The Journal of Immunology

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J Immunol 2005; 175:5596-5600; ;
doi: 10.4049/jimmunol.175.9.5596
http://www.jimmunol.org/content/175/9/5596

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: MyD88 Controls Phagocyte NADPH Oxidase Function and Killing of Gram-Negative Bacteria[1]

F. Stephen Laroux, Xavier Romero,* Lee Wetzler,‡ Pablo Engel,† and Cox Terhorst[2]**

***MyD88 is an adaptor protein for the TLR family of proteins that has been implicated as a critical mediator of innate immune responses to pathogen detection. In this study, we report that MyD88 plays a crucial role in killing Gram-negative bacteria by primary macrophages via influencing NADPH oxidase function. Peritoneal macrophages from MyD88$^{-/-}$ mice exhibited a marked inability to kill* Escherichia coli *(F18) or an attenuated strain of* Salmonella typhimurium *(sseB) in vitro. This defect in killing was due to diminished NADPH oxidase-mediated production of superoxide anion in response to bacteria by MyD88$^{-/-}$ phagocytes as a consequence of defective NADPH oxidase assembly. Defective oxidase assembly in MyD88-deficient macrophages resulted from impaired p38 MAPK activation and subsequent phosphorylation of p47^{phox}. Together these data demonstrate a pivotal role for MyD88 in killing Gram-negative bacteria via modulation of NADPH oxidase activity in phagocytic cells. The Journal of Immunology,* 2005, 175: 5596–5600.**

Recognition of, and the first line of defense against, bacterial infection falls under the purview of the innate arm of the immune system and is the specific domain of phagocytic leukocytes such as macrophages and neutrophils. Toward this end, phagocytes sense bacteria, or their components, via a set of cell surface proteins collectively termed the TLR family (1, 2). In addition to these surface receptors, the immune system has evolved several adaptor proteins that associate with the TLR family in various combinations. These adaptors determine the cellular responses necessary for dealing with the particular bacteria, or bacterial component, sensed by the surface receptors. Among these adaptor proteins, MyD88 plays a prominent and ubiquitous role in TLR signaling (3–5).

In addition to sensing the presence of bacteria, the innate immune system has also developed several strategies for killing and processing bacteria as well as presenting their antigenic components to the adaptive immune system for subsequent humoral responses (6, 7). One of the key methods in which phagocytic cells are able to efficiently kill engulfed bacteria is through the production of bactericidal reactive oxygen species (ROS;[3] superoxide; O_2^-) via the NADPH oxidase enzyme complex (6, 8). The NADPH oxidase complex is comprised of both membrane-bound (i.e., gp91^{phox} and p22^{phox}) and cytosolic (i.e., p47^{phox}, p67^{phox}, p40^{phox}, and Rac-1/2) components (8, 9). Receptor-mediated activation of the oxidase complex involves activation of secondary signaling intermediates that culminates in the phosphorylation and recruitment of the cytosolic components to the membrane-bound components (9–12).

Although it is well appreciated that both MyD88 and NADPH oxidase each contribute to the sensing and resolution of bacterial infection (1, 2, 5, 6, 13), it is as yet unclear and, in some cases, controversial (14–16), if and how these two individually studied components of innate immunity interact in phagocytic cells. The study reported here was conducted to determine whether MyD88 deficiency would alter NADPH oxidase activity and consequently bacterial killing.

Materials and Methods

Isolation of mouse macrophages and neutrophils

Elicited macrophages were obtained from wild-type C57BL/6 (The Jackson Laboratory) and MyD88$^{-/-}$ (B6.129P2-MyD88^{tm1Aki}) mice by i.p. injection of 2 ml of 4% sterile Brewer's thioglycolate medium. On the fifth day postinjection, peritoneal lavage was performed with 20 ml of ice-cold RPMI 1640 supplemented with 5% FCS. Cells were washed three times with RPMI 1640/5% FCS before enumeration and plating.

Neutrophils were isolated from bone marrow as described previously (17). Briefly, bone marrow was washed three times in HBSS supplemented with 5% FCS and neutrophils were then isolated by discontinuous Percoll gradient centrifugation. Cells suspended in 2 ml of HBSS were layered on the top of a 15-ml conical tube containing a gradient consisting of (bottom to top) 4 ml of 75% Percoll in PBS, 3 ml of 65% Percoll, and 3 ml of 55% Percoll. The gradient was centrifuged at 1600 rpm for 30 min. Neutrophils were isolated from the 75/65 interface, washed, and enumerated. Using this technique, >95% purity was routinely obtained as assessed by Wright-Giemsa staining.

Gentamicin protection assay

Macrophage bactericidal activity was measured using a gentamicin protection assay. Macrophages were plated in 24-well plates at 1×10^6/well in triplicate for each condition and time point. Cells were incubated with bacteria at a 10:1 ratio of bacteria:macrophages for 1 h at 37°C to allow phagocytosis to occur. After 1 h, gentamicin was added to the medium at 100 μg/ml for 1 h to kill

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Received for publication June 9, 2005. Accepted for publication September 20, 2005.

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[1] This work was supported by National Institutes of Health Grants DK52510 and AI15066. F.S.L. is supported by National Institutes of Health Grant 5T32 DK07760.

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[3] Abbreviation used in this paper: ROS, reactive oxygen species.

0022-1767/05/$02.00

extracellular bacteria. At 2 h, the medium was replaced with fresh medium containing 10 μg/ml gentamicin. At 2, 6, and 24 h, cells were washed with PBS and lysed with 1 ml of 0.5% Triton X-100 in sterile water for 15 min at room temperature. Various dilutions were plated directly onto Lennox-Bertani agar plates and colonies were counted after overnight incubation at 37°C.

Flow cytometric measurement of macrophage phagocytosis

Peritoneal macrophages (4×10^6/ml in HBSS supplemented with 5% FCS) were incubated for various periods with 4×10^8 paraformaldehyde-fixed and opsonized GFP-expressing *Escherichia coli* strain MS589 (a gift from Dr. P. Klemm, Technical University of Denmark Lyngby, Denmark) or 4×10^8 fixed and opsonized GFP-expressing *Salmonella typhimurium* strain *sseB* (a gift from Dr. M. E. H. Bashir, Massachusetts General Hospital, Boston MA). Following incubation cells were washed three times in ice-cold PBS followed by a 60-s wash in 0.4% trypan blue to quench extracellular GFP and a final wash in PBS before data acquisition on a FACScan flow cytometer (BD Biosciences). As a negative control for nonspecific bacterial adhesion, a portion of the macrophages were fixed for 10 min in 2% paraformaldehyde before the assay.

Measurement of superoxide generation

Neutrophil and macrophage superoxide production was measured with the fluorogenic substrate lucigenin. Neutrophils and macrophages were resuspended in HBSS with 5% FCS at 2.5×10^5 and 1×10^6/ml, respectively. Cells were stimulated for 2 h with 8×10^7 heat-killed, opsonized *E. coli* or *S. typhimurium*. Alternatively, to measure maximal receptor-independent ROS production, cells were stimulated with PMA (Sigma-Aldrich) at 1 μg/ml. Luminescence was measured at various time points throughout the stimulations with a TD2020 luminometer (Turner Designs).

Results and Discussion

Defective bactericidal activity by MyD88-deficient macrophages is due to impaired NADPH phagocyte oxidase function

Since MyD88 plays a pivotal role in TLR-mediated signaling, we wished to determine whether deficiency in this key adaptor protein impacted the ability of peritoneal macrophages to kill Gram-negative bacteria upon phagocytosis. As shown in Fig. 1, A and *B*, MyD88$^{-/-}$ macrophages are severely impaired in killing both commensal and attenuated pathogenic Gram-negative bacteria (i.e., F18 *E. coli* and the *sseB*$^-$ variant of *S. typhimurium*, respectively) as judged using the in vitro gentamicin protection assay. This defect in bacterial killing was not due to either impaired phagocytosis of bacteria, because both wild-type and MyD88$^{-/-}$ macrophages engulfed GFP-expressing *E. coli* and *S. typhimurium sseB*$^-$ with similar efficiencies (Fig. 1*C*)

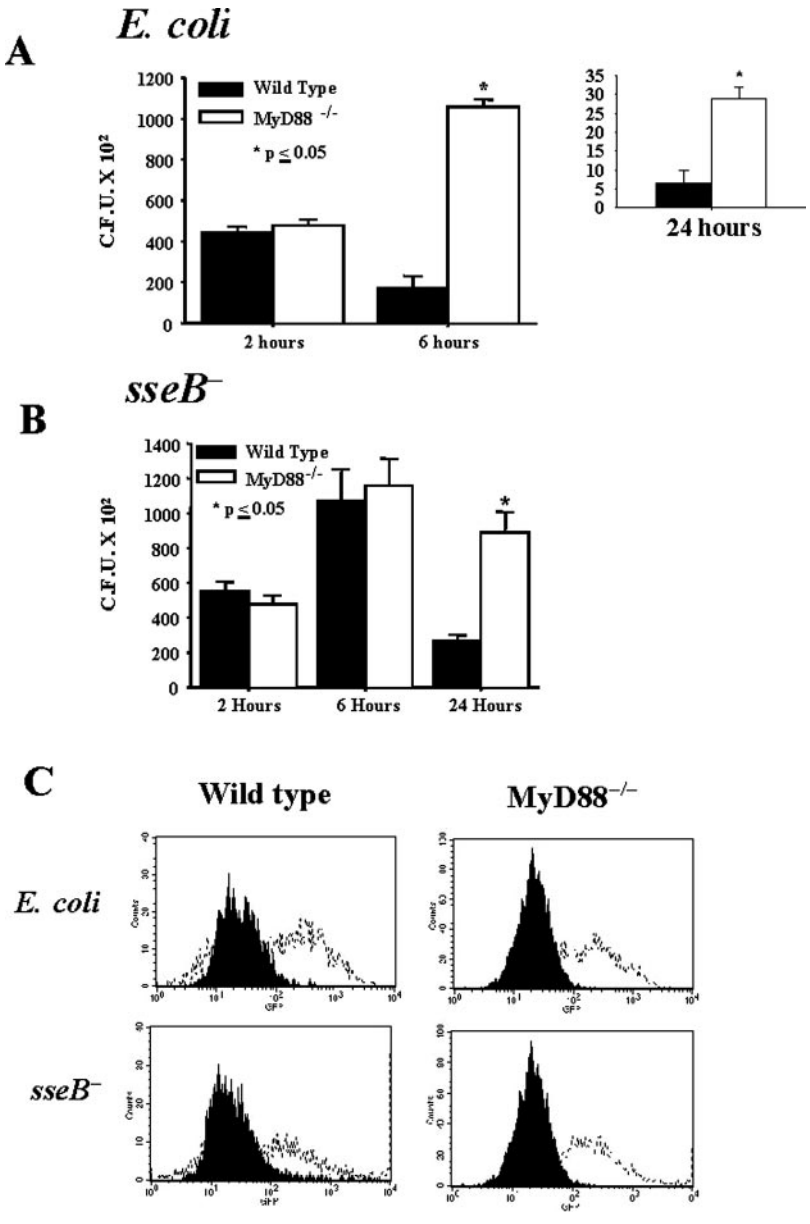

FIGURE 1. MyD88 deficiency impairs killing but not phagocytosis of Gram-negative bacteria. *A* and *B,* Isolated peritoneal macrophages from wild-type B6 and MyD88$^{-/-}$ mice were exposed to either F18 *E. coli* or attenuated *S. typhimurium* (*seB*$^-$) for 1 h and 100 μg/ml gentamicin for an additional hour followed by 10 μg/ml gentamicin for the duration of the assay. Viable intracellular bacteria were quantitated by gentle lysis of the macrophages and subsequent plating on Luria-Bertani agar. MyD88$^{-/-}$ macrophages displayed equivalent phagocytic capacity (*A* and *B,* 2-h time point) but a significant impairment in killing ability for both *E. coli* and *S. typhimurium* (*A,* 6 and 24 h, and *B,*24 h, respectively). Experiments were performed five times. *C,* FACS-based analysis confirmed that MyD88$^{-/-}$ deficiency does not impair engulfment of GFP-expressing Gram-negative bacteria (performed three times).

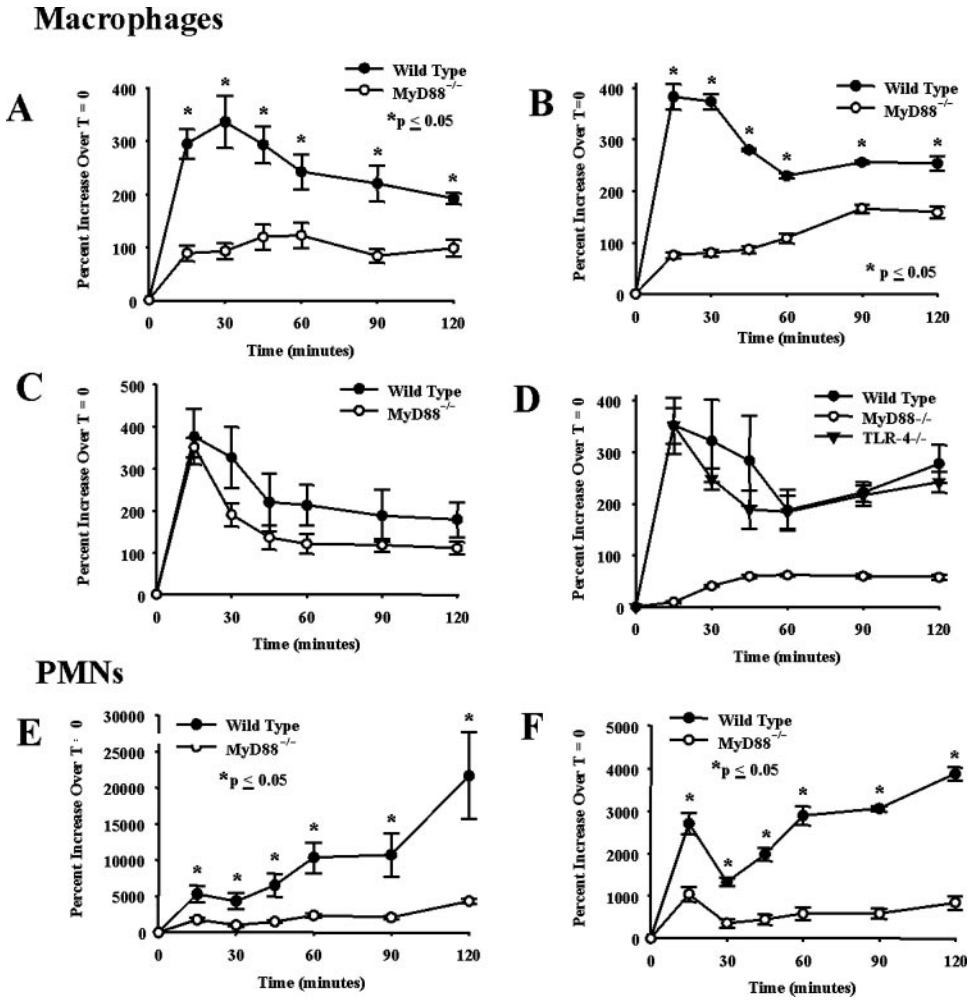

FIGURE 2. MyD88-deficient macrophages and neutrophils have impaired ROS production in response to Gram-negative bacteria. *A* and *B,* Isolated peritoneal macrophages from MyD88$^{-/-}$ mice display a severe impairment in their ability to produce superoxide in response to both *E. coli* and *S. typhimurium*. *C,* Nonspecific stimulation by PMA elicited an equivalent respiratory burst from both wild-type and MyD88$^{-/-}$ macrophages. *D,* TLR-4$^{-/-}$ macrophages do not exhibit bacteria-mediated impairment of NADPH oxidase. *E* and *F,* MyD88$^{-/-}$ PMNs also display defective ROS production in response to *E. coli* and *S. typhimurium*. All experiments were performed four times.

or differences in the activation state of the macrophages as assessed by surface marker expression (TLR-4, MHC class II, F4/80, Mac-1, and CD11c; data not shown). This is in contrast to a recent study showing that MyD88$^{-/-}$ macrophages have impaired phagocytic capacity (18). The difference between our findings may be due to the use of bone marrow-derived macrophages (18) vs primary elicited macrophages used in this study.

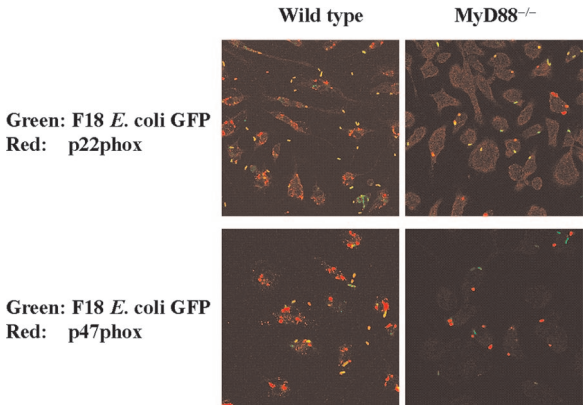

FIGURE 3. MyD88 is critical for assembly of NADPH oxidase. Confocal analysis of isolated peritoneal macrophages from wild-type and MyD88$^{-/-}$ mice exposed to *E. coli* demonstrated impaired concentration of both membrane as well as cytosolic components of NADPH oxidase to bacteria-containing phagosomes (e.g., p22^{phox}, *upper panels*, and p47^{phox}, *lower panels,* respectively). Experiments were conducted three times.

As stated earlier, one of the key enzymatic systems that contributes to efficient killing of intracellular bacteria by phagocytic cells is the NADPH oxidase complex. Therefore, production of ROS (e.g., singlet oxygen, superoxide, O_2^-) by NADPH oxidase in macrophages and neutrophils isolated from both wild-type and MyD88$^{-/-}$ mice was determined using a lucigenin-based bioluminescence assay. Indeed, MyD88-deficient macrophages were profoundly impaired in their ability to produce NADPH-derived ROS in response to Gram-negative bacteria (Fig. 2A, *E. coli* F18 and Fig. 2*B, S. typhimurium sseB*$^-$). This impaired respiratory burst was not due to an inherent inability to produce ROS as both wild-type and MyD88$^{-/-}$ macrophages produced equivalent amounts of superoxide in response to PMA stimulation (Fig. 2*C*). Surprisingly, TLR-4$^{-/-}$ macrophages did not exhibit the same defect in oxidant production in response to bacteria (Fig. 2*D*), suggesting that phagocytosis-mediated NADPH oxidase activity is MyD88 dependent but TLR-4 independent.

MyD88-dependent activation of the NADPH oxidase system in response to bacterial engulfment was not restricted to

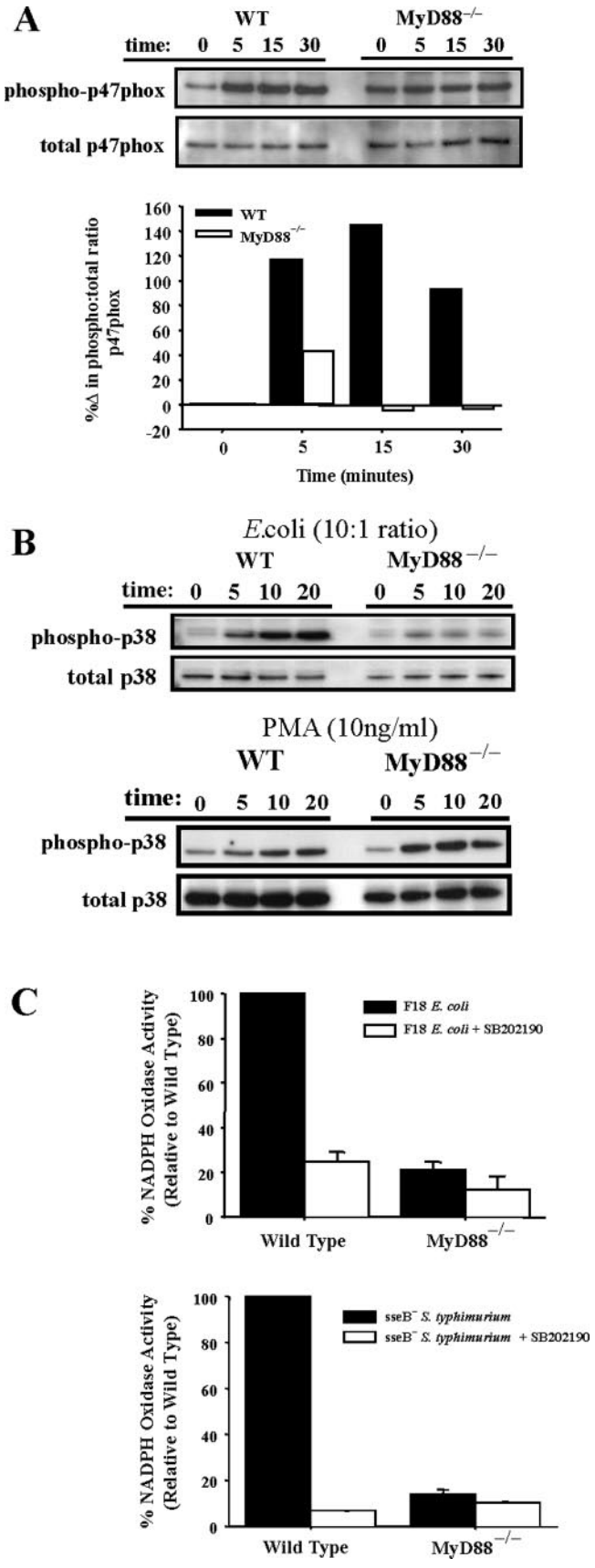

FIGURE 4. MyD88 is required for efficient activation of secondary signaling intermediates necessary for p47^{phox} phosphorylation and cellular activation. *A,* Phospho-p47^{phox} levels remain unchanged in MyD88$^{-/-}$ macrophages upon exposure to bacteria compared with wild type (WT) macrophages. Macrophages were stimulated and probed via Western blot for phospho- and total p47^{phox}. Representative of two separate experiments. *B,* Bacterial stimulation induces up-regulation of p38 MAPK activity in wild-type but not in MyD88$^{-/-}$ macrophages. Isolated macrophages were exposed to heat-killed bacteria for the indicated times, and lysates were analyzed for phospho- and total p38 MAPK by Western blot. Representative of three separate experiments. *C,* Bacteria-induced ROS generation is sensitive to p38 MAPK inhibition in wild-type but not MyD88$^{-/-}$ macrophages. Macrophages were exposed to bacteria for 120 min to allow engulfment and up-regulation of NADPH oxidase activity. The p38 MAPK inhibitor SB202190 was then added and production of ROS was measured for an additional 60 min (three experiments conducted).

macrophages since bone marrow-derived PMNs from MyD88$^{-/-}$ mice also exhibit reduced ROS production in vitro (Fig. 2, *E, E. coli*, and *F, S. typhimurium sseB*$^{-}$).

We conclude that macrophages and neutrophils derived from mice that are deficient in the TLR adaptor protein MyD88 are impaired in their ability to kill bacteria. This killing defect arises as a result of impaired NADPH oxidase function.

Assembly of the NADPH oxidase enzyme complex is impaired in MyD88$^{-/-}$ phagocytes

We next determined whether the observed defect in ROS production in MyD88$^{-/-}$ macrophages was due to impaired activity or assembly. To this end, wild-type and MyD88$^{-/-}$ peritoneal macrophages were examined for distribution of both the membrane-bound p22^{phox} protein and the cytosolic p47^{phox} component of NADPH oxidase after exposure to GFP-expressing *E. coli*. As shown in Fig. 3, clustering of p22^{phox} to bacteria-containing phagosomes is impaired in the MyD88$^{-/-}$ macrophages (Fig. 3, *upper panels*). In addition, mobilization of cytosolic p47^{phox} to bacteria-containing vesicles is also inefficient in MyD88$^{-/-}$ cells (Fig. 3, *lower panels*). This suggests that a defect in assembly is the underlying cause of reduced ROS production by the NADPH oxidase enzyme complex in MyD88-deficient phagocytes.

Inefficient p38 MAPK-mediated phosphorylation of p47^{phox} causes improper assembly of NADPH oxidase in MyD88$^{-/-}$ macrophages

It is well appreciated that serine phosphorylation of the cytosolic components of NADPH oxidase, particularly p47^{phox}, is required for mobilization to the membrane-bound cytochrome *b*558 (i.e., gp91-p22^{phox}) complex and subsequent production of ROS by NADPH oxidase. Since a defect in assembly of NADPH oxidase in MyD88$^{-/-}$ macrophages was apparent, we next determined whether this was due to impairment of one or more of the signaling cascades known to play a role in oxidase assembly via phosphorylation of p47^{phox} (11, 12, 19, 20). To this end, we examined macrophage responses to whole bacteria, which is considered more physiologically relevant, while using LPS stimulation as a reference. Examining levels of phospho-p47^{phox} in response to bacteria, we found that although wild-type macrophages show a robust up-regulation, MyD88$^{-/-}$ macrophages were unable to increase the level of phospho-p47^{phox} relative to the unstimulated state (Fig. 4*A*). This correlated with a lack of up-regulation of p38 MAPK activity in MyD88$^{-/-}$ macrophages exposed to bacteria (Fig. 4*B*, *top*). As with functional superoxide generation (Fig. 2), lack of p38 MAPK activation in response to bacteria was not intrinsic to MyD88$^{-/-}$ macrophages because PMA stimulation induced similar levels of phospho-p38 MAPK in both wild-type and MyD88$^{-/-}$ macrophages (Fig. 4*B, bottom*). This was indirectly

confirmed by studies showing that the residual NADPH oxidase activity of MyD88$^{-/-}$ macrophages was largely insensitive to p38 MAPK kinase inhibitors (Fig. 4*C*).

Thus, the levels of phosphorylated p38 MAPK, known to be critical for NADPH oxidase activation (11, 19), are severely compromised in MyD88$^{-/-}$ macrophages, in agreement with reduced ROS production by these cells. In addition, IκBα degradation and activation of the SAPK/JNK pathway, both of which are important for cytokine production, are also impaired in MyD88$^{-/-}$ macrophages following exposure to Gram-negative bacteria (data not shown). The latter suggests that MyD88 plays a role not only in the immediate oxidant-mediated killing of bacteria, but also in later cytokine-mediated events important for resolution of infection.

Taken together, the results of this study demonstrate a previously unappreciated role for MyD88, and potentially other TLR adaptor proteins, in mediating killing of intracellular bacteria via influencing assembly and thus activity of the NADPH oxidase complex. As such, further study of the mechanisms underlying the interplay between TLR adaptor proteins and oxidant-producing enzyme complexes would contribute greatly to our understanding of how the innate immune system resolves bacterial infection as well as form a basis for the development of therapeutic strategies to enhance clearance of pathogenic bacteria.

Acknowledgments

We thank Dr. Per Klemm for his generous gift of GFP expressing *E. coli* as well as Dr. Xiuping Liu and Heather Macleod for technical assistance. We especially thank Dr. Shizuo Akira for the generous gift of MyD88$^{-/-}$ mice.

Disclosures

The authors have no financial conflict of interest.

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