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SIRPα Controls the Activity of the Phagocyte NADPH Oxidase by Restricting the Expression of gp91^{phox}

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SUMMARY

The phagocyte NADPH oxidase mediates oxidative microbial killing in granulocytes and macrophages. However, because the reactive oxygen species produced by the NADPH oxidase can also be toxic to the host, it is essential to control its activity. Little is known about the endogenous mechanism(s) that limits NADPH oxidase activity. Here, we demonstrate that the myeloid-inhibitory receptor SIRPα acts as a negative regulator of the phagocyte NADPH oxidase. Phagocytes isolated from SIRPα mutant mice were shown to have an enhanced respiratory burst. Furthermore, overexpression of SIRPα in human myeloid cells prevented respiratory burst activation. The inhibitory effect required interactions between SIRPα and its natural ligand, CD47, as well as signaling through the SIRPα cytoplasmic immunoreceptor tyrosine-based inhibitory motifs. Suppression of the respiratory burst by SIRPα was caused by a selective repression of gp91^{phox} expression, the catalytic component of the phagocyte NADPH oxidase complex. Thus, SIRPα can limit gp91^{phox} expression during myeloid development, thereby controlling the magnitude of the respiratory burst in phagocytes.

INTRODUCTION

One of the most important antimicrobial activities of phagocytes is the abrupt formation of reactive oxygen species (ROS), a process known as the respiratory burst. This is mediated by the phagocyte NADPH oxidase complex, and its importance is best illustrated by patients with chronic granulomatous disease (CGD) and dysfunctional NADPH oxidase, and as a result are hypersusceptible to a variety of bacterial and fungal infections (Roos et al., 2003; Bedard and Krause, 2007). The phagocyte NADPH oxidase is a multisubunit enzyme complex composed of (1) the membrane proteins gp91^{phox} (NOX2, the catalytic component of the oxidase) and p22^{phox}; (2) the cytosolic proteins p40^{phox}, p47^{phox}, and p67^{phox}; and (3) the small GTPase Rac. Activation of the oxidase involves translocation of the cytosolic subunits p40^{phox}, p47^{phox}, p67^{phox}, and Rac to the plasma membrane and assembly of the oxidase complex. Once assembled, the NADPH oxidase generates superoxide (O_2^-) formation by transferring electrons from NADPH in the cytosol over the plasma membrane to molecular oxygen. Superoxide produced by the oxidase forms the basic compound from which other ROS, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), are formed. High concentrations of ROS are directly toxic to microbes, and may also be indirectly toxic due to the liberation of hydrolytic proteases (Reeves et al., 2002).

The mechanisms of NADPH oxidase activation have been relatively well characterized, but essentially nothing is known about whether and how the magnitude of the respiratory burst is controlled. The latter is important because ROS not only play a critical role in host defense but can also be toxic to the host. The tight control over NADPH oxidase activity is illustrated in part by the observation that there is only very little interindividual variation in the respiratory burst (Zhao et al., personal communication). The magnitude of the respiratory burst is likely to be primarily determined by the protein expression levels of the various NADPH oxidase components, which are expressed in a developmentally regulated fashion in phagocytes. Although the developmental pathways and transcription factors that trigger the expression of the different NADPH oxidase components during myeloid development have been established (Lindsey et al., 2007; Zhu et al., 2008; Kautz et al., 2001), the putative regulatory mechanisms that counterbalance these factors and prevent excessive, potentially harmful expression of the various NADPH oxidase components within phagocytes have remained unknown.

SIRPα is an inhibitory immunoreceptor that is predominantly expressed on myeloid and neuronal cells (Fujioka et al., 1996; Adams et al., 1998). The cytoplasmic region of SIRPα contains four immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that upon ligand binding become phosphorylated and mediate the recruitment and activation of the SH2-domain-containing

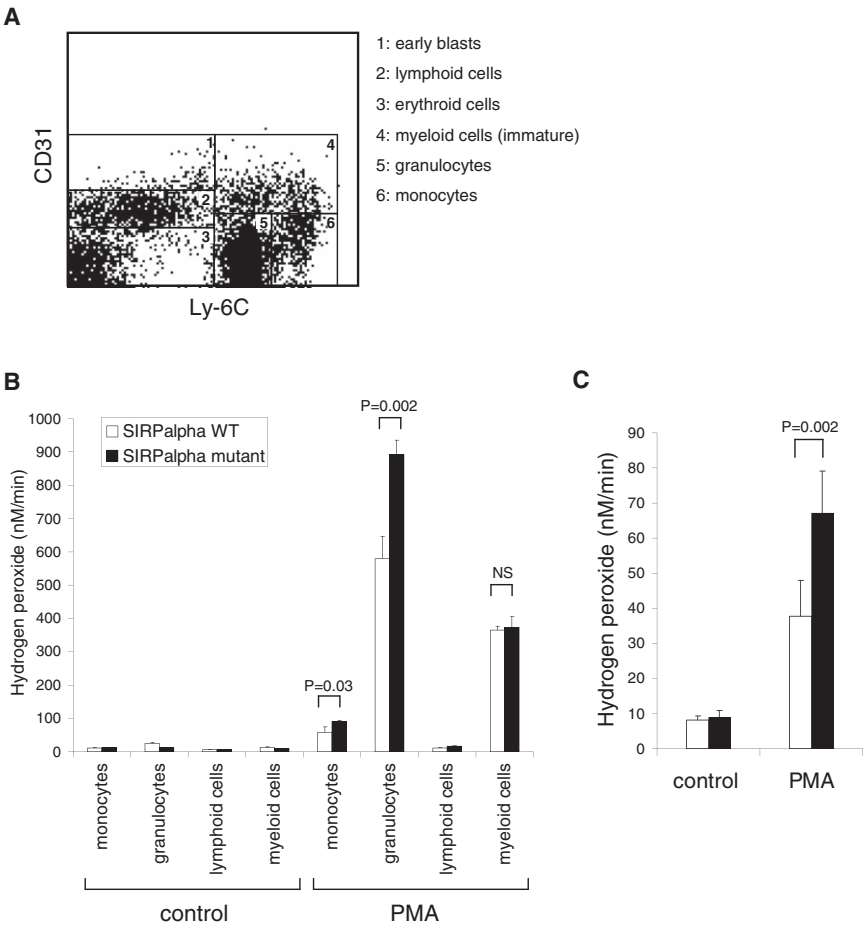

Figure 1. Phagocyte NADPH Oxidase Activity Is Enhanced in Macrophages and Granulocytes from SIRPα Mutant Mice

(A) Flow-cytometric double labeling of bone-marrow cells for CD31 and Ly-6C, identifying the major subpopulations of hematopoietic cells.

(B and C) Sorted populations of (B) monocytes, granulocytes, lymphocytes, and immature myeloid cells, or (C) cultured bone-marrow-derived macrophages from WT (white bars) or SIRPα mutant (black bars) C57BL/6 mice were evaluated for PMA-induced NADPH oxidase activity.

Data are the means ± SD of five animals, with each measurement performed in triplicate. Significance was determined by Student's t test. See also Figure S1.

tyrosine phosphatases (PTPase) SHP-1 and SHP-2. SHP-1 and SHP-2 can in turn dephosphorylate specific protein substrates and thereby mediate various biological functions, generally in a negative fashion. The N-terminal V-like Ig domain mediates recognition of the broadly expressed transmembrane glycoprotein CD47 (Jiang et al., 1999; Seiffert et al., 1999; Vernon-Wilson et al., 2000; Liu et al., 2007; Lee et al., 2007; Hatherley et al., 2008). SIRPα has been implicated in the regulation of a number of functions in myeloid cells (van Beek et al., 2005; Barclay and Brown, 2006). One of the best-documented functions of SIRPα is its inhibitory role in the phagocytosis of host cells by macrophages. In particular, the ligation of SIRPα on macrophages by the "don't eat me" signal CD47 expressed on host cells, such as erythrocytes and platelets, generates an inhibitory signal that negatively regulates phagocytosis (Oldenborg et al., 2000; Yamao et al., 2002). This suggests that CD47 acts as a molecular signature of self that limits immune-mediated damage against normal and healthy host cells during infection and inflammation by interacting with the self sensor SIRPα on phagocytes and other myeloid cells. However, until now, a direct involvement of CD47-SIRPα interactions in the regulation of inflammatory mediators and antimicrobial functions has not been reported.

Here we demonstrate that SIRPα acts as a critical negative regulator of the respiratory burst. Inhibition of the phagocyte NADPH oxidase by SIRPα involves interactions between SIRPα and the self molecule CD47, as well as signaling through the SIRPα ITIMs, which result in a selective suppression of gp91^{phox} expression. This mechanism may help prevent collateral oxidative damage to the host during infection and other inflammatory conditions.

RESULTS AND DISCUSSION

Enhanced NADPH Oxidase Activity in SIRPα Mutant Phagocytes

To investigate whether SIRPα signaling regulates the phagocyte NADPH oxidase, we performed studies with cells from SIRPα mutant mice. These mice express a SIRPα molecule lacking the cytoplasmic tail and signaling capacity (Yamao et al., 2002). We sorted distinct bone-marrow cell populations of SIRPα mutant and control mice, including granulocytes, monocytes, immature myeloid cells, and lymphoid cells, by fluorescence-activated cell sorting (FACS) using CD31 and Ly-6C as markers (Figure 1A) as described previously (Nikolic et al., 2003), and analyzed their phorbol myristate acetate (PMA)-induced respiratory burst. As reported previously (van Beek et al., 2009), there were no detectable differences in bone-marrow composition between the mutant and control mice, essentially excluding a prominent nonredundant role of SIRPα signaling in myeloid differentiation. We observed a significantly (50%–75%) enhanced respiratory burst activity in granulocytes and monocytes from SIRPα mutant mice in comparison with cells from wild-type (WT) mice (Figure 1B). A similar difference was seen in bone-marrow-derived macrophages (Figure 1C). The respiratory burst in immature myeloid cells (Figure 1B) appeared unaffected. A comparison of WT and SIRPα mutant mice showed that the production of other inflammatory mediators, including nitric oxide (NO), tumor necrosis factor α (TNFα), interleukin 1β (IL1β), IL6, and IL10, by bone-marrow-derived macrophages in response to lipopolysaccharide (LPS) was not significantly affected (Figure S1). Collectively,

these data indicate a selective inhibitory role for SIRPα signaling in the regulation of the respiratory burst.

The lack of difference between WT and SIRPα mutant phagocytes appears to be in contrast to previous reports (Kong et al., 2007; Dong et al., 2008), in which knockdown of SIRPα in macrophages was shown to enhance cytokine production in response to TLR ligands. A possible explanation for this apparent discrepancy is that inhibition of cytokine production can only be caused by a complete absence of SIRPα, and not by selective deletion of its cytoplasmic tail. A more trivial explanation could relate to differences in the method used for interference. We used macrophages from gene-targeted knockout mice, whereas the other studies used small hairpin RNA (shRNA)- and small interfering RNA (siRNA)-mediated knockdown, which may have triggered macrophage danger pathways that contributed to the response (Stacey et al., 2000). Our current findings essentially exclude a regulatory role for SIRPα signaling in TLR-induced cytokine production, and support the idea that SIRPα signaling regulates selected inflammatory mediators, such as ROS.

SIRPα Overexpression Inhibits the NADPH Oxidase in Human Phagocytic Cells

To investigate the mechanism by which SIRPα regulates the phagocyte respiratory burst, we tested the effect of overexpression of SIRPα in human myeloid PLB-985 cells. PLB-985 cells are suitable for studying NADPH oxidase activity (Zhen et al., 1993), and were found to express relatively low levels of endogenous SIRPα as shown by western blotting (Figure S2A) and flow cytometry (Figure S4A). A chimeric rat-human SIRPα protein was expressed in PLB-985 cells because it allows for selective monitoring and manipulation by the agonistic monoclonal antibody (mAb) ED9 specifically directed against the rat SIRPα extracellular domain (Adams et al., 1998; Alblas et al., 2005). PLB-985 cells, or mutants with a targeted mutation of the gp91^{phox} gene (PLB-985 X-CGD; Zhen et al., 1993), were retrovirally transduced with full-length chimeric rat-human SIRPα protein (SIRPα-WT) or a SIRPα deletion mutant (SIRPα-Δ87) that is unable to signal because it lacks the cytoplasmic tail. A flow-cytometric analysis showed that the levels of SIRPα expression were comparable for the different cell lines generated (Figure 2A) and similar to those generally seen in rat myeloid cell lines or primary rat myeloid cells, such as macrophages or granulocytes (Adams et al., 1998; not shown). Western blotting with an antibody against the cytoplasmic tail of SIRPα identified both endogenous and chimeric SIRPα proteins, and confirmed that SIRPα-Δ87 cells express a truncated SIRPα (Figure S2A).

We studied the respiratory burst in the different PLB-985 cells after in vitro granulocytic or monocytic differentiation using dimethylformamide (DMF) or vitamin D3 (VitD3), respectively. PMA-induced NADPH oxidase activity was normal in control cells but was abolished in cells expressing WT SIRPα (Figures 2B and 2C). This effect occurred after either granulocytic or monocytic differentiation. Clearly, the inhibitory effect was not observed in the SIRPα-Δ87 mutant, suggesting that SIRPα signaling was required. In fact, the SIRPα-Δ87 cells generated a considerably higher response than the empty vector cells, suggesting that the mutant SIRPα protein acted as a dominant-negative protein by competing with, e.g., endogenous SIRPα for CD47 binding (see below). Of importance, all responses were entirely attributable to the gp91^{phox} (NOX2)-containing phagocyte NADPH oxidase, because they were completely absent in the PLB-985 X-CGD cells, which have a targeted mutation of the gp91^{phox}-encoding *CYBB* gene. Among several other tested stimuli of NADPH oxidase activation (i.e., serum-treated zymosan [STZ], formyl-methionyl-leucyl-phenylalanine [fMLP], and human IgG complexes), only STZ generated a measurable response in the PLB-985 cells, and again this was completely abrogated by SIRPα-WT expression (Figure S2B). This is consistent with a generalized effect of SIRPα on the NADPH oxidase.

SIRPα Selectively Represses gp91^{phox} Expression during Myeloid Differentiation

Activation of the multisubunit NADPH oxidase complex requires assembly of its individual components, which are expressed during myeloid differentiation (Roos et al., 2003). To establish the basis for SIRPα-dependent regulation of the respiratory burst, we investigated the expression levels of the different components of the NADPH oxidase complex upon myeloid differentiation. Expression of the membrane component gp91^{phox}, which forms the enzymatic core of the phagocyte NADPH oxidase, was evaluated in undifferentiated or granulocytic or monocytic differentiated PLB-985 cells by western blotting. The differentiation-induced rise in gp91^{phox} expression was completely absent in cells that express the full-length SIRPα protein (SIRPα-WT; Figure 2D). The same was observed when surface levels of gp91^{phox} were analyzed by flow cytometry with 7D5 mAb (Figure S3A). Also, the enhanced respiratory burst activity in cells that express the truncated receptor (SIRPα-Δ87) was associated with a higher gp91^{phox} expression. Of importance, the levels of two other NADPH oxidase components, p67^{phox} and p47^{phox}, remained unaffected by overexpression of full-length or truncated SIRPα (Figure 2D), suggesting that SIRPα was selectively regulating gp91^{phox} expression. However, the similar levels of upregulation of p47 and p67 observed in the empty vector, SIRPα-WT, and SIRPα-Δ87 cells also indicated that differentiation was unaffected by the introduction of SIRPα-WT or SIRPα-Δ87, suggesting that SIRPα was not regulating differentiation in general. Furthermore, SIRPα did not affect the upregulation of other myeloid differentiation markers, such as CD11b and CD14, during granulocytic or monocytic differentiation (not shown). The upregulation of endogenous SIRPα on PLB-985 cells coincided with that of gp91^{phox} at days 1–3 of neutrophilic differentiation (Figure S3B).

To demonstrate that gp91^{phox} was indeed the only relevant factor downregulated by SIRPα, gp91^{phox} was reconstituted by retroviral expression into SIRPα-WT cells (Figure 2E), and this resulted in a full restoration of the respiratory burst (Figure 2F). A similar restoration was observed when such reconstitution was performed in PLB-985 X-CGD cells. This shows that SIRPα suppresses phagocyte NADPH oxidase activity by a selective repression of gp91^{phox} protein expression during myeloid differentiation.

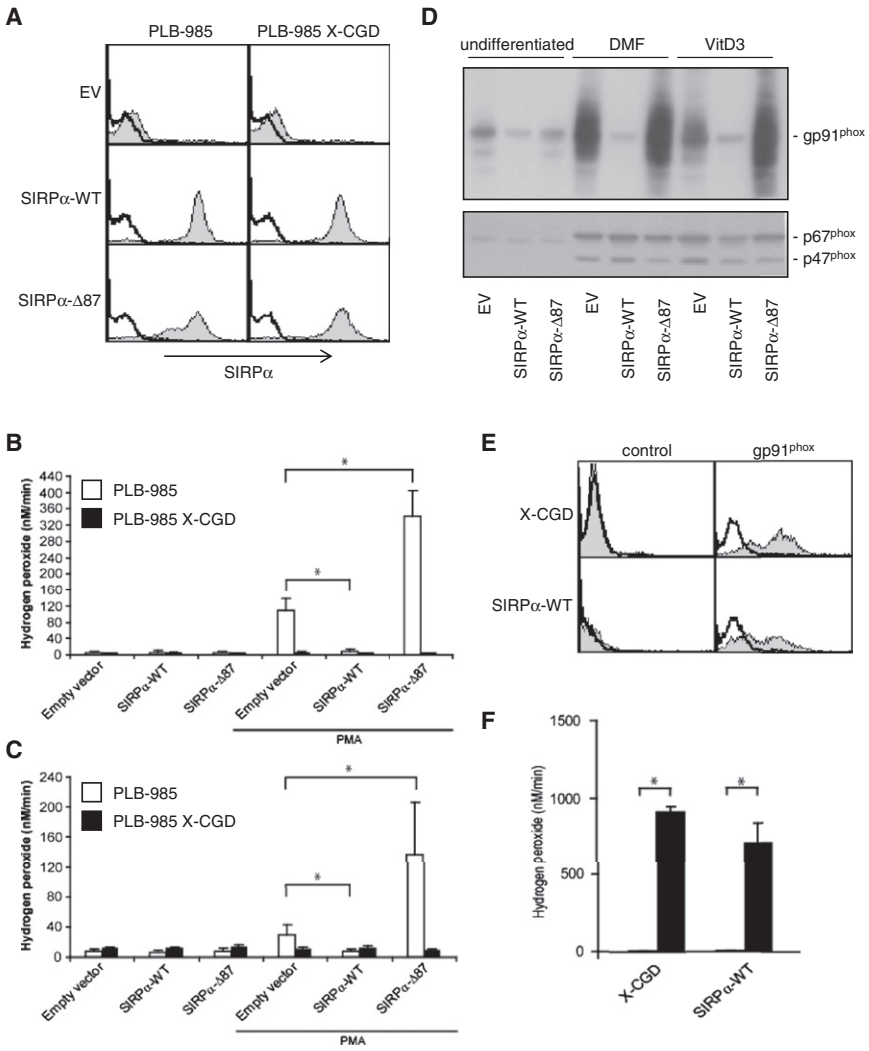

Figure 2. SIRPα Overexpression in PLB-985 Cells Inhibits the Respiratory Burst by Repressing gp91^{phox} Expression

PLB-985 and gp91^{phox} PLB-985 X-CGD cells were stably transduced with empty vector, chimeric rat-human SIRPα (SIRPα-WT), or an N-terminal truncated SIRPα protein lacking 87 amino acids of the cytoplasmic tail of SIRPα (SIRPα-Δ87).

(A) SIRPα surface expression was evaluated by flow cytometry with Alexa 633-conjugated ED9 directed against rat SIRPα antibody (solid histograms). Unstained cells are indicated as control (open histogram).

(B and C) PMA-induced NADPH-oxidase activity in the indicated PLB-985 (white bars) and PLB-985 X-CGD (black bars) cell lines after 5–6 days of either granulocytic (B) or monocytic (C) differentiation induced with DMF or VitD3, respectively. Values shown in (B) and (C) represent means ± SD of 15 measurements from five independent experiments; *p < 0.001, by Student's t test.

(D) Expression levels of gp91^{phox}, p67^{phox}, and p47^{phox} determined by western blotting in undifferentiated PLB-985 cells or those differentiated with DMF or vitamin D3 into granulocytic or monocytic cells, respectively. Note the lack of gp91^{phox}, but not p67^{phox} or p47^{phox}, in SIRPα-WT cells.

(E) Restoration of gp91^{phox} expression after reconstitution of gp91^{phox} in SIRPα-WT and X-CGD empty vector cells by retroviral transduction. The expression of gp91^{phox} before (left panel) and after (right panel) retroviral transduction was evaluated by flow cytometry after incubation with mAb 7D5 and goat-anti-mouse-IgG1 Alexa 633 antibody (solid histogram) or stained with isotype-matched antibody (open histogram).

(F) PMA-induced NADPH-oxidase activity in granulocytic SIRPα-WT and X-CGD empty vector cells (white bars) in which gp91^{phox} was reconstituted (black bars).

Data are presented as the mean ± SD of three independent experiments each performed in triplicate; *p < 0.001, by Student's t test. See also Figures S2 and S3.

Inhibition of the NADPH Oxidase Involves Signaling via the SIRPα Cytoplasmic ITIMs

The experiments described above suggested that direct signaling through the cytoplasmic tail is involved in the regulation of the NADPH oxidase and gp91^{phox} protein expression. The SIRPα cytoplasmic tail harbors ITIMs responsible for the recruitment of the cytosolic tyrosine phosphatases SHP-1 and SHP-2 (Fujioka et al., 1996). Studies with dominant-negative SHP-1 in myeloid cells (Dong et al., 1999) and with phagocytes from *motheaten* SHP-1-deficient mice (Kruger et al., 2000) demonstrated that at least SHP-1 acts as a negative regulator of the respiratory burst in myeloid cells. To investigate whether SHP-1 and/or SHP-2 recruitment by SIRPα plays a critical role in suppressing the respiratory burst by SIRPα, we mutated each of the four tyrosines from the SIRPα ITIMs, or combinations thereof, into phenylalanines and expressed the resultant proteins in PLB-985 cells. To evaluate the binding of SHP-1 and SHP-2 to the SIRPα mutants, we performed immunoprecipitation experiments. An analysis of the precipitates by western blotting demonstrated constitutive binding of SHP-1 and SHP-2 to SIRPα, and this was eliminated or at least strongly reduced by mutation of the ITIM tyrosines (Figure 3A). The same was observed in the reverse experiment, i.e., SHP-1 or SHP-2 immunoprecipitation followed by western blotting with SIRPα specific antibody (not shown). Mutation of all four ITIM tyrosines (SIRPα-Y1, SIRPα-Y2, SIRPα-Y3, and SIRPα-Y4F) completely restored the respiratory burst (Figure 3B) and gp91^{phox} protein expression (Figure 3C) to levels seen with the SIRPα-Δ87 cytoplasmic deletion mutant, suggesting that the ITIMs were responsible for the inhibitory activity. The level of inhibition obtained with the individual mutants correlated very well with their capacity to recruit SHP-1 and SHP-2. For instance, the membrane proximal Y1 appeared to be more important for the negative regulation of the NADPH oxidase than its membrane distal counterpart Y3.

To obtain insight into the level of gp91^{phox} regulation by SIRPα, we analyzed its mRNA levels by quantitative PCR (qPCR) in the various PLB-985 mutants. Similarly to the gp91^{phox} protein

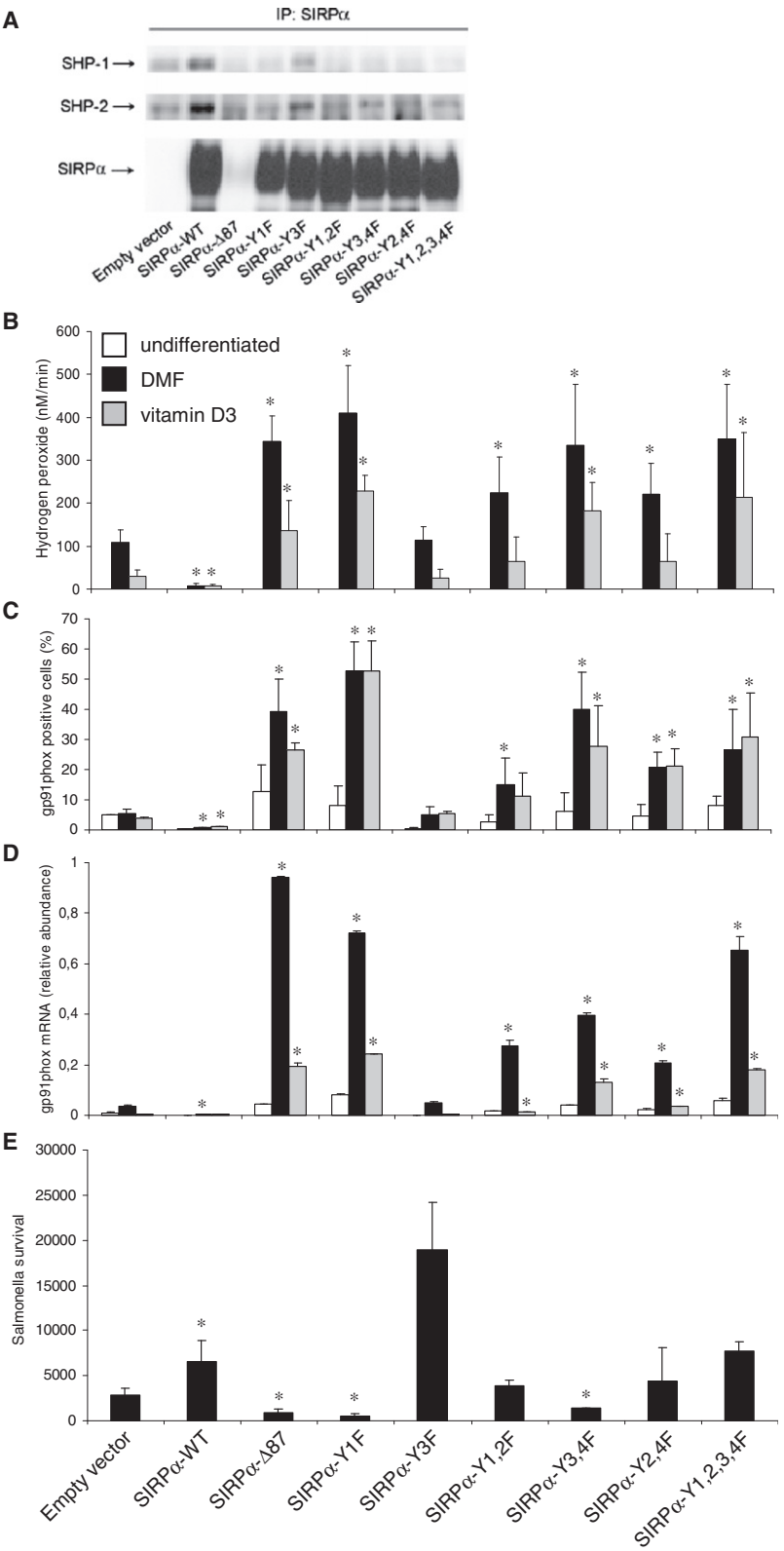

Figure 3. The Cytoplasmic ITIMs of SIRPα Are Required for Inhibition of the NADPH Oxidase

(A) SHP-1 and SHP-2 binding to SIRPα-WT, SIRPα-Δ87, and SIRPα tyrosine mutants was evaluated by immunoprecipitation of SIRPα, with mAb ED9 against rat SIRPα, and western blotting with antibodies against SHP-1, SHP-2, and SIRPα.

(B) PMA-induced NADPH oxidase activity in differentiated granulocytic or monocytic PLB-985. Data are presented as the mean ± SD of three independent experiments, each performed in triplicate.

(C) Expression of surface gp91^{phox} in differentiated granulocytic or monocytic, or undifferentiated PLB-985 cells analyzed by flow cytometry using 7D5 mAb. Data are presented as the mean ± SD of three independent experiments.

(D) mRNA level of gp91^{phox} in granulocytic or monocytic differentiated, or undifferentiated cells detected by RT-qPCR. Data are presented as the mean ± SD of two independent experiments, each performed in duplicate.

(E) Intracellular killing of *Salmonella* bacteria. The different cell lines were allowed to ingest *S. enterica* serovar Typhimurium 14028s, and the numbers of intracellular bacteria were determined 24 h after challenge. The levels of *Salmonella* uptake at the start of the experiment were comparable for all cells (not shown).

Data are presented as the mean ± SD of three independent experiments, each performed in triplicate; *p < 0.05, by Student's t test between the indicated conditions and the empty vector control. See also Figure S3.

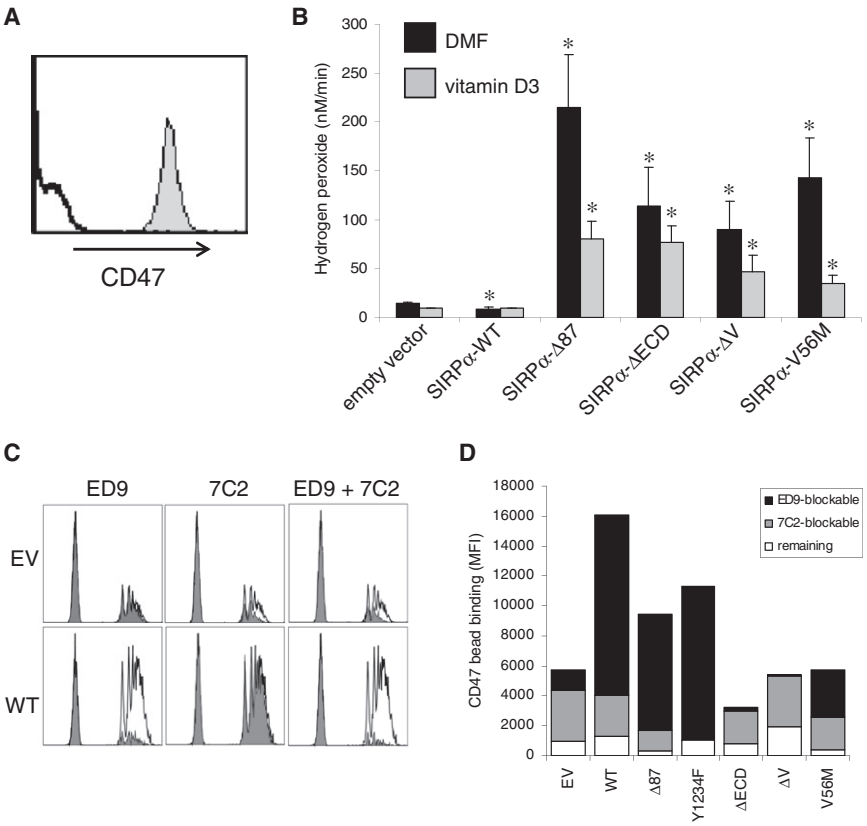

Figure 4. CD47-SIRPα Interactions Are Instrumental in Suppressing the Respiratory Burst by SIRPα

(A) CD47 expression on PLB-985 cells as demonstrated by flow cytometry with mAb B6H12.

(B) PMA-induced NADPH-oxidase activity in granulocytic (black bars) or monocytic (gray bars) PLB cells expressing each of the SIRPα extracellular mutants (SIRPα-ΔECD, SIRPα-ΔV, or SIRPα-V56M). Data are presented as the mean ± SD of three independent experiments, each performed in triplicate; *$p < 0.05$, by Student's t test between the indicated conditions and the empty vector control.

(C) Binding of fluorescent beads coated with human CD47-Fc protein to PLB-985 cells expressing rat-human chimeric SIRPα (WT) or empty vector (EV) cells. Histograms show the total CD47-bead binding (white area) and CD47-bead binding after preincubation of the cells with blocking mAb anti-rat SIRPα (ED9), anti-human SIRPα (7C2), or both (ED9 + 7C2) (grey area).

(D) CD47-bead binding to the indicated mutants. The bars show total binding in the absence of antibodies, as well as the effects of preincubation with anti-rat SIRPα mAb ED9 alone (ED9-blockable), the calculated difference between preincubation with both mAb ED9 plus the anti-human mAb 7C2 and preincubation with ED9 (7C2-blockable), and preincubation with both ED9 and 7C2 mAb (remaining).

See also Figure S4.

expression, mRNA levels were low in the cells that express the full-length SIRPα protein (Figure 3D), suggesting suppression of transcriptional activity of the gp91^{phox}/*CYBB* gene and/or increased mRNA turnover. There was a strong correlation among gp91^{phox} mRNA, protein, and NADPH oxidase activity.

Finally, to investigate whether SIRPα expression and signaling also affected the intracellular microbial killing, we evaluated the various PLB-985 mutants for their capacity to kill intracellular *Salmonella* bacteria. Enhanced *Salmonella* outgrowth was observed in the PLB-985 X-CGD cells (Figure S3C), directly indicating a role for the NADPH oxidase in *Salmonella* killing. There was a good inverse relation between NADPH oxidase activity and intracellular bacterial survival (Figure 3E). For instance, the overexpression of SIRPα-WT tended to enhance bacterial survival, whereas the SIRPα-Δ87 cytoplasmic deletion mutant resulted in lower bacterial survival compared with that observed with the empty vector cells. Also, the various Y mutants displayed a pattern of microbial survival generally corresponding to their NADPH oxidase capacity. The various mutants displayed similar levels of *Salmonella* uptake (Figure S3D).

Collectively, these results show that SIRPα signaling via the ITIMs negatively regulates the respiratory burst by controlling the expression of gp91^{phox}. It should be noted that we have not yet been able to characterize the relevant downstream signaling pathway(s). Of note, two transcription factor complexes, ICSBP and HoxA10, were previously shown to play a key role in the regulation of gp91^{phox} gene expression, and both of these complexes are also subject to regulation by SHP-1 and/or SHP-2 (Zhu et al., 2008; Lindsey et al., 2007; Kautz et al., 2001). Both complexes are obvious candidates to mediate the effects of SIRPα signaling on gp91^{phox} gene expression, but our analysis of their activity by electrophoretic mobility shift assay in the cell panel studied here did not provide any evidence for their involvement (J.A.Z., unpublished data). We are currently exploring alternative possibilities.

Inhibition of the NADPH Oxidase by SIRPα Involves CD47-SIRPα Interactions

SIRPα has been shown to interact via its N-terminal Ig-like domain with the broadly expressed CD47 molecule, and the molecular basis for CD47-SIRPα interactions has been established by mutagenesis and crystallography (Liu et al., 2007; Hatherley et al., 2008). SIRPα ligation by CD47 triggers SIRPα ITIM phosphorylation and SHP-1 and/or SHP-2 recruitment and signaling, which in turn regulates downstream cellular responses. As indicated above, the observation that SIRPα mutants, such as the SIRPα-Δ87 and several of the ITIM tyrosine mutants, display an enhanced oxidase activity and gp91^{phox} expression suggested a dominant-negative effect. We anticipated that the rat extracellular domain of the chimeric SIRPα molecule that was introduced into the PLB-985 cells would compete with the endogenous human SIRPα for CD47 binding, thereby reducing inhibitory signaling through the latter. Indeed, PLB-985 cells expressed CD47 on their surface (Figure 4A), and CD47 expression levels were not significantly affected by

expression of SIRPα or its mutants (Figure S4A). To directly address whether CD47-SIRPα interactions contribute to the inhibitory effect of SIRPα on the phagocyte respiratory burst, we constructed several SIRPα variants in which the extracellular ligand-binding domain was mutated. The mutants included deletions of the entire extracellular region (SIRPα-ΔECD), the N-terminal V-like Ig domain (SIRPα-ΔV), or a single point mutation V56M within the N-terminal V-like Ig region that was previously demonstrated to abolish CD47 binding (Liu et al., 2007). The resulting cell lines were analyzed by FACS with antibodies against rat and human SIRPα (Figure S4A) and western blotting (Figure S4B), and were found to express comparable surface levels of the various SIRPα molecules, with the expected sizes. In all of the cells with SIRPα extracellular domain mutations, the respiratory burst was strongly enhanced compared with empty vector controls, yielding activities close to those of the Δ87 mutant (Figure 4B). This suggested that similarly to the mutants that affected SIRPα signaling, the mutants that affected ligand binding were acting as dominant-negative molecules, and in addition were sequestering relevant downstream signaling molecules such as SHP-1 and/or SHP-2.

It was clearly important to demonstrate a direct interaction between the rat extracellular domains of our chimeric SIRPα constructs and human CD47. For this purpose, we generated a fusion protein of the extracellular domain of human CD47 and the Fc part of IgG1 (CD47-Fc), and developed a fluorescent bead assay to measure cellular CD47 binding. Analysis of the mutants demonstrated enhanced CD47 binding in SIRPα-WT cells as compared with empty vector cells (Figure 4C). This enhanced binding was prevented by blocking with the anti-rat SIRPα-specific mAb ED9, directly demonstrating that the chimeric rat-human SIRPα molecules are capable of binding human CD47. In addition, this analysis also demonstrated detectable CD47 binding by the endogenous human SIRPα that could be inhibited by the mAb 7C2. Analysis of the other mutants confirmed CD47 binding to SIRPα-WT, SIRPα-Δ87, SIRPα-Y1, SIRPα-Y2, SIRPα-Y3, and SIRPα-Y4 cells, but not to any of the SIRPα extracellular domain mutants (Figure 4D).

Collectively, these results indicate that CD47-SIRPα interactions contribute to the inhibitory activity of SIRPα on the respiratory burst. Clearly, we are formally unable to distinguish whether *cis* (i.e., on the same cell) and/or *trans* (i.e., between different cells) interactions are involved. However, considering the relatively low culture density of our cells and the low number of interactions between cells that occur during culture, we think it is most likely that the observed effects occur primarily as a result of *cis* interactions. However, it would seem that in the context of a hematopoietic tissue such as the bone marrow in vivo, where myeloid cells develop normally, the propensity for *trans* interactions would be much higher, and such interactions could also contribute to restricting gp91^{phox} expression.

Taken together, these results demonstrate that CD47-SIRPα interactions and ITIM-dependent downstream signaling via SIRPα control the magnitude of the phagocyte respiratory burst by regulating the expression levels of gp91^{phox}. We propose that CD47-SIRPα interactions participate in a homeostatic pathway that acts on developing phagocytes and functions to control excessive NADPH oxidase activity, and this serves to protect host cells and tissues against collateral oxidative damage during infection and other inflammatory conditions. Among the next challenges will be to provide insight into the mechanism(s) by which SIRPα-signaling regulates gp91^{phox} expression, and to establish a contribution of CD47-SIRPα-dependent regulation of the NADPH oxidase during inflammation and infection. The latter issue may not be straightforward to address, because other relevant processes, such as leukocyte transendothelial migration, may also be regulated by CD47 and SIRPα (Liu et al., 2002; de Vries et al., 2002).

EXPERIMENTAL PROCEDURES

Mice, Cells, and Culture

C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region were described previously (Yamao et al., 2002). PLB-985 and PLB-985 X-CGD cells (Zhen et al., 1993), kindly provided by Dr. M. Dinauer (Indianapolis, IN, USA), were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) at 37°C and 5% CO_2. For granulocytic differentiation, the cells were exposed to 0.65% (v/v) DMF (Sigma-Aldrich, St. Louis, MO, USA) for 5–6 days. Monocytic differentiation was achieved by culturing in the presence of 100 nM VitD3 (1α, 25-dihydroxyvitamin D3; Sigma-Aldrich) for 5–6 days.

Measurement of Respiratory Burst Activity

Activity of the respiratory burst after PMA (Sigma-Aldrich) or STZ (ICN Biochemicals, Cleveland, OH, USA) stimulation in transduced PLB-985 or PLB-985 X-CGD cells was measured with the Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) Hydrogen Peroxide Assay kit (Molecular Probes, Eugene, OR, USA) as previously described (Kuijpers et al., 2007). For information about antibodies, mice, isolation and culture of other cells, retroviral transductions, flow-cytometric analysis, qPCR, immunoprecipitation, western blotting, and intracellular killing of *Salmonella*, see Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.08.027.

LICENSING INFORMATION

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