Exocytosis acts as a modulator of the ILT4-mediated inhibition of neutrophil functions

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Neutrophils play a major role in inflammatory responses and immune defense against pathogens. Even though expression of inhibitory receptors has been reported on neutrophils, their role remains poorly defined. Here we show that primary human neutrophils expressed immunoglobulin-like transcript 4 (ILT4) inhibitory receptor and that this expression was induced during differentiation of the myelomonoblast PLB-985 cell line into "neutrophil-like" cells. Functional assays indicated that human leukocyte antigen G, the preferred ligand of ILT4, inhibited the phagocytic function of neutrophils. ILT4 engagement also impaired reactive oxygen species production induced through CD32a and both receptors were found colocalized into neutrophil lipid rafts. Moreover, neutrophil degranulation induced through inflammatory stimuli increased ILT4 expression as a result of the rapid translocation of an intracellular pool to the cell surface. Consequently to this ILT4 up-regulation, the human leukocyte antigen G-mediated inhibition of neutrophil phagocytic function was enhanced. Finally, we found that ILT4 up-regulation induced on healthy donor neutrophils following stimulation was impaired in presence of plasma from patients with sepsis. Similarly, ILT4 up-regulation was inhibited in neutrophils from septic patients. Altogether, our results reveal a unique mechanism of regulation of neutrophil functions through ILT4 and its exocytosis that may have implications in inflammatory disorders.

granulocytes | LILRB2 | HLA-G | inflammation

Neutrophils constitute more than 50% of circulating leukocytes in humans and play a major role in host defense against invading pathogens. The release of chemotactic signals from inflamed or infected tissues triggers neutrophil migration from the bloodstream to inflammatory foci (1). They thus recognize pathogens through pattern recognition receptors or Fc gamma receptors (FcγR) such as CD32a (FcγRIIa) when microbes have previously been opsonized with immunoglobulins (Ig) (2). Pathogens are then engulfed by phagocytosis and killed through fusion with neutrophil intracellular toxic granules and reactive oxygen species (ROS) production (3). The release of toxic granules by exocytosis constitutes another function of neutrophils that is critical for their antimicrobial activity. Three granule subsets have been characterized: gelatinase, specific, and azurophil granules. They enclose proteolytic enzymes and antimicrobial peptides in their luminal space whereas their membranes contain receptors. Neutrophils also have secretory vesicles, carrying mainly receptors, which fuse easily with the plasma membrane upon stimulation. Granule subset release depends on stimulus strength and follows a hierarchical order of mobilization. The most easily mobilized are secretory vesicles, followed by gelatinase, specific, and, to a lesser extent, azurophil granules (4).

Neutrophils have a short life span and undergo apoptosis during the resolution of inflammation (5). Even though this process has been considered as the main regulator of their activity, growing evidence indicates that inhibitory receptors are also involved in the control of neutrophil function preventing exacerbated responses and tissue damages (6). In this regard, we focused on the Ig-like transcript 4 (ILT4/LILRB2/CD85d) inhibitory receptor, which is composed of four extracellular Ig-like domains and three immuno-receptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic part (7). ILT4 interacts with several HLA class I molecules but binds with the highest affinity the nonclassical HLA class I molecule HLA-G (8). The triggering of ILT4 leads to ITIM motifs phosphorylation, which then become docking sites for SH2 domain-containing tyrosine phosphatases (SHP)-1 and SHP-2. Once recruited, these phosphatases can in turn deactivate activatory cascades. The interaction of ILT4 with HLA-G has been shown to inhibit the differentiation of monocytes into dendritic cells, suggesting a role of this receptor in the attenuation of inflammatory responses (9, 10).

Sepsis is a systemic inflammatory syndrome developed during infection and may lead to remote noninfected organs failure (11). Septic shock remains the main cause of death in intensive care medicine (12). Recent studies indicate that neutrophil survival and migration to infectious sites are dysregulated in septic conditions (13, 14). Moreover, exacerbated activity of neutrophils within tissues contributes to severe organ dysfunctions

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Significance

Neutrophils are key components of inflammatory responses and immune defense against pathogens. Neutrophil functions are tightly controlled by surface receptors. Our study shows that the engagement of immunoglobulin-like transcript 4 (ILT4) inhibitory receptor impairs phagocytosis and respiratory burst of neutrophils. Moreover, we found that following induction of neutrophil granule exocytosis, ILT4 expression increases as a result of the rapid translocation of an intracellular pool to the cell surface. This increase of ILT4 expression enhances the ILT4-mediated inhibition of neutrophil activity. Finally, ILT4 up-regulation on neutrophils is impaired in sepsis patients. These results reveal a unique mechanism of regulation of neutrophil functions through ILT4 and its exocytosis that may have implications in inflammatory disorders and immune responses.

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associated with poor prognosis (15). Although these studies suggest that modulation of neutrophil activity could be beneficial for septic patients, further investigations on the molecules involved in the control of neutrophil activation are required.

In this study, we report that ILT4 engagement inhibits neutrophil phagocytic function and ROS production. We also show that an intracellular pool of ILT4 is rapidly mobilized to the neutrophil surface following stimulation. This increase enhances inhibition of neutrophil phagocytosis, notably upon engagement with HLA-G. Furthermore, our study indicates that ILT4 mobilization on neutrophil surface is dysregulated in the context of human sepsis. These findings demonstrate that ILT4 is a potent regulator of neutrophil activity that might be considered as a therapeutic target to prevent neutrophil dysfunctions observed during inflammatory disorders.

Results

ILT4 is Expressed on Human Neutrophils and Differentiated Neutrophil-Like PLB-985 Cells. It is well established that ILT4 is expressed on myeloid but not on lymphoid cells. However, its expression on neutrophils remains poorly characterized (7). Thus, we analyzed ILT4 expression on neutrophils from healthy donors. As shown in Fig. 1A and Table 1, neutrophils, characterized by the expression of CD11b, CD15, and CD66b, always showed significant surface expression of ILT4 (68.8 ± 19.1%). Confocal microscopy analysis further confirmed the circular distribution of ILT4 on neutrophil surface (Fig. 1B). Because the preferred ligand for ILT4 is HLA-G, the expression of ILT2 and KIR2DL4, the

Table 1. Cell surface expression of ILT4, ILT2, and KIR2DL4 receptors for HLA-G on human peripheral blood neutrophils

Receptor	Percentage mean ± SD, %	n
ILT4	68.8 ± 19.1	75
ILT2	0.3 ± 0.7	13
KIR2DL4	0.7 ± 0.9	5

Percentages of positive neutrophils are presented as mean ± SD; n represents the number of independent experiments.

two other well-known receptors for HLA-G, was also assessed. As shown in Table 1 and Fig. 1A, neither ILT2 nor KIR2DL4 were expressed on neutrophils.

The myelomonoblast PLB-985 cell line is considered as an appropriate model to generate neutrophil-like cells upon differentiation with Dimethylformamide (DMF) or Dimethylsulfoxide (DMSO) treatment. As shown in Fig. 1C, most of undifferentiated PLB-985 cells did not express ILT4 on their surface. However, ILT4 expression was detected on neutrophil-like cells after treatment with DMF or DMSO (70.05 ± 1.55% and 88.10 ± 1.27%, respectively, at day 5, vs. 0.78 ± 0.45% at day 0; P < 0.0001). The expression level of CD11b was used as a control of differentiation efficiency. Finally, ILT4 or CD11b surface expression analysis throughout time showed that the acquisition of their expression was progressive during PLB-985 cell differentiation (Fig. 1D).

These results show that ILT4 is the only known receptor for HLA-G expressed on human neutrophils and that this expression is induced during neutrophil differentiation.
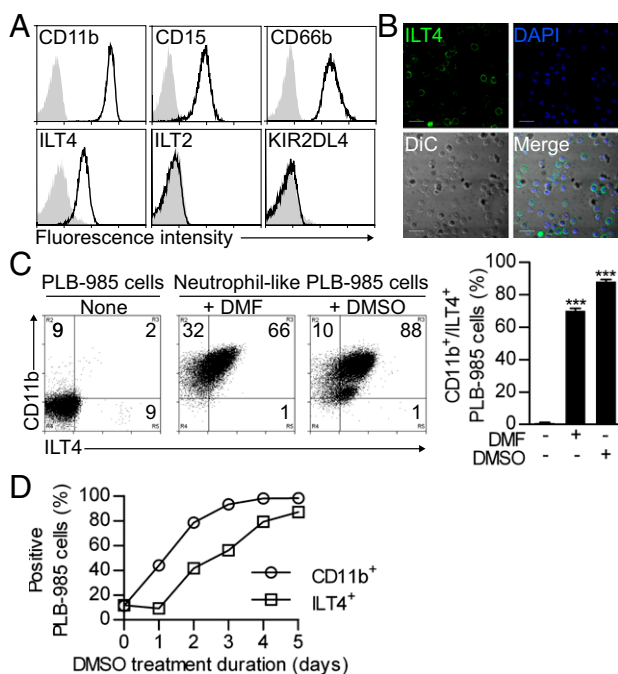
The Phagocytic Function of Neutrophils Is Inhibited by HLA-G. Having shown that ILT4 was the only receptor for HLA-G expressed on neutrophils, we next investigated the role of ILT4/HLA-G interaction on neutrophil phagocytic function. For this purpose, we used soluble HLA-G precoated on nanobeads (Fig. 2A) to mimic HLA-G multimerization, which is required to efficiently bind ILT4 and induce inhibitory signals (16). Neutrophils were coincubated with opsonized fluorescent *Escherichia coli*, and phagocytosis was measured by flow cytometry. As shown in Fig. 2 B and C, control nanobeads had no effect on neutrophil phagocytic function. By contrast, in presence of HLA-G, the capacity of neutrophils to phagocyte opsonized *E. coli* was highly inhibited (mean 3.80%, range 0–10.53% with HLA-G5 nanobeads vs. mean 13.90%, range 2.47–35.62% with control nanobeads; P < 0.0001). Since neutrophil-like cells expressed CD32a (Fig. 2D), we measured the impact of HLA-G on their phagocytic function. In agreement with data obtained on primary neutrophils, the phagocytic function of neutrophil-like cells was inhibited in the presence of HLA-G (Fig. 2E). In addition, flow cytometry analysis indicated that only ILT4 expression was induced on neutrophil-like cells, ruling out the implication of ILT2 or KIR2DL4 on the HLA-G−mediated inhibition of neutrophil-like PLB-985 cells phagocytosis (Fig. S1).

These results show that the phagocytic function of neutrophils is inhibited by HLA-G probably by interacting with ILT4.

ILT4 Ligation Inhibits the CD32a-Mediated Production of ROS, and both ILT4 and CD32a Colocalize Within Neutrophil Lipid Rafts. Production of ROS is one of the earliest events induced in neutrophils upon recognition of opsonized pathogens through FcγR receptors (17). As shown in Fig. 3 A and B, stimulation of neutrophils through CD32a induced ROS production which was abrogated in presence of NADPH oxidase inhibitor diphenyleneiodonium (DPI) (7.67 ± 2.46% vs. DMSO 79.34 ± 4.73% of control anti-CD32a + isotype alone; P = 0.0286). When CD32a and ILT4 were simultaneously engaged, the production of ROS was also inhibited (70.67 ± 13.09% of control; P = 0.0042). Similar results were obtained using luminescence technique (Fig. S2 A and B).

Assessment of ILT4 localization during neutrophil activation showed that stimulation through CD32a induced the colocalization of CD32a and ILT4 within lipid rafts (Fig. 3C).

Fig. 1. ILT4 inhibitory receptor is expressed on peripheral blood neutrophils and neutrophil-like PLB-985 cells. (A) Flow cytometry analysis of CD11b, CD15, and CD66b markers, and ILT2, ILT4, and KIR2DL4 inhibitory receptors (open histograms) expression on neutrophils. Gray-filled histograms represent isotype controls. Data are representative of at least five experiments. (B) ILT4 expression on neutrophils by confocal microscopy. Neutrophils were stained for ILT4 (green) and nucleus (DAPI, blue). (Scale bars: 20 μm.) Images are representative of three independent experiments. (C) CD11b and ILT4 expression on undifferentiated, DMF-, and DMSO-differentiated PLB-985 cells. Percentages of positive PLB-985 cells for CD11b or ILT4 or CD11b and ILT4 are presented in each quadrant. Histograms on the right represent percentages of CD11b⁺/ILT4⁺ PLB-985 cells (n = 4). (D) CD11b or ILT4 surface expression during 5 d of differentiation of PLB-985 cells with DMSO. Curves are representative of three independent experiments. ***P < 0.001.
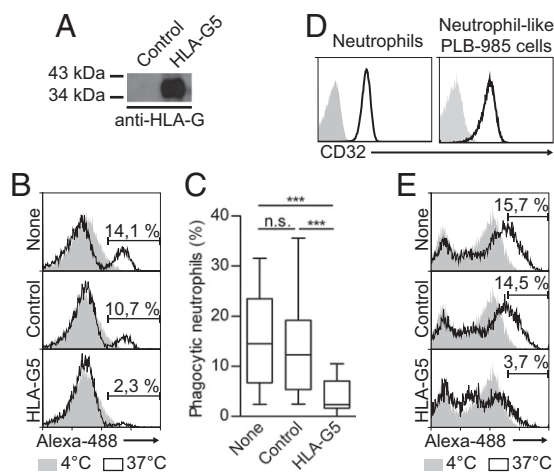
Fig. 2. ILT4 inhibitory receptor ligand HLA-G inhibits the phagocytic function of neutrophils. (*A*) Western blot of HLA-G5-coated nanobeads. (*B* and *C*) Phagocytosis of fluorescent *E. coli* by neutrophils. In *B*, percentage of fluorescent neutrophils at 37 °C was compared with the 4 °C control. Pretreatment with control or HLA-G5 nanobeads and percentages of fluorescent neutrophils are indicated. *C* shows percentage of fluorescent neutrophils from 17 independent experiments. (*D*) Surface CD32 expression (open histograms) on neutrophils and neutrophil-like cells. Gray-filled histograms represent isotype controls. Histograms are representative of three independent experiments. (*E*) Phagocytosis of fluorescent *E. coli* by neutrophil-like cells. Percentage of fluorescent neutrophil-like cells at 37 °C was compared with the 4 °C control. Pretreatment with control or HLA-G5 nanobeads and percentages of fluorescent neutrophil-like cells are indicated. Histograms are representative of three independent experiments. ***P < 0.001.

These results indicate that ILT4 inhibits CD32a-mediated production of ROS and colocalizes with CD32a within lipid rafts of activated neutrophils.

ILT4 Is Rapidly Mobilized on Cell Surface Through Granule Exocytosis Following Neutrophil Stimulation. Several membrane receptors are contained in neutrophil granules and released on cell surface upon exocytosis. Similarly, Fig. 4*A* shows that a granular expression of ILT4 was found inside neutrophils. We then assessed if stimulation of neutrophil could induce the mobilization of this intracellular pool to the cell surface. Fig. 4*B* indicates that, after neutrophil stimulation with either N-formyl-methionyl-leucyl phenylalanine (fMLF), LPS, or TNFα, the surface expression level of ILT4 was increased. The transmembrane molecule CD11b, which is contained in neutrophil granules, was used as control (Fig. 4*B*). The increase of ILT4 expression was also induced on neutrophil-like cells following stimulation with fMLF (Fig. S3).

Comparative analysis of numerous donors showed that neutrophil activation with fMLF induced ILT4 expression [Mean Fluorescence Intensity (MFI) 8.13 ± 0.69 vs. 5.35 ± 0.41 mock-treated; *P* < 0.0001; Fig. 4*C*] with a similar extent to CD11b (MFI 141.70 ± 18.89 vs. 72.82 ± 6.75 mock-treated; *P* < 0.0001). Moreover, increase of ILT4 and CD11b levels on neutrophil surface was already detected from 5 min stimulation and reached a plateau after 15 min (Fig. 4*D*).

Neutrophil actin cytoskeleton forms a barrier controlling granules exocytosis (18). Neutrophil treatment with cytochalasin D (CytD) confirmed that ILT4 up-regulation was mediated through granule exocytosis since it enhanced ILT4 up-regulation on neutrophils stimulated with fMLF (MFI 7.95 ± 0.89 with control DMSO plus fMLF vs. 11.02 ± 1.22 with CytD plus fMLF; *P* = 0.0039; Fig. 4*E*). The efficiency of CytD on granule exocytosis was assessed by surface staining of the azurophil granule marker CD63 (Fig. 4*F*). Finally, neutrophil pretreatment with cycloheximide (CHX) or the kinase inhibitor PP2 showed that ILT4 up-regulation

did not result from protein neosynthesis and was dependent on kinase activation (Fig. S4 *A* and *B*).

These results indicate that a pool of ILT4 receptors contained inside neutrophil granules is rapidly mobilized to the cell surface after stimulation.

ILT4 Up-Regulation Enhances the HLA-G−Mediated Inhibition of Neutrophil Phagocytic Function. We next assessed the functional outcome of ILT4 up-regulation by comparing the sensitivity of neutrophils to HLA-G before and after granule exocytosis. To rule out any implication of other HLA-G receptors than ILT4 in our experimental system, we measured the surface levels of ILT2 and KIR2DL4 on neutrophils after induction of granule exocytosis. As shown in Fig. 5*A* and Table 2, neutrophil treatment with fMLF induced the up-regulation of ILT4 but not of ILT2 or KIR2DL4, even when associated with CytD. We next compared the effect of HLA-G on the phagocytic function of untreated neutrophils expressing middle ILT4 levels (ILT4^{mid}) and neutrophils expressing higher ILT4 levels (ILT4^{high}) because of exocytosis induced by fMLF pretreatment. In absence of HLA-G, no significant difference of phagocytosis was observed between ILT4^{mid} and ILT4^{high} neutrophils (Fig. 5*B*). However, in the presence of HLA-G, the inhibition of phagocytosis was enhanced in ILT4^{high} neutrophils compared with ILT4^{mid} neutrophils (71.80 ± 4.05% vs. 54.84 ± 6.83%; *P* = 0.0009; Fig. 5 *B* and *C*).

These results show that ILT4 is the only known receptor for HLA-G up-regulated on stimulated neutrophils and that this up-regulation enhances the HLA-G−mediated inhibition of neutrophil phagocytic function.

ILT4 Up-Regulation Induced During Neutrophil Stimulation Is Impaired in Sepsis Conditions. Neutrophil inflammatory activity has been reported to be dysregulated during sepsis (15). Therefore, we investigated if ILT4 up-regulation induced upon neutrophil stimulation was modified in sepsis conditions.
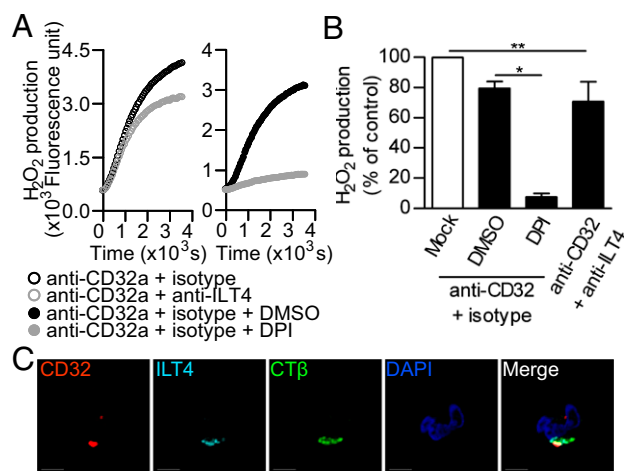

Fig. 3. ILT4 inhibits the CD32a-mediated production of ROS, and both ILT4 and CD32a colocalize within neutrophil lipid rafts. (*A*) Neutrophils were stimulated with coated anti-CD32 mAb in presence of either coated anti-ILT4 or isotype control mAb (*n* = 5). When indicated, neutrophils were previously incubated with 1 μM DPI or control DMSO (*n* = 4). The amount of ROS produced by neutrophils was detected using Amplex Red method. Fluorescence was measured every 30 s for 60 min. A representative experiment is shown. (*B*) Total areas under curves from the independent experiments were measured. Results obtained after stimulation with anti-CD32 + isotype control mAb were assigned a value of 100%. Mann–Whitney test was performed, and *P* values < 0.05 (*) and < 0.01 (**) were taken to be significant. (*C*) ILT4 colocalized with CD32a within neutrophil lipid rafts. CD32a (red) was cross-linked followed by ILT4 (blue), lipid rafts (CTβ, green), and nucleus (DAPI, dark blue) stainings. (Scale bars: 5 μm.) Images are representative of three independent experiments.

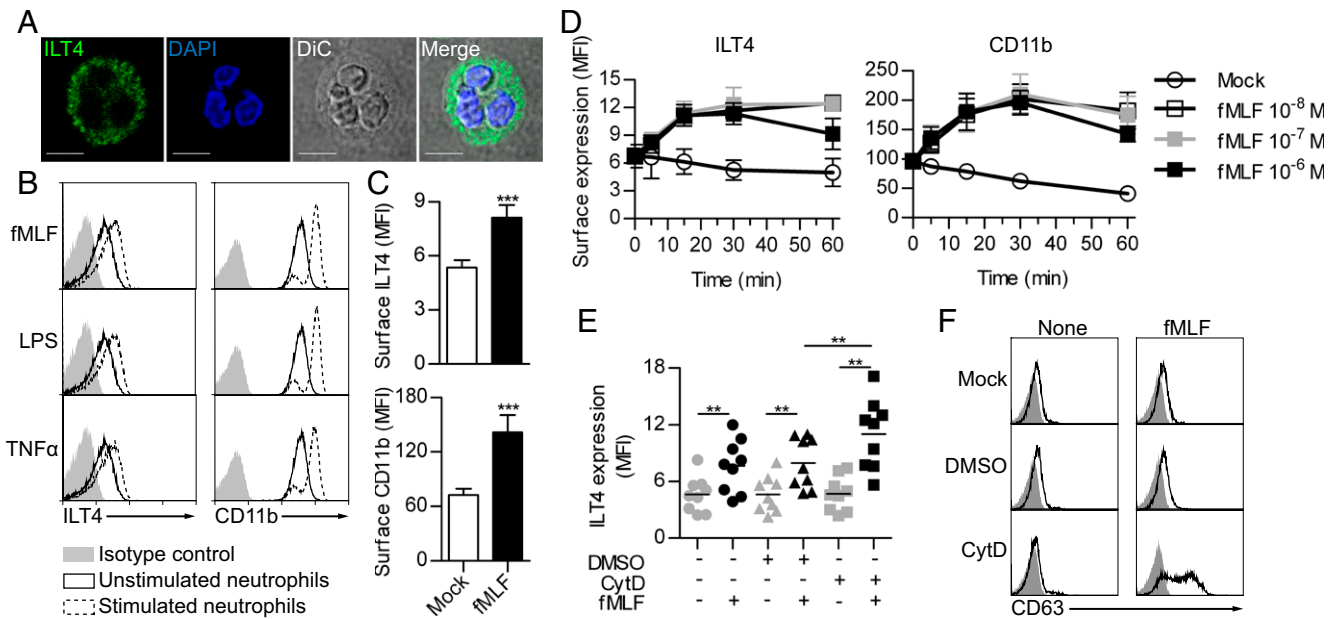IMMUNOLOGY

Fig. 4. ILT4 is localized within neutrophil granules and is up-regulated on cell surface upon stimulation. (*A*) Confocal microscopy analysis of ILT4 localization. Permeabilized neutrophils were stained for ILT4 (green) and nucleus (DAPI, blue). (Scale bars: 5 μm.) (*B*) Surface ILT4 and CD11b expression on neutrophils after stimulation with 10^{-7} M fMLF, 1 μg/mL LPS, or 2 ng/mL TNF-α. (*C*) ILT4 and CD11b surface expression on resting and fMLF-stimulated neutrophils. Histograms represent ILT4 ($n = 36$) and CD11b ($n = 23$) MFI ratios. (*D*) Time course analysis of surface ILT4 and CD11b increase. Neutrophils were stimulated with 10^{-6}, 10^{-7}, or 10^{-8} M fMLF for 5, 15, 30, and 60 min before CD11b/ILT4 expression analysis ($n = 3$). (*E*) The fMLF-induced surface ILT4 increase is enhanced by actin polymerization inhibition. Neutrophils were stimulated or not with 10^{-7} M fMLF following pretreatment with 10 μM cytochalasin D (CytD), control DMSO, or PBS 1×. Surface ILT4 expression (MFI ratio) was analyzed by flow cytometry ($n = 9$). (*F*) Azurophil granules marker CD63 surface expression on neutrophils following previous treatments. Histograms are representative of three independent experiments. **$P < 0.01$; ***$P < 0.001$.

Apoptosis assays were performed to determine if septic plasma was toxic for neutrophils. A high level of apoptosis was observed in healthy neutrophils incubated with control healthy donor plasma (HP in Fig. 6*A*). However, in the presence of septic plasma (SP in Fig. 6*A*), apoptosis of healthy neutrophils was highly inhibited, indicating that septic plasma was not toxic ($25.91 \pm 4.82\%$ vs. $44.56 \pm 4.55\%$ with healthy plasma; $P = 0.0078$). The prosurvival activity of septic plasma was confirmed by monitoring the loss of mitochondrial transmembrane potential (Fig. S5).

Next, we compared the effect of septic and healthy plasma on ILT4 expression of healthy neutrophils. As shown in Fig. 6*B*, ILT4 expression on healthy neutrophils was slightly increased in the presence of septic plasma compared with healthy plasma (MFI 10.20 ± 1.51 vs. 8.77 ± 1.08; $P = 0.0064$; representative analysis in Fig. S6*A*). However, ILT4 up-regulation induced by fMLF stimulation was significantly inhibited in presence of septic plasma (induction 1.34 ± 0.07 with septic plasma vs. 1.74 ± 0.08 with healthy plasma; $P = 0.0001$; Fig. 6*C*; representative analysis in Fig. S6*B*). Similar results were obtained for CD11b and CD66b and when neutrophils were stimulated for a longer time (Fig. S6 and Fig. S7 *A* and *B*), strongly suggesting that septic plasma impaired neutrophil granule exocytosis induced by fMLF stimulation.

Finally, we compared the impact of fMLF stimulation on ILT4 up-regulation from healthy and septic neutrophils. As shown in Fig. 6*D*, treatment of septic neutrophils with fMLF induced a lower up-regulation of ILT4 than for healthy neutrophils (MFI 6.47 ± 1.47 through 7.24 ± 1.81; P value not significant vs. MFI 5.62 ± 0.40 through 9.09 ± 0.59; $P < 0.001$).

These results indicate that ILT4 up-regulation on neutrophils is dysregulated in sepsis conditions probably through impairment of granule exocytosis.

Discussion

In this study, we have investigated the expression levels of the inhibitory receptor ILT4 on neutrophils and its role on their functions. Our data showed that ILT4 is expressed on peripheral blood neutrophils and that this surface expression rapidly increased upon their activation. Besides ILT4, we also investigated the expression levels of ILT2 and KIR2DL4, two others receptors for HLA-G. Only ILT4 was detected on resting or activated neutrophils.

Expression of inhibitory receptors on immune effector cells occurs during their differentiation (19). In this regard, we found that ILT4 expression was induced on PLB-985 cells upon their

Table 2. Surface expression of ILT4, ILT2, and KIR2DL4 after neutrophil exocytosis induced with fMLF and Cytochalasin D

	Mock		DMSO		Cytochalasin D	
	None	fMLF 10^{-7} M	None	fMLF 10^{-7} M	None	fMLF 10^{-7} M
ILT4 ($n = 9$)	49.8 ± 21.9	76.1 ± 18.4	46.7 ± 23.1	77.0 ± 15.7	46.3 ± 21.1	86.9 ± 9.9
ILT2 ($n = 3$)	0.5 ± 0.6	0.4 ± 0.5	0.3 ± 0.3	0.5 ± 0.4	0.3 ± 0.3	0.9 ± 0.8
KIR2DL4 ($n = 3$)	2.4 ± 2.0	4.2 ± 4.3	2.3 ± 1.5	5.2 ± 5.1	1.9 ± 1.2	6.9 ± 9.6

Quiescent and fMLF-stimulated human peripheral blood neutrophils were previously mock-, control DMSO-, or cytochalasin D-treated. Percentages of positive neutrophils are presented as mean ± SD; n represents the number of independent experiments.
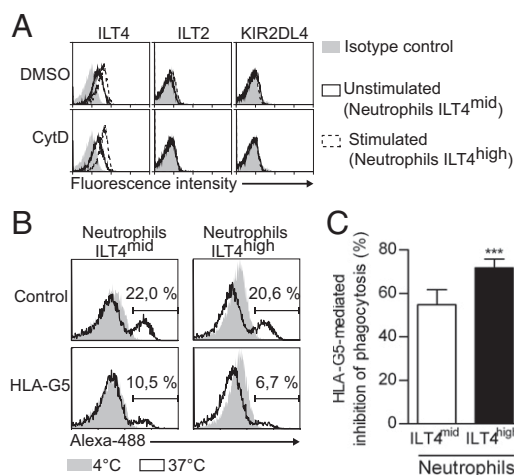
Fig. 5. ILT4 up-regulation increases neutrophil sensitivity to HLA-G−mediated inhibition. (*A*) ILT4, ILT2, and KIR2DL4 surface expression after PBS 1×, DMSO or 10 μM CytD treatment followed or not by 10^{-7} M fMLF treatment. Open and dash histograms represent stainings of unstimulated (ILT4^{mid}) and fMLF-stimulated (ILT4^{high}) neutrophils, respectively. Gray-filled histograms represent isotype controls. (*B*) Phagocytosis of fluorescent *E. coli* by neutrophils after pretreatment (Neutrophils ILT4^{high}) or not (Neutrophils ILT4^{mid}) with 10^{-7} M fMLF for 10 min followed by incubation with control (*Upper*) or HLA-G5-coated nanobeads (*Lower*). Percentages of phagocytic neutrophils are indicated in each panel. (*C*) HLA-G5-mediated inhibition of untreated Neutrophils ILT4^{mid} (open bar) and fMLF-pretreated Neutrophils ILT4^{high} (black bar) from 10 independent experiments. Inhibition of phagocytosis was calculated as [100 − (% fluorescent neutrophils $_{HLA-G5}$/% fluorescent neutrophils $_{control}$ × 100)]. ***$P < 0.001$.

Our results also indicated that ILT4 is contained within neutrophil granules and that it is rapidly up-regulated upon stimulation with fMLF, LPS, or TNF-α through an exocytosis process. No other receptor for HLA-G was up-regulated, even after strong treatment leading to exocytosis of all granule subsets. In agreement with our findings, proteomic studies showed that ILT4 is present in neutrophil gelatinase and specific granules whereas ILT2 and KIR2DL4 were not found in all granules subsets (26).

The ILT4 expression increase following neutrophil activation was shown to enhance HLA-G−mediated inhibition of phagocytosis. This rheostat mechanism could also be implicated in the control of neutrophil pro-inflammatory cytokine production and thus help to prevent an exacerbated inflammation. It could also serve to increase the activation threshold required for neutrophil extracellular traps (NET)osis, known as being harmful for tissues and thus requiring stringent control (27, 28).

We found that spontaneous apoptosis of neutrophils from healthy donors was reduced in the presence of septic plasma, in comparison with neutrophils incubated with healthy plasma. These results concord with previous studies showing that inflammatory mediators released during the early phase of sepsis promote neutrophil survival (29).

Our results showed that ILT4, CD11b, and CD66b levels were slightly increased on healthy resting neutrophils in the presence of septic plasma. This may result from fusion of secretory vesicles with cytoplasmic membrane induced by priming agents present in septic plasma (30). Conversely, fMLF stimulation induced a much lower increase of ILT4, CD11b, or CD66b levels on neutrophils when incubated with septic plasma compared with healthy plasma. This discrepancy could illustrate the sepsis-induced disabling of neutrophil granule release, whereas secretory vesicles apparently rely
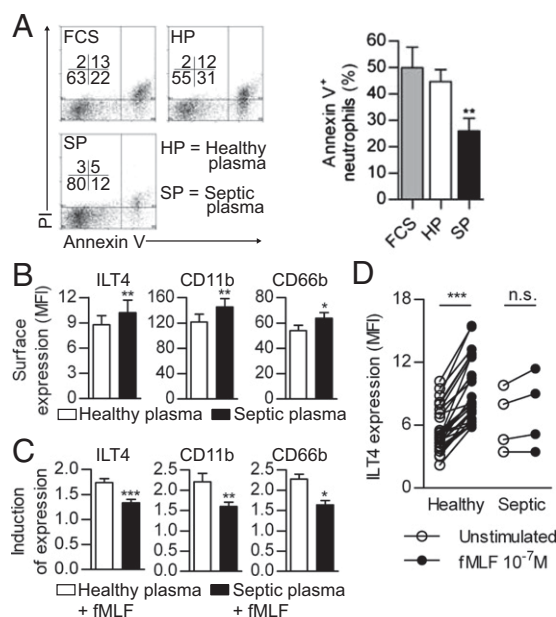
differentiation into neutrophil-like cells. The surface expression of ILT4 could be detected after 2 d of differentiation, suggesting that the induction of ILT4 expression may correspond to transcriptional rather than posttranscriptional events. In favor of this hypothesis, we could observe an increase of ILT4 mRNA levels during differentiation of PLB-985 cells into neutrophil-like cells (Fig. S8).

ROS production and phagocytosis are essential functions of neutrophils required for pathogen killing. As recently reported for the inhibitory receptor CD300a (20), we found that ILT4 ligation impaired ROS production induced by CD32a stimulation. The signaling events involved in this inhibition remain unknown but could depend on lipid rafts reorganization, as both CD32a and ILT4 were found colocalized there. Neutrophil lipid rafts could favor the proximity of CD32a and ILT4 receptors to rapidly deactivate signaling pathways. Even though the role of CD16b on neutrophil activation remains unclear, aggregation of CD16b also induced colocalization with ILT4 within lipid rafts (Fig. S9). Based on previous studies performed in monocytes, we propose that, in the presence of ligand, ILT4 may recruit SHP-1 that in turn would deactivate the spleen tyrosine kinase (Syk) required for calcium mobilization and neutrophil activation (7, 21). We also investigated the effect of ILT4 engagement on neutrophil ROS production induced by fMLF, but no inhibition was observed (Fig. S10). Our study demonstrates that HLA-G inhibits the phagocytosis of opsonized bacteria by neutrophils in two different models. Phagocytosis requires cytoskeleton remodeling, and it is likely that the interaction of ILT4 with HLA-G may act on neutrophil signaling components involved in this process (22). In this regard, we have previously shown that the interaction between ILT2 and HLA-G impairs natural killer (NK) cell cytoskeleton reorganization and thus cytolytic function (23, 24). Moreover, actin remodeling depends on Syk and Lyn tyrosine kinases activation which are triggered by CD32a translocation into lipid rafts (25). Since unopsonized phagocytosis implies other molecular mechanisms, future studies will be helpful to determine how it is affected by ILT4 engagement.

Fig. 6. The up-regulation of ILT4 expression on neutrophils mediated through exocytosis is inhibited in sepsis conditions. (*A*) (*Left*) Neutrophil apoptosis was measured by Annexin V-FITC/PI staining after incubation with 5% FCS, healthy plasma (HP), or septic plasma (SP). Percentages are indicated in each panel. (*Right*) Quantifications of apoptotic (Annexin V-FITC$^+$) neutrophils ($n = 8$). (*B*) ILT4 ($n = 13$), CD11b ($n = 8$), or CD66b ($n = 6$) expression (MFI) on neutrophils after 15 min incubation in 5% healthy plasma (white bars) or 5% septic plasma (black bars). (*C*) The fMLF induction of ILT4 ($n = 13$), CD11b ($n = 8$), and CD66b ($n = 6$) surface expression on neutrophils incubated in 5% healthy plasma (white bars) or 5% septic plasma (black bars). (*D*) Comparison of fMLF-induced ILT4 exocytosis on neutrophils from healthy ($n = 27$) and septic patients ($n = 4$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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on distinct mechanisms (31). Moreover, the reduced response of isolated septic neutrophils despite the absence of septic plasma indicates the long-term effect of the septic microenvironment. One could argue that leukocyte metabolism is modulated under sepsis conditions and that this energetic failure could affect neutrophil granule release (32). On the other hand, microarray studies showed that inflammatory disorders induced an immune-regulatory transcriptional profile in leukocytes (33). In this regard, IL-10 produced during sepsis is proposed as a major modulator of neutrophil functions (34). Furthermore, a high amount of HLA-G5 was found in plasma from surviving septic patients and could thus help to dampen neutrophil proinflammatory activity (35).

In conclusion, our study provides unique insights into the molecular mechanisms regulating neutrophil functions through ILT4 inhibitory receptor that will be helpful in the understanding of their dysregulation in inflammation.

Materials and Methods

Detailed materials and methods and Figs. S1–S10 are provided in *SI Materials and Methods*.

Peripheral Blood Neutrophil Isolation and Cell Line. Neutrophils were isolated by centrifugation over a double Ficoll Histopaque density gradient. The myelomonoblast PLB-985 cell line was grown in complete RPMI medium and differentiation in neutrophil-like cells was induced by 1.25% (vol/vol) DMSO or 0.5% DMF for 5 d with medium renewal on day 3.

Patients' Samples. Informed consent was obtained from the human subjects involved in our research. Septic patients sampling for cell and plasma have been authorized by the Comité de Protection des Personnes of Saint Louis Hospital (N° European Union Drug Regulating Authorities Clinical Trials 2010-A0004039), May 27, 2010. Twenty-three septic plasmas were collected at the early phase of patients' hospitalization for a severe septic syndrome and pooled. Seventeen control healthy plasmas were collected and pooled.

Flow Cytometry Stainings. $Fc\gamma R$ were blocked with 20 µg/mL human IgG prior specific staining. All steps were performed on ice, and isotype control antibodies (Abs) were systematically used.

Phagocytosis Assay. Neutrophils (2×10^5) were incubated with Alexa 488-conjugated *E. coli* Bioparticles opsonized in autologous plasma. When indicated, neutrophils were stimulated with 10^{-7} M fMLF and incubated with control or HLA-G5–coated nanobeads.

ROS Generation Assay. Neutrophils (1×10^5) were incubated in plates coated with anti-CD32 and anti-ILT4 monoclonal Abs or isotype-matched controls and ROS production was measured by Amplex Red method (Life Technologies).

Exocytosis Assays. Neutrophils (2×10^5) were stimulated with fMLF, LPS, or TNFα for 15 min in PBS 1× supplemented with 5% FCS or 5% healthy or septic plasma. When indicated, neutrophils were preincubated for 20 min with 10 µM cytochalasin D or DMSO.

Apoptosis Assay. Neutrophils (2×10^5) were resuspended in RPMI, 5% FCS supplemented with 5% FCS or plasma. After 20 h incubation at 37 °C, 5% CO_2, apoptosis was assessed by Annexin V-FITC/PI staining following manufacturer's instructions (Miltenyi Biotec).

Confocal Microscopy. Stainings were performed as previously described (36). For colocalization experiments, CD32 was capped on neutrophils surface before ILT4 and lipid rafts stainings.

Statistical Analyses. Analyses were performed using Graph-Pad Prism (version 5.01). Except when indicated, experimental data are expressed as mean ± SEM and Wilcoxon matched pairs test was used. "*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ were taken to be significant.

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