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Cutting Edge: Slamf8 Is a Negative Regulator of Nox2 Activity in Macrophages

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Slamf8 (CD353) is a cell surface receptor that is expressed upon activation of macrophages (MΦs) by IFN-γ or bacteria. In this article, we report that a very high NADPH oxidase (Nox2) enzyme activity was found in Slamf8−/− MΦs in response to Escherichia coli or Staphylococcus aureus, as well as to PMA. The elevated Nox2 activity in Slamf8−/− MΦs was also demonstrated in E. coli or S. aureus phagosomes by using a pH indicator system and was further confirmed by a reduction in the enzyme activity after transfection of the receptor into Slamf8-deficient primary MΦs or RAW 264.7 cells. Upon exposure to bacteria or PMA, protein kinase C activity in Slamf8−/− MΦs is increased. This results in an enhanced phosphorylation of p40phox, one key component of the Nox2 enzyme complex, which, in turn, leads to greater Nox2 activity. Taken together, the data show that, in response to inflammation-associated stimuli, the inducible receptor Slamf8 negatively regulates inflammatory responses. The Journal of Immunology, 2012, 188: 000–000.

Several members of the Slam family (Slamf) of cell surface receptors modulate macrophage (MΦ) functions (1–3). For example, we recently found that on the surface of MΦs, Slamf1 acts as a microbial sensor and modulates two bactericidal processes in the Escherichia coli phagosome: NADPH oxidase (Nox2) activity and phagosomal maturation (1). Because human and mouse SLAMF8 (BLAME, CD353) are expressed upon activation of macrophages (MΦs), but it can be induced by exposure to IFN-γ and bacteria. Using MΦs isolated from Slamf8-deficient (Slamf8−/−) mice or RAW 264.7 MΦs, Slamf8 was found to be a negative regulator of Nox2 activity in bacterial phagosomes. In Slamf8−/− MΦs, in contrast to Slamf8-sufficient cells, bacteria or PMA induce increased protein kinase C (PKC) activity, resulting in enhanced phosphorylation of p40phox, which increases Nox2 activity.

Materials and Methods

Mice

Slamf8−/− (Slamf8−/−) BALB/c mice were generated, as described in Supplemental Fig. 1A, and were generously donated by Drs. A. Coyle and J.C. Gutierrez-Ramos (Millennium Pharmaceuticals, Cambridge, MA). Slamf8−/− mice develop normally, and spontaneous disease or inflammation was not observed. In Slamf8−/− mice, T and B cells and MΦs develop similarly as in wild-type (w) mice (Supplemental Fig. 1). wt BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME); all mice were maintained in a specific pathogen-free facility. The Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee approved all animal care and experimental procedures.

Cells

F4/80CD11b+ bone marrow-derived, resident peritoneal or thioglycollate (TGC)-elicited pMΦs were obtained, as described (1). Slamf8−/− MΦs and RAW 264.7 MΦs were transfected with a Slamf8-mCherry vector using Amaxa (Lonza Group, Basel, Switzerland).

Real-time PCR (TagMan)

Cell RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA); 100 ng RNA was used as an internal reference gene to normalize the transcripts level, and the relative gene mRNA expression level was determined by the 2−ΔΔCt method.

Nox2 assays

Nox2 assays using lucigenin (Sigma-Aldrich, St. Louis, MO) were performed after exposure to stimulated heat-killed Escherichia coli F18 or Staphylococcus aureus bacteria (multiplicity of infection [MOI] 100) or 1 μg/ml PMA using a standard Glomax luminometer (Promega, Madison, WI) (1). Phagosomal pH was determined using pHrodo E. coli or S. aureus (MOI 15; Invitrogen), as described (1).

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Abbreviations used in this article: MΦ, macrophage; MOI, multiplicity of infection; Nox2, NADPH oxidase; PKC, protein kinase C; pMΦ, peritoneal macrophage; Slamf, Slam family; TGC, thioglycollate; wt, wild-type.
PKC activity
PKC activity was measured using the ADI-EKS-420A assay kit (Enzo Life Sciences, Plymouth Meeting, PA), as described in the manufacturer’s protocol. TGC-elicited MΦs were stimulated with 1 μg/ml PMA or heat-killed E. coli F18 (MOI 100) for the indicated times and then lysed with 500 μl RIPA buffer. Thirty microliters of lysate/sample was used to quantify PKC activity.

Western blotting
TGC-elicited MΦs from wt and Slamf8−/− mice were stimulated with 1 μg/ml PMA or E. coli F18 (MOI 100) at the indicated time points. Phospho-p40phox (Thr514) Ab (no. 4311; Cell Signaling Technology, Beverly, MA) and p40-phox Ab (sc-30087; Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect phosphorylation and protein expression of p40phox by standard immunoblotting procedures.

Statistical analysis
Statistical significance was determined by the Student t test (two-tailed distribution with a two sample equal variance). The p values < 0.05 were considered significant.

Results and Discussion
Slamf8 is upregulated in MΦs upon stimulation
To evaluate whether expression of Slamf8 was induced by inflammatory agents, resident pMΦs isolated from BALB/c mice were incubated with LPS, IFN-γ, E. coli, S. aureus, or PMA, followed by TaqMan analysis. Slamf8 mRNA was virtually undetectable in the resting cells, but it increased up to 100-fold after exposure to IFN-γ (Fig. 1A, 1B). E. coli or PMA caused a 10-fold induction of expression, whereas LPS and S. aureus each had a lesser effect (Fig. 1B). IFN-γ also significantly induced Slamf8 mRNA expression in bone marrow-derived MΦs (Fig. 1C). Slamf8 expression was up to 200 times higher in TGC-elicited MΦs than in resident cells (Fig. 1D). Nonetheless, in response to IFN-γ, Slamf8 expression was still upregulated further in cell sorter-purified TGC-elicited monocytes (CD11b+Ly6ClowF4/80low) and MΦs (CD11b+F4/80+) (Fig. 1E). This upregulation of Slamf8 in MΦs, particularly by IFN-γ, could indicate that this receptor functions in late responses to innate stimuli. Thus, Slamf8 expression in MΦs depends upon the presence of stimuli associated with bacterial infections.

Slamf8 is a negative regulator of Nox2 activity
To assess whether Slamf8 would have an effect on Nox2 functions, Nox2 enzyme activity in Slamf8−/− BALB/c MΦs was tested. Unexpectedly, in response to E. coli and S. aureus, a very significantly elevated Nox2 activity was found in Slamf8−/− bone marrow-derived MΦs (Fig. 2A). Surprisingly, this phenotype was also observed in response to the receptor-independent PMA stimulus. Importantly, Slamf8−/− TGC-elicited MΦs had a phenotype similar to that of the bone marrow-derived MΦs (Fig. 2B), in contrast to resident resting MΦs (data not shown).

To determine whether the increased Nox2 activity in Slamf8−/− MΦs occurs in the bacterial phagosome, we used a fluorescence-based assay based on the principle that conversion of the reactive oxygen species that are produced by Nox2 to HO2 and H2O2 consumes protons, which enter the phagosome via a proton pump. Thus, a defective Nox2 activity results in less proton consumption in the bacterial phagosome, and, hence, the pH is decreased more rapidly (1). Therefore, we assumed that a “hyperactive” Nox2 enzyme in the bacterial phagosome would result in increased proton consumption and impaired acidification. To test this, a pH-sensitive dye, pHrodo, coupled to either E. coli or S. aureus, was used in conjunction with flow cytometry. Consistent with the Nox2 phenotype, Slamf8−/−-resident MΦs had no detectable changes in phagosomal acidification in comparison with wt cells (Fig. 2C). As predicted, Slamf8−/− TGC-elicited MΦs displayed subnormal phagosomal acidification compared with Slamf8+ cells (Fig. 2D). We conclude that Slamf8 negatively modulates phagosomal Nox2 activity and acidification in a cargo-independent manner.

To confirm that Slamf8 negatively modulates Nox2 activity, RAW 264.7 MΦs were transfected with Slamf8, which greatly reduced Nox2 activity in response to bacteria and PMA (Fig. 3A). Furthermore, transfection of Slamf8 into Slamf8−/−-primary MΦs also resulted in a significant reduction in the production of reactive oxygen species by Nox2 (Fig. 3B).

Because we found that IFN-γ significantly enhances Slamf8 expression in RAW 264.7 MΦs (Figs. 1F, 3C), we tested the dependence of Nox2 activity in these MΦs. Indeed, the presence of Slamf8 suppressed Nox2 activity in these cells (Fig. 3D). Taken together, these studies demonstrate that
Slamf8 is a negative regulator of Nox2 activity in MΦs. Bone marrow-derived (A) and TGC-elicited (B) MΦs were stimulated with *E. coli* F18 (MOI 100), *S. aureus* (MOI 100), or PMA (1 μg/ml) and assessed for Nox2 activity using lucigenin. Resident peritoneal MΦs (C) or TGC-elicited pMΦs (D) were examined for phagosomal acidification using *E. coli* or *S. aureus* (MOI 15) pHrodo bacteria. The data are representative of three independent experiments.

**FIGURE 2.** Slamf8 is a negative regulator of Nox2 activity in MΦs. Bone marrow-derived (A) and TGC-elicited (B) MΦs were stimulated with *E. coli* F18 (MOI 100), *S. aureus* (MOI 100), or PMA (1 μg/ml) and assessed for Nox2 activity using lucigenin. Resident peritoneal MΦs (C) or TGC-elicited pMΦs (D) were examined for phagosomal acidification using *E. coli* or *S. aureus* (MOI 15) pHrodo bacteria. The data are representative of three independent experiments.

**Slamf8 negatively controls phosphorylation of p40 upon induction by E. coli or PMA**

To elucidate how Nox2 activity in *Slamf8*−/− MΦs could be increased by bacteria and PMA, we reasoned that PKC activity in these cells might be increased, because this enzyme is known to phosphorylate p40phox (6). To this end, we first determined that the protein expression of the key components of Nox2 (i.e., gp91phox, p22phox, p40phox, p47phox, and p67phox) was similar in wt and *Slamf8*−/− MΦs. Western blotting of wt and *Slamf8*−/− MΦ lysates showed that the basal expressions of all subunits are comparable (Supplemental Fig. 2A). Furthermore, after LPS, *E. coli* F18, *S. aureus*, or PMA activation, the mRNA expression level of the membrane-bound enzyme components gp91phox and p22phox was identical in wt and *Slamf8*−/− MΦs (Supplemental Fig. 2B).

The isoforms of the serine/threonine kinase PKC in mouse MΦs, which are regulated by PMA, are PKC-β, -d, -ε, and -η (5). PKC-δ is known to phosphorylate p40phox on threonine 154 and serine 315 (7). Phospho-p40phox stabilizes the assembled Nox2 enzyme complex (8). We first determined the kinetics of overall PKC activity in response to various stimuli in wt and *Slamf8*−/− MΦs (Fig. 4A). The PKC activity reached a peak at 5 min after PMA stimulation and was significantly higher in *Slamf8*−/− MΦs than in wt cells, which correlates with Nox2 activity (Fig. 2). Similarly, upon exposure to *E. coli*, enzyme activation was higher in *Slamf8*−/−.

**FIGURE 3.** Introduction of Slamf8 into cells by transfection or IFN-γ stimulation rescues the Nox2-associated phenotype. (A) RAW 264.7 cells were transfected with Slamf8-mCherry or a mock construct, stimulated with *E. coli* F18 (MOI 100), *S. aureus* (MOI 100), or PMA (1 μg/ml), and assessed for Nox2 oxidase activity. (B) *Slamf8*−/− primary MΦs were transfected with Slamf8-mCherry, stimulated with *E. coli* F18 (MOI 100), and assessed for Nox2 activity. (C) RAW 264.7 cells were incubated for 12 h with IFN-γ (10 ng/ml) and assessed for Slamf8 expression, as in Fig. 1. (D) Nox2 activity in IFN-γ-stimulated RAW 264.7 cells was determined as in (A). The data are representative of three independent experiments.
Because Slamf8 expression is increased in monocytes and could be designed to mitigate inflammatory conditions.

Slamf8 is a novel negative regulator of Nox2 activity in Mφs. Because Slamf8 is coupled to the PKC activated by bacteria and PMA, cell biology experiments are required to understand how Slamf8, Nox2 activity increases. Further biochemical and three independent experiments.

Relative p40phox phosphorylation level was normalized to total p40phox (β-actin), which is representative of three independent experiments.

Mφs than in Slamf8+/+ Mφs, but it peaked at 15 min (Fig. 4A). Consistently with enhanced PKC activity in Slamf8−/− Mφs, the level of p40phox phosphorylation was significantly higher in Slamf8−/− Mφs, and phosphorylation in response to PMA preceded that of E. coli (Fig. 4B, 4C).

These data indicated that Slamf8 negatively controls PKC activity in Mφs, which is known to phosphorylate p40phox, a key component of Nox2 in Mφs. Therefore, in the absence of Slamf8, Nox2 activity increases. Further biochemical and cell biology experiments are required to understand how Slamf8 is coupled to the PKC activated by bacteria and PMA in Mφs and phagosomes, in particular.

Taken together, the outcomes of these studies show that Slamf8 is a novel negative regulator of Nox2 activity in Mφs. Because Slamf8 expression is increased in monocytes and Mφs upon IFN-γ stimulation and/or exposure to bacteria, the data suggest that Slamf8 signaling could become operational to dampen an ongoing innate immune response. This notion is supported by the previous observation that the degree of Mφ activation has a differential effect on phagosome activity (9–11). Because Slamf8 is a negative regulator of Nox2 activity in Mφs, Slamf8-based therapeutic strategies could be designed to mitigate inflammatory conditions.

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Disclosures

The authors have no financial conflicts of interest.

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