Circulating neutrophils of septic patients constitutively express IL-10R1 and are promptly responsive to IL-10

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Abstract
Previous studies have demonstrated that neutrophils isolated from the blood of healthy donors do not respond to IL-10 in terms of either activation of signal transducer and activator of transcription-3 (STAT3) tyrosine phosphorylation or induction of suppressor of cytokine signalling (SOCS)-3 protein, unlike autologous mononuclear cells. This was explained by the fact that circulating neutrophils of healthy donors express only IL-10R2, but not IL-10R1, the latter IL-10R chain being essential for mediating IL-10 responsiveness. In this study, we report that peripheral blood neutrophils of septic patients constitutively display, besides IL-10R2, also abundant levels of surface IL-10R1. Consequently, septic neutrophils are promptly responsive to IL-10 in vitro, as revealed by a direct IL-10-mediated induction of STAT3 tyrosine phosphorylation and SOCS-3 gene transcription, mRNA and protein expression. Consistent with the presence of a fully functional IL-10R, modulation of LPS-induced CXCL8, CCL4, tumour necrosis factor-α and IL-1ra gene expression was also rapidly induced by IL-10 in septic, but not normal, neutrophils. Collectively, these data uncover that neutrophils of septic patients are predisposed to be promptly responsive to IL-10, presumably to help limiting their pro-inflammatory state. They also fully validate our previous observations, herein in the context of a human disease, that responsiveness of human neutrophils to IL-10 is strictly dependent upon the modulation of IL-10R1 expression.

Introduction
Sepsis and its more dangerous consequence, septic shock, are the major causes of morbidity and mortality in critically ill patients. During the onset of sepsis, several pro-inflammatory mediators, including cytokines and chemokines, are released systemically to mediate most of the subsequent pathophysiological events (1). This so-called ‘cytokine storm’, if not efficiently counterregulated by anti-inflammatory feedback mechanisms, causes dysfunctions of various immunological functions, which, in turn, result in the inability to ward off the infection and consequently lead to multiple organ failure and death. It would be therefore extremely important to expand our understanding on the pathological components causing the aberrant development of an anti-inflammatory/immunosuppressive response because it would greatly help to develop more appropriate therapeutic strategies (2).

The neutrophil has emerged as a central effector cell in sepsis, as it possesses a formidable armamentarium of enzymes and oxygen radicals that might inflict damage to cells (3). During sepsis, for instance, the number of circulating neutrophils is often increased, their survival is extended and their functional responses are enhanced (4). Neutrophils also represent an important source of pro-inflammatory cytokines (5), a novel function which offers to these cells additional possibilities, not only for acting in the early phases of acute inflammation but also for conditioning and influencing the progression of subsequent inflammatory and immune events (6). However, very little is known on the factors and the mechanisms that modulate the production of cytokines in neutrophils. IL-10, for instance, is well known to negatively influence pro-inflammatory cytokine and chemokine expression by neutrophils stimulated in vitro with LPS (7-9). Accordingly, previous in vitro studies have shown that the inhibitory effects of IL-10 on pro-inflammatory cytokine mRNA expression and production induced by LPS only start...
after 3–4 h of neutrophil culture (6, 9), in contrast with the more rapid action exerted by IL-10 on mononuclear cells (10). The delayed action of IL-10 was explained, in part, by the fact that one of the two subunits composing the IL-10R, namely IL-10R1 (the signalling chain of the IL-10R complex) (11), is substantially undetectable on the surface of circulating or freshly isolated neutrophils (12), a fact that renders neutrophils poorly responsive or completely unresponsive to IL-10 (12). On the other hand, expression of IL-10R1 was shown to become markedly elevated in neutrophils incubated with LPS for 3–4 h (12). Such a phenomenon, in conjunction with other protein synthesis-dependent events triggered by the LPS itself (10), was shown to render cultured neutrophils able to promptly respond to IL-10 (10, 12). In other words, these observations have made it clear that responsiveness of neutrophils to IL-10 is controlled by the regulated expression of IL-10R1, consistent with what was observed in other cell types (13–15). However, despite of intensive molecular analyses on the mechanisms utilized by IL-10 to modulate LPS-induced gene expression (10, 12, 16, 17), no information exists on the expression and/or functional role of IL-10R1 (and IL-10R2) in neutrophils isolated from patients affected by inflammatory diseases, including sepsis.

In this study, we report that circulating neutrophils of septic patients, differently from those of healthy donors, constitutively display high levels of surface IL-10R1 and IL-10R2. We also show that, after their isolation from the blood, septic neutrophils promptly respond to IL-10 in terms of signal transducer and activator of transcription-3 (STAT3) activation and modulation of target gene expression. These findings not only are consistent with our previous in vitro studies (10, 12) but also suggest that neutrophils of septic patients in an acute phase of the disease are predisposed to be readily responsive to exogenous IL-10, presumably to help limiting their pro-inflammatory state.

Methods

Patients

Eight patients (three females and five males, ranging between 54 and 84 years) fulfilling the criteria of systemic inflammatory response syndrome (SIRS) according to the American College of Chest Physicians and the Society of Critical Care Medicine (18), admitted to the medical surgical intensive care unit of the Verona University Hospital, were enrolled in this study. Six healthy subjects (three females and three males) were also enrolled as controls. SIRS was related to infection in six of the eight patients: two were affected by septic shock of abdominal origin, while the other four were affected by severe sepsis (one related to pneumonia and three to abdominal infection). In the two remaining patients, SIRS was related to trauma and to cardiac arrest. Five of the eight patients survived (four of these belonging to the ‘septic group’) and were discharged from the intensive care unit. Cells from these individuals were collected and processed as described below. This study was approved by the Institutional Review of Verona University, with informed consent obtained from each patient’s family.

Cell purification and culture

Whole blood was collected in BD Vacutainer® Plus plastic whole blood tube (BD Bioscience, Franklin Lake, NJ, USA), from either healthy donors or SIRS patients, and then used either for direct flow cytometric analysis or for cell purification. In the latter case, highly purified granulocytes (>98.5%) were isolated under endotoxin-free conditions exactly as previously described (12). Immediately after purification, neutrophils were suspended in standard culture medium (RPMI 1640 medium; BioWhittaker, Walkersville, MD, USA) supplemented with 10% low endotoxin FCS (<0.06 EU ml⁻¹) by Limulus ameboocyte lysate assay, from BioWhittaker and cultured at 5 × 10⁶ ml⁻¹ in the absence or in the presence of 200 U ml⁻¹ IL-10 (from Schering-Plough Biopharma, Palo Alto, CA, USA) and/or 100 ng ml⁻¹ ultrapure LPS (from Escherichia coli serotype 0111:B4) purchased from Invitrogen (San Diego, CA, USA) in six-tissue culture well plates (BioWhittaker) for 30 min. Cells were then collected, spun at 350 × g for 5 min and used either for protein extraction or for total RNA purification. All reagents used were of the highest available grade and were dissolved in clinical grade pyrogen-free water.

Flow cytometry analysis

Determination of antigenic surface expression was performed by a two-colour flow cytometric assay in whole blood. Discrimination among the various leucocyte populations was based on side scatter versus CD33–FITC (Biolegend, San Diego, CA, USA) expression plot analysis. The following were the antibodies used: 20 μg ml⁻¹ of anti-IL-10R1 (clone 3C2) and anti-IL-10R2 (clone 4B2) (kindly provided by Rene de Waal Malefyt, Biopharma Schering-Plough) (12), 20 μg ml⁻¹ of isotype controls (IgG1 or IgG2a, Biolegend), 2 μg ml⁻¹ of a second biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) and 0.5 μg ml⁻¹ streptavidin-PE (BD Bioscience). Cytofluorimetric analysis was performed on a FACScan (BD Bioscience) using CellQuest software.

Immunoblots

Preparation of neutrophil lysates and subsequent immunoblot analysis were conducted as previously described (10, 19). For the direct detection of tyrosine-phosphorylated and total STAT3, antigenic suppressor of cytokine signalling (SOCS)-3 (molecular weight of 29 kDa) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), two-colour western blots were performed with, respectively, rabbit anti-phospho-STAT3 (Tyr705) antibodies (Cell Signaling, Denver, MA, USA), mouse anti-STAT3 mAbs (clone 124H6, Cell Signaling), rabbit anti-SOCS-3 polyclonal antibodies (ImmunologyBiological laboratories, Tokyo, Japan) and mouse anti-GAPDH mAbs (clone 6C5, Ambion, Austin, TX, USA), as already described (17). Detection was simultaneously carried out with Alexa Fluor®680 goat anti-rabbit antibodies (Molecular Probes™, Invitrogen, Carlsbad, CA, USA) and IRDye®680-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA, USA) secondary antibodies. Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) (17).
Real-time reverse transcription–PCR and primary transcript real-time reverse transcription–PCR

Real-time reverse transcription (RT)–PCR and primary transcript (PT) real-time RT–PCR have been performed as described (17), using 1 μg total RNA (usually extracted from 10⁷ neutrophils), with gene-specific primer pairs (purchased from Invitrogen) available in the public database RTPrimerDB (http://medgen.ugent.be/rtprimerdb/) under the following entry code: β2m (3534), GAPDH (3539), SOCS-3 (3828), PT SOCS-3 (3829), IL-1ra (3544), PT IL-1ra (3650), tumour necrosis factor (TNF)-α (3551), CCL4/MIP1β (3535) and CXCL8/IL-8 (3553) Data were calculated with Q-Gene software (http://www.BioTechniques.com) and are expressed as mean normalized expression units after β2m normalization (20).

Statistical analysis

Data are expressed as means ± SEMs. Statistical evaluation was performed using Student’s t-test and considered to be significant if P < 0.05.

Results

IL-10R1 expression in circulating neutrophils of septic patients

Previous studies have uncovered that circulating neutrophils of healthy donors constitutively express only one of the two IL-10R subunits, namely IL-10R2 (12). Since expression of IL-10R1 is up-regulated in neutrophils cultured with either LPS or IL-4 (12, 21), enabling them to promptly respond to IL-10 (10), we questioned here whether this receptor chain could be expressed in septic neutrophils. We therefore performed indirect immunofluorescence flow cytometry analysis on whole blood leukocytes of six subjects affected by sepsis and control individuals. As shown in Fig. 1, these experiments revealed that septic neutrophils display readily detectable IL-10R1-binding sites, at variable, but significantly greater levels [mean fluorescence intensity (MFI) = 32.8 ± 13.3, n = 6, P = 0.037] than those observed in peripheral blood neutrophils of healthy individuals (MFI = 0.8 ± 0.2) (Fig. 1A and B) or non-septic SIRS patients (MFI = 1.4, n = 2, not shown). By comparison, the levels of IL-10R2 expression in neutrophils of septic and normal donors were quantitatively comparable (Fig. 1A and B). Taken together, these data show that neutrophils of septic patients selectively display a markedly up-regulated expression of membrane IL-10R1.

Up-regulation of IL-10R1 expression in neutrophils of septic patients is functional

Since the responsiveness of neutrophils to IL-10 is conditioned by the presence of surface IL-10R1 (10), we examined whether septic neutrophils are able to rapidly and directly respond to IL-10 in terms of STAT3 activation (22). The latter was the case, as revealed by the potent IL-10-mediated stimulatory effect on STAT3 tyrosine phosphorylation, provoked in neutrophils of all three septic patients that we could examine (Fig. 2), and even when STAT3 was already tyrosine phosphorylated at low levels (Fig. 2). By contrast, no detectable IL-10-induced STAT3 activation was observed in neutrophils isolated from healthy donors (Fig. 2), as expected from their lack of IL-10R1 expression (Fig. 1) (12).

Because IL-10-induced STAT3 tyrosine phosphorylation is fundamental for the direct transcriptional induction of IL-10R1 expression in septic neutrophils

\[ IL-10 \]

\[ STAT3 \]

Fig. 1. Surface expression of IL-10R1 and IL-10R2 in circulating neutrophils of septic patients. Whole blood of septic patients and healthy donors were stained with anti-IL-10R1 and anti-IL-10R mAbs for flow cytometric analysis. As negative controls, cells were also stained with corresponding isotype control mAbs. Panel (A) depicts a representative pattern of IL-10R1 and IL-10R2 expression in neutrophils of septic patients and healthy donors. Panel (B) shows the MFI values for each antigen detected in granulocytes of all septic and normal individuals examined. MFI was calculated by subtracting the MFI of the IgG1/IgG2a-stained neutrophils from the corresponding IL-10R-stained cells. Horizontal bars indicate the mean MFI values for each group.

Fig. 2. IL-10-mediated STAT3 tyrosine phosphorylation in septic neutrophils. Neutrophils from three septic patients and one representative healthy donor were incubated, immediately after isolation, in the presence or absence of 200 U ml⁻¹ IL-10 for 30 min and then lysed for western blot analysis using antibodies specific for tyrosine-phosphorylated forms of STAT3. Simultaneous blotting with anti-STAT3 antibodies was carried out to ensure that similar amounts of material were deposited in each lane.
a number of target genes, including SOCS-3 (23, 24), we subsequently investigated whether SOCS-3 expression was rapidly modulated by IL-10 in septic neutrophils. Real-time RT-PCR experiments revealed that, following a 30-min treatment, IL-10 greatly increases the levels of SOCS-3 mRNA accumulation in neutrophils of septic patients, but not of healthy donors (Fig. 3A). Such induction of SOCS-3 mRNA expression was mirrored by an equivalent increase of SOCS-3 PTs (Fig. 3A)—demonstrating its transcriptional induction—as well as SOCS-3 protein (Fig. 3B). Interestingly, the IL-10-mediated up-regulation of SOCS-3 protein occurred even if septic neutrophils constitutively contained detectable amount of SOCS-3 (Fig. 3B).

Finally, because IL-10 is also a potent modulator of cytokine gene expression in LPS-activated neutrophils (9), we analyzed whether IL-10 was also able to rapidly modulate CXCL8, CCL4, TNF-α and IL-1ra mRNA expression in neutrophils of two septic patients, freshly purified and immediately treated with LPS. Real-time RT-PCR studies revealed that, within 30 min, IL-10 was able to inhibit the mRNA expression of CXCL8 (by 37.3 and 33.9%), CCL4 (by 18 and 35.7%) and TNF-α (by 16.6 and 27% inhibition by IL-10) (Fig. 4A), without affecting (25) the mRNA expression of IL-1ra (Fig. 4B). However, by PT real-time RT-PCR, IL-10 was found to strongly up-regulate the induction of IL-1ra-PT in LPS-treated neutrophils of septic patients, but not of healthy donors (Fig. 4B). Taken together, these data clearly demonstrate that neutrophils freshly isolated from septic patients rapidly and directly respond to IL-10 in terms of STAT3 activation, SOCS-3 transcriptional induction and mRNA expression. When coincubated with LPS, septic neutrophils also respond to IL-10 in terms of CXCL8, CCL4, TNF-α and IL-1ra gene modulation.

Discussion

In this work, we report that peripheral blood neutrophils of septic patients display measurable surface levels of IL-10R1. Unlike neutrophils of non-septic SIRS patients or healthy donors. In contrast, the levels of IL-10R2 expression, which are remarkable in normal neutrophils (12), were found to be unchanged in septic neutrophils. Interestingly, the amounts of surface IL-10R1 and IL-10R2 in mononuclear cells of the same septic and normal individuals were substantially comparable (F. Calzetti et al., unpublished observations), pointing for a specific up-regulation of IL-10R1 membrane levels in septic neutrophils only. Regrettably, the low number of patients examined in this study, in combination with their heterogeneity, does not allow us to definitively classify IL-10R1 as a potential marker of septic SIRS, in addition to those already identified, such as CD64 (26, 27) and PR3 (28). An analysis of a larger cohort of clinical cases might certainly give useful hints to clarify whether the detection of surface IL-10R1 in septic neutrophils could be utilized as a potential indicator of the disease outcome.

Irrespective of this, septic neutrophils isolated from the blood were found to promptly respond to IL-10, consistent with their constitutive and functional expression of both IL-10R1 and IL-10R2. Cell responsiveness was, accordingly, verified in terms of capacity of IL-10 to rapidly (within 30 min) activate STAT3 and up-regulate SOCS-3 gene and protein expression and to rapidly modulate LPS-induced CXCL8, CCL4, TNF-α mRNA expression and IL-1ra transcription in septic neutrophil themselves. A regulatory effect of IL-10 on the LPS-induced IL-1ra accumulation was not observed, likely because the time point at which our studies were performed was too early to uncover a potential action of IL-10 at the level of IL-1ra mRNA expression, other than IL-1ra transcription. Unfortunately, due to the limited amount of blood that could be drawn from these severely ill patients, and the very low RNA content present in human neutrophils (5), we could not obtain sufficient material to extend our gene expression analysis to more delayed time points. But despite of this, the behaviour of septic neutrophils, after treatment with IL-10, is fully reminiscent of the behaviour that normal neutrophils acquire when cultured with LPS in vitro (10, 12). The latter consists, in fact, in an acquisition of a prompt ability to respond to IL-10 in terms of STAT3...
activation and modulation of target gene expression (10, 12), in concomitance with the up-regulation of IL-10R1 expression. Viewed under the latter perspective, the data reported in this study fully validate our previous observations (12): they in fact demonstrate that, under specific inflammatory settings such as sepsis, the up-regulation of IL-10R1 expression in neutrophils may occur also in vivo and it is essential to render neutrophils rapidly responsive to IL-10. In view of our present and previous findings (10, 12), we could suggest that, at least during sepsis, circulating neutrophils are induced to up-regulate their IL-10R1 expression in response either to pathogen-derived pro-inflammatory mediators, possibly including LPS (12) and/or other toll like receptor (TLR) ligands, or to cytokines, such as for instance IL-4 (21). If so, TLR4 and/or IL-4 would function via an induction of IL-10R1 gene and de novo IL-10R1 synthesis (12, 21).

Alternatively, up-regulation of IL-10R1 expression in septic neutrophils might simply be the result of a TNF-α or granulocyte macrophage colony-stimulating factor-triggered mobilization of neutrophil-specific granules (that appear to contain preformed IL-10R1) (29) to the membranes. Further studies are obviously necessary to elucidate these issues. Similarly, it would be also important to clarify what is the real biological meaning of the up-regulated expression of surface IL-10R1 in neutrophils during sepsis. One possible explanation might be that this phenomenon represents a sort of safety mechanism by which the host is attempting to render neutrophils readily responsive to circulating IL-10, in order to attenuate their pro-inflammatory status. Given its well established anti-inflammatory actions (22), several clinical investigations have already examined the potential benefits of IL-10 administration in human models of endotoxemia, demonstrating beneficial inhibitory effects of such a therapeutic strategy on the generation of the inflammatory cytokines TNF-α, IL-6 and CXCL8, (30) and the chemokines CCL2, CCL3 and CCL4 (31) or on the coagulant/fibrinolytic activity (32). However, these experiments gave no information about the specific reactivity of individual leukocyte subtypes to circulating IL-10. In consideration of the fact that neutrophils represent the most numerous circulating leukocyte type, and that they produce and release cytokines and chemokines in response to LPS or other TLR ligands (5, 9), neutrophils may certainly represent, within the blood compartment, one of the major target cells in patients receiving IL-10. In accord with this latter hypothesis, it has been shown that circulating neutrophils from septic patients stimulated in vitro with LPS have a significantly reduced capacity to release IL-1β (33) and CXCL8 (34) as compared with neutrophils from healthy subjects. Consequently, it has been suggested that anti-inflammatory mediators such as IL-10 may be responsible for the observed ‘anergy’ of septic neutrophils (34). The latter notion has been further supported by a recent analysis of gene-expression profiling of septic neutrophils, which identified a set of 50 signature genes whose degree of expression was considerably lower than in normal neutrophils (35). An alternative explanation for the up-regulation of IL-10R1 expression in septic neutrophils might be referred in the context of the mechanisms controlling neutrophil survival. Some
studies have, in fact, reported that IL-10 attenuates the capacity of LPS and other cytokines to delay neutrophil apoptosis in vitro (36). While we were unable to confirm those findings (37), we could not exclude that in pathological conditions in which neutrophils are activated by a number of agonists, such as sepsis (1), IL-10 may effectively decrease their survival. If so, the up-regulation of IL-10R1 might certainly favor a pro-apoptotic action of IL-10. Regardless of their survival. If so, the up-regulation of IL-10R1 might certainly favor a pro-apoptotic action of IL-10. Regardless of their survival.

References


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