusion, we demonstrated that Toso is a regulator of granulocyte activation. Toso thus plays a key role in the coordination of granulocyte phagocytosis, ROS production, and degranulation and is critical for the normal control of bacterial infection in mice.

Experimental Procedures

Mice. Genomic DNA fragments containing the *Toso* gene were isolated from a mouse genomic DNA library (129J/Ola; J. Rossant, SickKids Hospital, University of Toronto, Toronto, Canada) using a mouse full-length *Toso* cDNA as a probe. The KO construct was assembled using two PCR-generated fragments: a 6.5-kb fragment found within an intron located in the 5′ leader sequence of the gene and a 0.65-kb fragment downstream of the last methionine. Both fragments were inserted on either side of a neomycin (*neo*) expression cassette, such that homologous recombination caused the replacement of almost all of the *Toso* coding region by *neo* (Fig. S1*A*). An artificial EcoRI site was added to reduce the WT 6-kb EcoRI fragment to a 2.3-kb size when probed on a Southern blot with a flanking probe. The KO construct was electroporated into D3 embryonic stem (ES) cells, and a clone demonstrating homologous recombination was injected into E3.5 C57/BL6 blastocysts, which were implanted in pseudopregnant ICR mice. The chimeric progeny were bred until germ-line transmission was achieved, and heterozygotes bearing the KO allele were intercrossed to generate homozygous progeny. Deletion of *Toso* was confirmed by PCR and RT-PCR analyses of various tissues (Fig. S1 *B*–*D*). $Toso^{-/-}$ mice were backcrossed into the C57BL/6 background for more than nine generations before use in experiments. $Toso^{del/del}$ mice, an independent line of Toso KO mice on the C57BL/6 background, have been previously described (7). $sIgM^{-/-}$ mice were also maintained on the C57BL/6 genetic background. All experiments were performed in single ventilated cages. Animal experiments were carried out in Germany and Canada under the authorization of the Veterinäramt of Duesseldorf and in accordance with German laws for animal protection and according to institutional guidelines at the Ontario Cancer Institute of the University Health Network.

***Listeria* Infection and In Vitro Phagocytosis.** *L. monocytogenes* (ATCC strain 43251) bacteria were the kind gift of Klaus Pfeffer (Institute of Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University Duesseldorf, Duesseldorf, Germany) and were maintained in heart infusion agar as previously described (34). If not otherwise indicated, mice were infected i.v. with 2×10^4 CFU of *Listeria*. For CFSE labeling, 10^9 CFUs *Listeria* were incubated for 10 min with 50 μM CSFE (Invitrogen) in 10 mL PBS. After two washes in PBS/10% (vol/vol) FCS and an additional three washes in medium, CSFE-labeled *Listeria* were incubated for indicated time points with granulocytes in antibiotic-free, FCS-free medium.

Granulocyte Activation and FACS. Stimuli for granulocyte activation were fMLP (Sigma Aldrich), LPS (Sigma), and GM-CSF (self-made from GM-CSF–producing cells [X63O]). For granulocyte activation studies, mouse peripheral blood was incubated with DMEM/2% FCS containing fMLP plus anti-Gr1,

anti-CD11b antibody (eBiosciences) and dihydro-rhodamine (Alexis). For priming studies, granulocytes were pretreated for 30 min with cytokines followed by 15-min activation with fMLP. Granulocytes, which produced enhanced levels of ROS and showed degranulation in SSC-A, were considered activated granulocytes. Toso expression was analyzed by an anti-Toso antibody generated previously in the laboratory of K.-H.L. Absolute counts of bone marrow cells were determined on a Z2 cell counter (Beckman Coulter). FACS analysis was performed on a LSRII flow cytometer (Becton Dickinson) after staining with fluorochrome-conjugated antibodies (Becton Dickinson, eBioscience, Biolegend) as previously described (35, 36). Toso expression was analyzed by a monoclonal antibody against murine Toso (clone B68) that was generated in the laboratory of K.-H.L. (7).

Bone Marrow–Derived Macrophages. Bone marrow cells were treated with macrophage colony-stimulating factor (M-CSF) derived from L929 cells in very low endotoxin DMEM 10% FCS. After 8–10 d, cells were used for experiments.

LPS Treatment. WT and Toso-deficient animals were injected i.p. with a 25-mg/kg dose of LPS (026:B6; Sigma).

Myeloperoxidase. Levels of myeloperoxidase protein in serum were quantified by ELISA (Hycult Biotech) according to the manufacturer's instructions.

Quantitative RT-PCR. RNA extraction and cDNA synthesis were performed using Trifast (peqlab) and the QuantiTec Rev. Transcription Kit (Qiagen) according to the manufacturers' instructions. Analysis of specific gene expression was performed using Toso (Faim3) kits (Applied Biosystems or Qiagen). The mRNA expression levels of all target genes were normalized against GAPDH or 18sRNA.

Temperature Shock Experiment. Mouse peripheral blood (10 μL) was incubated with 100 μL DMEM/2% FCS plus anti-Gr1 and anti-CD11b for 30 min either at 4 °C, 15 °C, 24 °C, 37 °C, or 42 °C. Degranulated granulocytes were determined by SSC-A.

Immunoblotting. Bone marrow–derived macrophages were seeded in six-well tissue-culture plates and treated as indicated. Cells were washed with PBS and lysed using 1.1% SDS, 11% glycerol, and 0.1 M Tris, pH 6.8, with 10% β-mercaptoethanol. Blots were probed with anti–phospho-NF-κB p65 (Ser536), anti–phospho-IκBα (Ser32), anti-IκBα, anti-GAPDH (Cell Signaling), and anti–NF-κB p65 (Santa Cruz).

Histology. Histological analyses were performed on snap-frozen tissues as previously described (34). Slides were stained for anti–Gr1-FITC (eBiosciences) and rabbit anti-*Listeria* (Abcam), followed by staining with anti-rabbit phycoerythrin (Jackson Laboratories).

ELISAs. Supernatant or serum cytokine levels were detected using the IL-6 (eBioscience), TNFα (eBioscience), and INFγ (eBioscience) detection kits.

Statistical Analyses. Where appropriate, data are expressed as the mean ± SEM. Statistically significant differences between two groups were analyzed using the unpaired Student *t* test. Analyses of multiple groups were performed using one-way ANOVA in conjunction with the Bonferroni or Dunnett test. Statistically significant differences between experimental groups over multiple time points were calculated using two-way ANOVA (repeated measurements). $P < 0.05$ was considered statistically significant.

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