The phagocyte inhibitory receptor sirpα in the immune system

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SIRPα CONTROLS THE ACTIVITY OF THE PHAGOCYTE NADPH OXIDASE BY RESTRICTING THE EXPRESSION OF gp91^PHOX
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 controlling its activity is essential. Little is known about the endogenous mechanism(s) that limit 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 ITIM motifs. Suppression of the respiratory burst by SIRPα was caused by a selective repression of gp91phox expression, the catalytic component of the phagocyte NADPH oxidase complex. Thus, SIRPα can limit gp91phox expression during myeloid development and thereby controls the magnitude of the respiratory burst in phagocytes.

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INTRODUCTION

One of the most important anti-microbial 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) that have a dysfunctional NADPH oxidase and as a result are hypersusceptible to a variety of bacterial and fungal infections(1;2). The phagocyte NADPH oxidase is a multi-subunit enzyme complex composed of: i) the membrane proteins, gp91phox (NOX2), the catalytic component of the oxidase, and p22phox, ii) the cytosolic proteins p40phox, p47phox, p67phox and iii) the small GTPase Rac. Activation of the oxidase involves translocation of the cytosolic subunits p40phox, p47phox, p67phox and Rac to the plasma membrane and assembly of the oxidase complex. Once assembled, NADPH oxidase generates superoxide (O$_2^-$) formation, by transferring electrons from NADPH in the cytosol over the plasmamembrane to molecular oxygen. Superoxide produced by the oxidase forms the basic compound from which other ROS, such as hydrogen peroxide (H$_2$O$_2$) and hypochlorous acid (HOCl), are formed. High concentrations of ROS are directly toxic to microbes, and perhaps also indirectly by the liberation of hydrolytic proteases(3).

The mechanisms of NADPH oxidase activation have been relatively well characterized, but essentially nothing is known on whether and how the magnitude of the respiratory burst is controlled. The latter is important since ROS do 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 amongst other things by the observation that there is only very little interindividual variation in the respiratory burst (Zhao et al, unpublished). 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. While the developmental pathways and transcription factors that trigger the expression of the different NADPH oxidase components during myeloid development have been established (4-6), putative regulatory mechanisms that counterbalance these and that prevent excessive potentially harmful expression of the various NADPH oxidase components within phagocytes have remained unknown.

SIRPα is a inhibitory immunoreceptor predominantly expressed on myeloid and neuronal cells (7;8). The cytoplasmic region of SIRPα contains four immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which upon ligand binding become phosphorylated, and mediate the recruitment and activation of the SH2-domain-containing 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 (9-14). SIRPα has been implicated in the regulation of a number of functions in myeloid cells (15;16). 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 (17;18). Based on this is appears that CD47 acts as a molecular signature of ‘self’ that by interacting with the ‘self sensor’ SIRPα on phagocytes and...
other myeloid cells limits immune-mediated damage against normal and healthy host cells during infection and inflammation. However, until now a direct involvement for CD47-SIRPα interactions in the regulation of inflammatory mediators and anti-microbial 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 gp91phox expression. This mechanism may help to 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 (18). Distinct bone marrow cell populations of SIRPα-mutant and control mice, including granulocytes, monocytes, immature myeloid cells, and lymphoid cells, were FACS-sorted using CD31 and Ly-6C as markers (Fig.1A) as described previously (19), and their PMA-induced respiratory burst was analyzed. As reported before there are no detectable differences in bone-marrow composition between the mutant and control mice, essentially excluding a prominent non-redundant role of SIRPα signaling in myeloid differentiation(20). We observed a significantly (50-75%) enhanced respiratory burst activity in granulocytes and monocytes from SIRPα-mutant mice in comparison to cells from wild-type mice (Fig.1B). A similar difference was seen in bone marrow-derived macrophages (Fig.1C). The respiratory burst in immature myeloid cells (Fig.1B) appeared unaffected. The production of other inflammatory mediators, including nitric oxide, TNFα, IL1β, IL6 and IL10, by bone marrow-derived macrophages in response to LPS was not significantly affected when comparing wild-type and SIRPα-mutant mice (Fig.2). 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 in contrast to previous reports (21;22), in which knock-down of SIRPα in macrophages was shown to enhance cytokine production in response to TLR ligands. One way to explain this apparent discrepancy is that inhibition of cytokine production can only be caused by a complete absence of SIRPα, but not by selective deletion of its cytoplasmic tail. A more trivial explanation could relate to differences in the method of interference. We have used macrophages from gene-targeted knock-out mice, whereas the other studies used shRNA- and siRNA-mediated knock-down, which could also have triggered macrophage danger pathways that may have contributed to the response(23). At least our current findings essentially exclude a regulatory role for SIRPα signaling in TLR-induced cytokine production and support the idea that SIRPα signaling is regulating 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 (24) and were found to express relatively low levels of endogenous SIRPα as shown by Western blotting (Fig. 3A) and flow cytometry (Fig. 4A). A chimeric rat-human SIRPα protein was expressed in PLB-985 cells because it would allow selective monitoring and manipulation by the agonistic mAb ED9, specifically directed against the rat SIRPα extracellular domain (8;25). PLB-985 cells, or mutants with a targeted mutation of the gp91phox gene (PLB-985 X-CGD) (24), were retrovirally transduced with full-length chimeric rat-human SIRPα protein (SIRPα-WT), or a SIRPα deletion mutant (SIRPα-Δ87) that is unable to

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. Sorted populations of (B) monocytes, granulocytes, lymphocytes and immature myeloid cells, or (C) cultured bone marrow-derived macrophages from wild-type (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 being performed in triplicate. Significance was determined by Student’s t-test. See also Figure 2.
signal because it lacks the cytoplasmic tail. Flow cytrometric analysis showed that the levels of SIRPα expression were comparable for the different cell lines generated (Fig.5A) and similar to those generally seen on rat myeloid cell lines or primary rat myeloid cells, such as macrophages or granulocytes (8) (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 (Fig.3A).

Figure 2. NO and Cytokine Production by SIRPα Mutant Macrophages. (A–E) Bone-marrow-derived macrophages from WT and SIRPα mutant macrophages were cultured in the presence of 100 ng/ml E. coli LPS, or a combination of 400 U/ml of IFNγ and LPS. NO production and cytokines were measured as described in Materials and Methods at 4 h (TNFα) or 20 h (NO, IL1β, IL6, and IL10). Data are presented as the mean ± SD of triplicate measurements; n.s., nonsignificant. Note that there are no detectable differences between WT and SIRPα mutant mice.
Figure 3. Properties of PLB-985 Cell Lines Expressing WT and Δ87 Versions of Chimeric Rat-Human SIRPα. (A) SIRPα expression in PLB-985 cell lines. SIRPα protein expression in PLB cells was evaluated by western blotting with an antibody directed against the cytoplasmic tail of SIRPα. Differential glycosylation allows the distinction between the chimeric rat-human exogenous and human endogenous SIRPα, which carry 14 and three potential N-glycosylation sites in their extracellular regions, respectively. The lack of antibody reactivity with the exogenous molecule in the case of the SIRPα-Δ87 cell lines in combination with anti-rat SIRPα reactivity detected by flow cytometry (Figure 2A) confirms the absence of the cytoplasmic tail for the truncated SIRPα. β-actin was used as a loading control. (B and C) SIRPα is a negative regulator of both PMA- and STZ-induced respiratory bursts. NADPH oxidase activity in the indicated PLB-985 cell lines induced with buffer (white bars), PMA (black bars), or STZ (gray bars) after 5–6 days of either granulocytic (B) or monocytic (C) differentiation. Values shown represent the mean ± SD of three independent experiments, each performed in triplicate; *p < 0.05, **p < 0.001, by Student’s t test.
**Figure 4.** Characterization of PLB-985 Cell Lines Expressing WT and Mutant Versions of Chimeric Rat-Human SIRPα. (A) Flow-cytometric analysis of the surface expression of the exogenous rat-human SIRPα (using anti-rat SIRPα mAb ED9), endogenous human SIRPα (with anti-human SIRPα/β mAb 7C2 and anti-human SIRPα1 mAb 1.23A), and CD47 (with mAb B6H12) on the indicated PLB-985 transfectants. All cell lines express comparable levels of exogenous and endogenous SIRPα molecules and CD47. Also note that staining with mAb ED9 is absent in the ΔECD and ΔV cells (but not V56M) cells, demonstrating that ED9 recognizes the N-terminal V-type of domain of rat SIRPα. (B) Expression of the SIRPα extracellular region mutants in PLB-985 cells evaluated by western blotting with an antibody against the SIRPα cytoplasmic domain. Differential glycosylation allows discrimination between the WT chimeric rat-human exogenous and human endogenous SIRPα.
The respiratory burst was studied 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 wild type SIRPα (Fig. 5B, C). 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 response that was considerably higher than that of the empty vector cells, suggesting that the mutant SIRPα protein acted as a dominant-negative protein, by competing e.g. with endogenous SIRPα for CD47 binding (see below). Importantly, all responses were entirely attributable to the gp91phox (NOX2)-containing phagocyte NADPH oxidase, as they were completely absent in the PLB-985 X-CGD cells, which have a targeted mutation of the gp91phox-encoding CYBB gene. Among several other stimuli of NADPH oxidase activation tested (i.e. serum-treated zymosan, fMLP and human IgG complexes) only serum-treated zymosan (STZ) generated a measurable response in the PLB-985 cells, and again this was completely abrogated by SIRPα-WT expression (Fig. 3B). This is consistent with a generalized effect of SIRPα on the NADPH oxidase.

**SIRPα selectively represses gp91phox expression during myeloid differentiation**

Activation of the multi-subunit NADPH oxidase complex requires assembly of its individual components, which are expressed during myeloid differentiation(1). 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 gp91phox, which forms the enzymatic core of the phagocyte NADPH oxidase, was evaluated in undifferentiated, and in granulocytic and monocytic PLB-985 cells by Western blotting. The differentiation-induced rise in gp91phox expression was completely absent in cells that express the full-length SIRPα protein (SIRPα-WT) (Fig. 5D). The same was observed when surface levels of gp91phox were analyzed by flow cytometry with 7D5 mAb (Fig. 6A). Also, the enhanced respiratory burst activity in cells that express the truncated receptor (SIRPα-Δ87) was associated with a higher gp91phox expression. Importantly, the levels of two of the other NADPH oxidase components, p67 and p47, remained unaffected by overexpression of full-length or truncated SIRPα (Fig. 5D), suggesting that SIRPα was selectively regulating gp91phox 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 SIRPα-WT or SIRPα-Δ87 introduction, 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 gp91phox around day 1-3 of neutrophilic differentiation (Fig. 6B).

To demonstrate that gp91phox was indeed the only relevant factor down-regulated by SIRPα, gp91phox was reconstituted by retroviral expression into SIRPα-WT cells (Fig. 5E) and this resulted in a full restoration of the respiratory burst (Fig. 5F). A similar restoration was observed when
Figure 5. SIRPα overexpression in PLB-985 cells inhibits the respiratory burst by repressing gp91phox expression. PLB-985 and gp91phox 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 (filled histograms). Unstained cells are indicated as control (open histogram). (B,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 vitamin D3, respectively. Values shown in panels B and C represent means ± SD of 15 measurements from 5 independent experiments. *, p < 0.001, by Student’s t test. (D) Expression levels of gp91phox, p67phox and p47phox 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 gp91phox, but not that of p67phox or p47phox, in SIRPα-WT cells. (E) Restoration of gp91phox expression after reconstitution of gp91phox in SIRPα-WT and X-CGD empty vector cells by retroviral transduction. The expression of gp91phox 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 gp91phox 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 3 and 6.
such reconstitution was performed in PLB-985 X-CGD cells. This shows that SIRPα suppresses phagocyte NADPH oxidase activity by a selective repression of gp91phox protein expression during myeloid differentiation.

Inhibition of the NADPH oxidase involves signaling via the SIRPα cytoplasmic ITIM motifs

The experiments described above suggested that direct signaling through the cytoplasmic tail is involved in the regulation of the NADPH oxidase and gp91phox protein expression. The SIRPα cytoplasmic tail harbors ITIM motifs responsible for the recruitment of the cytosolic tyrosine phosphatases SHP-1 and SHP-2 (Fujikawa et al. 1996; Kharitonenkov et al. 1997). Studies with dominant-negative SHP-1 in myeloid cells (26) and with phagocytes from motheaten SHP-1-deficient mice(27) 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 the suppression of the respiratory burst by SIRPα, each of the 4 tyrosines from the SIRPα ITIMs, or combinations thereof, were mutated into phenylalanines, and the resultant proteins were expressed in PLB-985 cells. To evaluate the binding of SHP-1 and SHP-2 to the SIRPα mutants we performed immunoprecipitation experiments. Analysis of the precipitates by Western blotting demonstrated constitutive binding of SHP-1 and SHP-2 to SIRPα and this was absent, or at least strongly reduced, by mutation of the ITIM tyrosines (Fig.7A). 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 4 ITIM tyrosines (SIRPα-Y1,2,3,4F) completely restored the respiratory burst (Fig.7B) and gp91phox protein expression (Fig.7C) 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 more important for the negative regulation of the NADPH oxidase than its membrane distal counterpart Y3.

To obtain insight into the level of gp91phox regulation by SIRPα, we analyzed its mRNA levels by qPCR in the various PLB-985 mutants. Similar to the gp91phox protein expression, mRNA levels were low in the cells that express the full-length SIRPα protein (Fig.7D), suggesting suppression of transcriptional activity of the gp91phox/CYBB gene and/or increased mRNA turnover. There was a strong correlation between gp91phox mRNA, protein and NADPH oxidase activity.

Finally, to investigate whether SIRPα expression and signaling also affected the intracellular microbial killing, the various PLB-985 mutants were evaluated for their capacity to kill intracellular Salmonella bacteria. Enhanced Salmonella outgrowth was observed in the PLB-985 X-CGD cells (Fig.6C) directly implicating a role for the NADPH oxidase in Salmonella killing. There was a good inverse relation between NADPH oxidase activity and intracellular bacterial survival (Fig. 7E). For instance, the overexpression of SIRPα-WT tended to enhance bacterial survival, whereas the SIRPα-Δ87 cytoplasmic deletion mutant resulted in bacterial survival lower than that observed in 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 (Fig.6D)
**Figure 6. Regulation of gp91phox Surface Expression and Salmonella Survival and Phagocytosis by SIRPα, and the Regulation of Endogenous SIRPα during Myeloid Differentiation.**

(A) SIRPα suppresses surface gp91phox expression. The indicated PLB-985 cell lines were differentiated for 6 days with either DMF or VitD3 into granulocytic or monocytic cells, respectively. Surface gp91phox levels were evaluated by flow cytometry with the 7D5 mAb. (B) Coordinated regulation of SIRPα and gp91phox expression during granulocytic differentiation of PLB985 cells. PLB985 cells were differentiated into granulocytic cells in the presence of DMF as described in Materials and Methods, and the expression of SIRPα and gp91phox was determined by western blotting (Bi). The fluorescent signal was quantified relative to lamin B (Bii), and for SIRPα1 by flow cytometry (Biii). Note that the upregulation of (surface) SIRPα coincides with that of gp91phox. (C) Salmonella killing by PLB-985 cells is dependent on the phagocyte NADPH oxidase. The PLB-985 and PLB-985 X-CGD cells were allowed to ingest S. enterica serovar Typhimurium 14028s, and the numbers of intracellular bacteria were determined 24 h after challenge. Data are presented as the mean ± SD of three measurements; *p < 0.05, by Student’s t test. (D) Salmonella binding and phagocytosis determined by PLB-985 cells expressing SIRPα mutants. The indicated PLB-985 cell lines were incubated with FITC-labeled Salmonella bacteria for the indicated periods, and binding and phagocytosis were quantified by flow cytometry.
Figure 7. The cytoplasmic ITIMs of SIRPα are required for the inhibition of the NADPH oxidase. (A) SHP-1 and SHP-2 binding to SIRPα-WT, SIRPα-Δ87 and SIRPα tyrosine mutants 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 gp91phox in differentiated granulocytic, 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 gp91phox in granulocytic, monocytic or undifferentiated cells detected by quantitative RT-PCR. 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 at 24h after challenge. The level 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 performed in triplicate. *, p < 0.05, by Student’s t test between the indicated conditions and the empty vector control. See also Figure 6.
Collectively, these results show that SIRPα signaling via the ITIMs negatively regulates the respiratory burst by controlling the expression of gp91phox. 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, have previously been shown to play a key role in the regulation of gp91phox gene expression, and both of these are also subject to regulation by SHP-1 and/or SHP-2(4-6). Both complexes constitute obvious candidates to mediate the effects of SIRPα signaling on gp91phox 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 (JAZ, unpublished). 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 (12;14). SIRPα ligation by CD47 triggers SIRPα ITIM phosphorylation, SHP-1 and/or SHP-2 recruitment and signaling, and this 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 gp91phox 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 express CD47 on their surface (Fig.8A) and CD47 expression levels were not significantly affected by expression of SIRPα or its mutants (Fig.4A). To directly address whether CD47-SIRPα interactions contribute to the inhibitory effect of SIRPα on the phagocyte respiratory burst, several SIRPα variants were constructed 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 (12). The resulting cell lines were analyzed by FACS with antibodies against rat and human SIRPα (Fig.4A) and Western blotting (Fig.4B) 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, as compared to empty vector controls, yielding activities close to those of the Δ87 mutant (Fig.8B). This suggested that similar to the mutants that affected SIRPα signaling, also the mutants that affect ligand binding were acting as dominant negative molecules, the latter likewise by 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 to empty vector cells
(Fig. 8C). 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 and SIRPα-Y1,2,3,4 cells, but not to any of the SIRPα extracellular domain mutants (Fig. 8D).

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 in trans- (i.e. between different cells) interactions

Figure 8. CD47-SIRPα interactions are instrumental in the suppression of 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 (grey 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 4.
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 of trans interactions would be much higher and these may be also contribute in restricting gp91phox 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 gp91phox. We propose that CD47-SIRPα interactions participate in a homeostatic pathway acting on developing phagocytes that functions to control excessive NADPH oxidase activity, and this is anticipated to serve in the protection of 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 gp91phox expression, and to establish a contribution of CD47-SIRPα-dependent regulation of the NADPH oxidase during inflammation and infection. The latter question may perhaps not be straightforward to answer, because other relevant processes, such as e.g. leukocyte transendothelial migration, might also be regulated by CD47 and SIRPα as well (28,29).

**EXPERIMENTAL PROCEDURES**

**Antibodies**

The following mAb were used in this study: ED9, mouse IgG1 directed against rat SIRPα (unconjugated or Alexa 633-labelled); 7C2, mouse IgG1 directed against human SIRPα and β, which blocks interactions between SIRPα and CD47 (Santa Cruz, CA, USA); 1.23A, mouse IgG1 directed against human SIRPα (29); 7D5, mouse IgG1 directed against human gp91phox, a kind gift of Dr. M. Nakamura (Nakasaki, Japan); B6H12, mouse IgG1 against human CD47 (kindly provided by Dr. E. Brown (San Fransisco, CA, USA); ED2, mouse IgG1 against rat CD163 (isotype control); FITC-conjugated ER-MP20, rat IgG2a against Ly-6C, and biotinylated ER-MP12, rat IgG2a against CD31, were generously provided by Dr. P. Leenen (Rotterdam, The Netherlands). Rabbit polyclonal Ab8120 (Abcam, Cambridge, United Kingdom) is directed against the cytoplasmic tail of human SIRPα. SH-PTP1, rabbit polyclonal antibodies against SHP-1 and lamain B, mouse monoclonal IgG1 directed against SHP-2, were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal antisera raised against recombinant human p47phox and p67phox were kindly provided by Dr. William Nauseef, (Iowa City, IA, USA). Alexa 633 goat-anti-mouse IgG (Molecular Probes, Eugene, OR, USA) and Alexa 633 streptavidin conjugate (Molecular Probes) were used as conjugates.

**Mice and isolation of cell populations**

C57BL/6 mice with a targeted deletion of the SIRPα cytoplasmic region have been described previously (18). The mice that were originally generated onto the 129/Sv background had been backcrossed onto the C57BL/6 mice for ten generations. Wild-type C57BL/6 mice of the same genetic background were maintained together with the SIRPα-mutant mice in the breeding facility of the VU Medical Center,
Amsterdam. All mice were specified pathogen free. Permission for animal experiments described here was obtained from the Animal Welfare committee of the Vrije Universiteit in Amsterdam.

Six- to eight-week-old SIRPα-mutant and wild-type mice were killed with a lethal intraperitoneal injection of Euthesate (8 mg sodium pentobarbital per mouse; Sanoﬁ Santé Animale Benelux B.V., Maassluis, The Netherlands). Tibiae were removed and cleaned of soft tissue and ground in a mortar with culture medium (α-Minimal Essential Medium (Gibco) supplemented with 5% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin b (Antibiotic antimyotic solution, Sigma-Aldrich) and heparin (170 IE/ml; Leo Pharmaceutical Products B.V., Weesp, The Netherlands) as described (20). The cell suspension was aspirated through a 21-gauge needle and filtered over a 100-µm pore size Cell Strainer filter (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA). Cells were washed twice in culture medium.

For FACS sorting of distinct bone marrow cell populations, including granulocytes, monocytes, myeloid cells and lymphoid cells, bone marrow cells were labeled with ER-MP12bio and ER-MP20FITC. After 1 hour incubation at 4°C, cells were washed three times with PBS-0,1% (v/v) BSA. ER-MP12 binding was detected with Alexa 633-streptavidin conjugate (Molecular Probes) for 30 min at 4°C. After three washes with PBS-0,1% (v/v) BSA, cells were analyzed and FACS sorted (FACS Star Plus Becton Dickinson), and subsequently used to measure the respiratory burst activity as described below. Purity of the cell populations was checked by May-Grünwald/Giemsa staining and was comparable to that described previously(31).

To obtain bone marrow-derived macrophages the freshly isolated bone-marrow cells were prepared as described above and were resuspended in RPMI-1640 containing 15% (v/v) L929-cell conditioned medium (as a source of CSF-1), 10% (v/v) fetal bovine serum, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cell suspension from one mouse was distributed evenly over two bacterial plastic Petri dishes. The plates were incubated at 37°C in 5% CO₂. Every 3 days medium was removed and replaced with fresh medium. After seven days of culturing cells were used to measure the respiratory burst activity as described above. Where indicated mouse bone-marrow-derived macrophages were cultured O/N at 0,3x10⁶/ml in complete medium containing 100 ng/ml LPS from E.coli (Sigma) and/or 400 U/ml IFNγ.

PLB-985 and PLB-985 X-CGD cells (24), 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% CO₂. For granulocytic differentiation, the cells were exposed to 0,65% (v/v) dimethylformamide (Sigma-Aldrich, St Louis, MO, USA) for 5 to 6 days. Monocytic differentiation was achieved by culturing in the presence of 100 nM vitamin D3 (1alpha, 25-dihydroxyvitamin D3, Sigma-Aldrich) for 5 to 6 days.

Measurement of respiratory burst activity
Activity of the respiratory burst after phorbol myristate acetate (PMA, Sigma-Aldrich) or serum-treated zymosan (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 described (32). For information on antibodies, mice, isolation and culture of other cells,
retroviral transductions, flow cytometric analysis, quantitative PCR, immunoprecipitation and Western blotting, and intracellular killing of *Salmonella* see extended experimental methods.

**Measurement of inflammatory mediators**

Culture supernatants were harvested and NO production was measured with the Giess assay as described before (25). Cytokines were measured by ELISA (R&D systems) according to instructions by the manufacturer.

**Retroviral vectors and transductions**

A rat-human SIRPα (SIRPα-WT) fusion construct was generated from cDNA and PCR fragments as follows: nt 1-1236 (count starts at first methionine) of the rat SIRPα cDNA(8) was fused to nt 1230-1509 of the human cDNA (prot. accession No NM_080792). The chimeric SIRPα protein contains amino acids 1-412 of rat and amino acids 411-503 of human SIRPα, resulting in a total length of 505 amino acids, including the signal sequence. The deletion construct SIRPα-delta87 (SIRPα-Δ87) was constructed by PCR from the rat-human fusion cDNA (Table 1). The amplified fragment was cloned into pcDNA3 (Invitrogen, Breda, The Netherlands) downstream of the BamH1-XhoI of the rat SIRPα cDNA.

The SIRPα tyrosine mutants were generated by PCR mutagenesis (Quikchange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA) (Table 1). The tyrosine → phenylalanine point mutations were introduced at positions Y430, Y454, Y471 and Y497 (representing the tyrosines of the four ITIMs of SIRPα and here termed Y1,2,3 and 4, respectively) of the human SIRPα-WT protein. The extracellular mutants, SIRPα-ΔV and SIRPα-ΔECD, were generated by the introduction of a Apal restriction site at aa 26 and 156 for the ΔV mutant and a XhoI restriction site at aa 26 and 351 for the ΔECD mutant. These restriction sites were generated by PCR mutagenesis. In the SIRPα extracellular mutant, SIRPα-V56M, a valine → methionine point mutation at position aa 56 was generated. The sequence of all constructs was confirmed by DNA sequencing.

For retroviral transduction, SIRPα-WT, the deletion mutant SIRPα-Δ87, the SIRPα extracellular domain mutants and SIRPα tyrosine mutants were cloned into the retroviral expression vector pLZRspMBN-linker-IRES-EGFP(NotI-)(33), which was obtained from Dr. H Spits (Amsterdam, The Netherlands).

The phoenix-A packing cell line(34), provided by Dr. G. Nolan (Stanford University, Stanford, CA, USA) was transfected, using calcium phosphate, with one of the different retroviral construct containing the SIRPα-WT, SIRPα-Δ87, SIRPα extracellular domain mutants, SIRPα tyrosine mutants or control construct containing no SIRPα insert. After selection of transfected cells with puromycin (1 µg/ml) (Sigma-Aldrich), virus was harvested as previously described (33) and used for retroviral transduction of PLB-985 and PLB-985 X-CGD cells. The transduced eGFP-positive cells were subsequently selected by FACS sorting (FACS Star Plus, Becton Dickinson). Cell surface expression of SIRPα was determined by FACS analysis with ED9 mAb as described below. FACS-sorted PLB-985 and PLB-985 X-CGD cells expressing eGFP only (termed empty vector) were used as a control in subsequent experiments. Expression of SIRPα and/or EGFP was tested regularly (i.e. at least once a month), and in the experiments described the proportion of cells expressing SIRPα exceeded 90%.
The retroviral expression vector LZRS-IRES-GFP with gp91phox was generated in our laboratory. PLB-985-SIRPα-WT and PLB-985 X-CGD empty vector cells were retrovirally transduced with this construct as described above. Cell surface expression of gp91phox was determined by flow cytometric analysis with 7DS mAb as described below.

**Flow cytometric analysis**

For flow cytometric analysis, cells of the PLB cell line panel were resuspended in PBS-0.1% BSA and stained with saturating concentrations of ED9-Alexa 633, 7DS, 7C2, 1.23A and/or B6H12-PE mAb. After 1 hour incubation at 4°C, cells were washed three times with PBS-0.1% BSA. 7DS, 7C2 or 1.23A binding was detected with goat-anti-mouse-IgG Alexa 633, for 30 min at 4°C. After three washes with PBS-0.1%B5A, cells were analyzed on a FACS Calibur (BD Biosciences, San Jose, CA, USA).

For the CD47-beads generation a fusion protein containing the extracellular domain of CD47 and a human Fc tail (CD47-Fc) was generated. The extracellular domain of human CD47(35) was amplified using the forward 5’-AGA TCG ATA TCC CAG CTA CTA TTT AAT AAA ACA AAA TC-3’ and reverse 5’-GAG ATC AGA TCT AAA CCA TGA AAC AAC ACG ATA TTT TAG-3’ and subcloned using EcoRV and Bgl-II into the pFUSE-hIgG1-Fc2 vector. For expression of the fusion protein Freestyle HEK cells (Invitrogen, Breda, The Netherlands) were co-transfected with the plasmid and p21, p27 and pSVLT. After 6 days the supernatant was collected and the CD47-Fc protein was purified on a protein G-sepharose beads column (Thermo Scientific, Breda, The Netherlands). The human CD47-Fc fusion protein was bound to streptavidin-coupled fluorescent beads. For the coupling to the beads, 20 µl goat-anti human Fc-biotin (0,5 mg/ml; Jackson Immuno Research Code 109-066-098) was added to a mix of 15 µl of streptavidin-coupled beads (6,7x10^6 beads/µl) and 350 µl PBS with 0.5% BSA and 0.02% azide (PBA). This was incubated for 2 hours at 37°C under shaking after which the beads were centrifuged 20,000g at 4°C for 2 minutes and washed twice with 500 µl PBA and resuspended in 100 µl PBA. 0.5 µl of the purified CD47-Fc protein in 300 µl PBA was added and this was incubated rotating overnight at 4°C. The beads with the coupled fusion protein were centrifuged and washed twice and resuspended in 100 µl PBA and stored at 4°C for a maximum of 6 months. For the beads binding assay 5x10^4 cells were incubated with 2 µl CD47-beads in 40 µl PBS/0.1%BSA at 37°C for 45 min. Cells and beads were then washed once and measured by FACS.

**Quantitative RT-PCR**

mRNA was isolated from monocytic, granulocytic or undifferentiated PLB-985 cells with an RNA isolation kit (Qiagen, Hilden, Germany). cDNA was prepared with the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer’s instruction. Primers used for gp91phox (CYBB gene) expression analysis were 5’-TGC AGA TCT GCC TCT CTT TTG TTG TTT CAG GCC TGT C-3’ and reverse 5’-CAA AGT CTT TTG TTT CAG GCC TGT C-3’. Primers 5’-AAA TAT GTG GTT GGA GAG CTC ATT-3’ and 5’-CCGAGTGAAGATCCCCTTTTA-3’ were used for β-glucuronidase (GUS), as a control. Amplification by PCR was performed on a LightCycler instrument (Roche, Almere, The Netherlands). All primer pairs used in qPCR amplified a single band with expected molecular weight as analyzed by agarose gel electrophoresis. The gp91phox (CYBB gene) band was sequenced and showed 100% homology to the published human CYBB sequence (Genebank acc. No NM_000397).
Immunoprecipitation and Western blot analysis

For immunoprecipitation of SIRPα, 1.5x10⁶ PLB-985 cells were washed twice in PBS and centrifuged, and the cell pellet was lysed with Igepal lysis buffer (Sigma-Aldrich) containing 1 mM Na₃VO₄ and protease inhibitor cocktail (Roche) for 1 hour at 4ºC. The lysates were clarified by centrifugation and precleared by incubation with protein A-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) for 1 hour. SIRPα was immunoprecipitated by addition of anti-SIRPα (ED9 mAb) bound to protein A beads for 1 hour at 4ºC. SHP-1 or SHP-2 were immunoprecipitated by addition of anti-SHP-1/2 (Santa Cruz Biotechnology). Beads were washed with Igepal lysis buffer containing 1 mM Na₃VO₄ and protease inhibitor cocktail and resuspended in SDS sample buffer. Western blot analysis was performed with anti-SHP-1/2 (Santa Cruz Biotechnology) and anti-SIRPα antibodies. The cellular expression level of gp91phox, p67phox and p47phox in granulocytic, monocytic or undifferentiated PLB-985 cells was determined by Western blot with mAb 48 (anti-gp91phox) and rabbit polyclonal antisera raised against p47phox and p67phox. Appropriate HRP- or IRDye®-conjugated secondary antibodies were used to visualize the proteins and the signal was detected with respectively Supersignal West Dura (Pierce, Rockford, IL, USA) and a Gelimag (Epi Chemi II Darkroom combined with a 12-bit SensiCam charged-coupled device camera driven by Labworks 4.0 (UVP, Inc., Upland, CA, USA), or by Odyssey (Li-Cor Biosciences).

Intracellular killing of Salmonella

Single colonies of Salmonella enterica serovar Typhimurium 14028s, obtained from Dr. A.M. van der Sar (Vrije Universiteit, Amsterdam, The Netherlands), were grown overnight in Luria-Bertani (LB) medium at 37ºC while being shaken (225 rpm). The CFU in the inoculum were determined by plating serial dilutions. PLB cell line cells were exposed to 0.65% DMF for granulocytic differentiation as described above. After 6 days, cells were washed with RPMI-1640 containing 10% (v/v) FCS and 1 mM glutamine, and cells (2*10⁵) were then incubated with S. enterica serovar Typhimurium 14028s at a 10:1 concentration of multiplicity of infection in Falcon round-bottom tubes (Becton Dickinson, Meylan Cedex, France) while rotating for 30 min at 37ºC, 5% CO₂. The cells were then washed with PBS and incubated for 1 h in medium supplemented with 100 µg of gentamicin/ml to kill extracellular bacteria, and washed again. This time point is designated time zero. Medium supplemented with 10 µg of gentamicin/ml was added to the cells to kill any remaining extracellular bacteria and to prevent reinfection. At 24 h, the cells were washed with PBS and lysed in milliQ. Serial dilutions of the lysate were plated for determination of the number of intracellular CFU. Numbers of phagocytosed bacteria were determined by sampling at time zero and were found to be similar for all cells tested. In addition, binding and phagocytosis was evaluated by incubation of cells with FITC-labelled Salmonella bacteria, after which binding and uptake was determined by flow cytometry and confocal microscopy.
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REFERENCES


