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Intracellular Pool of IL-10 Receptors in Specific Granules of Human Neutrophils: Differential Mobilization by Proinflammatory Mediators[1]

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IL-10 has a wide range of effects tending to control inflammatory responses. We used flow cytometry to study IL-10 binding at the polymorphonuclear neutrophil (PMN) surface and its modulation by various proinflammatory agents. Little IL-10 bound to the surface of resting PMN. However, binding was strongly increased after stimulation with LPS and proinflammatory cytokines such as TNF and GM-CSF. IL-1 and IL-8 did not significantly modify IL-10 binding. Cycloheximide had no effect on TNF-induced IL-10 binding, strongly suggesting the release of a pre-existing pool of IL-10R rather than de novo receptor synthesis by PMN. This was confirmed by the inhibitory effect of pentoxifylline, an inhibitor of degranulation. The existence of an intracellular pool of IL-10R was shown by flow cytometry, immunocytochemical staining, and Western blotting with several anti-human IL-10R Abs. In subcellular fractions of resting PMN, IL-10R was mainly located in the specific granule fraction, and was absent from azurophil granules and cytosol. We also tested the mobilization of specific granules by measuring the release of lactoferrin, their reference marker. The differential effects of the proinflammatory agents on IL-10 binding matched their effects on lactoferrin release and may therefore be related to differential mobilization of specific granules by these agents. Furthermore, the kinetics of TNF-induced up-regulation of IL-10 binding to PMN ran parallel to the kinetics of the inhibitory effect of IL-10 on the oxidative burst, suggesting a key role of IL-10R mobilization from specific granules to the membranes in optimal regulation of inflammatory responses. ***The Journal of Immunology*****, 2001, 166: 5201–5207.**

Polymorphonuclear neutrophils (PMN)[3] play a critical role in host defenses against invading microorganisms (1). In response to pathogens, PMN move from the circulating blood to infected tissues, where their activation triggers microbicidal mechanisms such as release of proteolytic enzymes and antimicrobial peptides, and rapid production of reactive oxygen species (ROS), a phenomenon known as the oxidative burst. PMN also participate in the regulation of immune and inflammatory responses by producing various mediators. Excessive or inappropriate stimulation of PMN can lead to tissue damage through excessive release of proinflammatory mediators into the extracellular medium (2, 3). Therefore, tight regulation of PMN responses, particularly by cytokines, is necessary for optimal antibacterial activity without detrimental consequences for host tissues. IL-10 plays an important role in the down-regulation of inflammatory and cell-mediated immune responses (4). This pleiotropic cytokine, mainly secreted by monocytic, T, and B cells, has a variety of cellular targets (5). Originally identified as an inhibitor of proinflammatory cytokine synthesis by monocytes and T lymphocytes (4, 6), IL-10 was subsequently found to have a wide range of additional effects, especially on PMN (7). For instance, recent studies have shown that IL-10 is a potent inhibitor of LPS-induced PMN production of proinflammatory cytokines such as TNF-α, IL-1, IL-8, MIP-1α, MIP-1β, and IL-12, whereas it potentiates LPS-elicited secretion of IL-1 receptor antagonist (8–13). IL-10 also down-regulates other PMN effector functions, such as phagocytic and bactericidal activities, Ab-dependent cellular cytotoxicity, and platelet-activating factor and ROS production (14–17).

IL-10 activities are mediated by a high-affinity cell surface receptor (IL-10R) that is structurally related to IFN receptors (18–20). IL-10R is a complex of a ligand-binding chain (IL-R1 or α) and a recently identified IL-10R2 (CRFB4/CRF-2) molecule whose linking to the ligand-binding chain results in a functional IL-10R (21–23). IL-10R1 has been found at the surface of a variety of cells, including several human lymphoid and myeloid cell lines (24–28). IL-10 binding to the PMN surface has recently been reported, albeit to a lesser extent than to monocytes and lymphocytes (29). However, no information is available on the regulation of IL-10 binding at the surface of mature PMN.

To further define the pro- and anti-inflammatory balance at an inflammatory site, we investigated IL-10 binding to the PMN surface during treatment with proinflammatory mediators (LPS, TNF-α, GM-CSF, IL-1, IL-8) that have different effects on PMN functions (8, 10, 30–34). We observed differential modulation of IL-10 binding to PMN by these proinflammatory agents, which was related to differential mobilization of the subset of specific granules, which was found to stock IL-10 receptors.

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil(s); ROS, reactive oxygen species; rhTNF-α, recombinant human TNF-α; DFP, diisopropylfluorophosphate; PTX, pentoxifylline; HE, hydroethidine; MFI, mean fluorescence intensity; SI, stimulation index.

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Materials and Methods

Reagents

The reagents and sources were as follows: recombinant human TNF-α (rhTNF-α; 10^5 U/ml), IL-1β (10^5 U/ml), and IL-8 (77 aa) produced by endothelial cells (Genzyme, Cambridge, MA); GM-CSF (1.2×10^5 ng/ml; Schering-Plough, Kenilworth, NJ); LPS endotoxin from *Escherichia coli* (O55:B5), PMA, diisopropylfluorophosphate (DFP), fMLP, primaquine, and a protease inhibitor mixture (Sigma, St. Louis, MO); pentoxifylline (PTX; Hoechst, Paris-La-Défense, France); rhIL-10 (5 μg/ml), biotinylated rhIL-10, FITC-avidin, and mouse biotinylated anti-human IL-10R polyclonal Ab (R&D Systems, Abingdon, U.K.); rat anti-human IL-10R mAb (3F9) (a gift from Kevin Moore, DNAX Research Institute, Palo Alto, CA); rabbit anti-human IL-10R polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA); peroxidase-conjugated swine anti-goat Ig (BioSource International, Camarillo, CA); Dako LSAB alkaline phosphatase kit (Dakopatts, Glostrup, Denmark); purified monoclonal mouse anti-human CD11b; FITC-streptavidin (Immunotech, Marseille, France); FCS (Life Technologies Laboratories, Grand Island, NY), and hydroethidine (HE; Fluka, Buchs, Switzerland). Stock solutions of HE (15 mg/ml) and fMLP (10^{-2} mol/L) were prepared in acetonitrile and DMSO, respectively, and stored at -20°C. The solutions were diluted in PBS (Pharmacia Fine Chemicals, Uppsala, Sweden) immediately before use. All of the recombinant cytokines were used as recommended by the manufacturers.

Study of IL-10 binding to the PMN surface

IL-10 binding to the PMN surface was measured by means of flow cytometry in whole blood, to minimize procedure-related changes in surface receptor expression (35). Whole blood (1 ml) from healthy donors was either kept on ice or incubated with TNF-α (0.1–1000 U/ml), GM-CSF (0.1–500 U/ml), IL-1β (0.1–500 U/ml), IL-8 (0.1–50 ng/ml), LPS (0.1–5 μg/ml), PMA (100 ng/ml), or PBS for 5–30 min (depending the stimulus) at 37°C with constant agitation. In some experiments, samples were preincubated with PTX (1 μM to 10 mM), primaquine (250 μg/ml), or a protease inhibitor mixture (10 μg/ml) for 5 min before adding TNF-α. IL-10 binding to the PMN surface was determined by using a biotinylated IL-10, with FITC-avidin revelation according to the manufacturer guidelines. Briefly, samples (100 μl) were incubated with biotinylated IL-10 for 60 min at 4°C. Nonspecific binding was determined by incubating samples with a negative control reagent consisting of a soybean trypsin inhibitor biotinylated to the same extent as the cytokine (5 μg/ml). The cells were then directly treated with FITC-avidin for 30 min at 4°C. The specificity of the reaction was checked by preincubating the cytokine reagent for 15 min with a blocking anti-hIL-10 Ab. After erythrocyte lysis with FACS lysing solution (BD Biosciences, Mountain View, CA) and one wash in PBS, cells were resuspended in washing buffer and kept on ice until flow cytometry.

Determination of intracellular IL-10R expression by flow cytometry

Erythrocytes were lysed with FACS lysing solution. Leukocytes were washed twice with PBS containing 2% FCS. Paraformaldehyde (0.25%) was then added while vortexing, and samples were incubated in the dark for 15 min at room temperature. After one wash with PBS, the leukocytes were incubated with ice-cold PBS-70% methanol in the dark for 60 min at 4°C to permeabilize the membranes as previously described (36). After one wash in PBS, the samples were incubated with a mouse biotinylated anti-human IL-10R Ab (R&D Systems) for 30 min at 4°C and then with FITC-streptavidin for 30 min at 4°C. After one wash with ice-cold PBS containing 2% FCS, cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Nonspecific Ab binding was determined on cells incubated with the same concentration of an irrelevant biotinylated Ab of the same isotype. The positive control used an anti-CD11b Ab.

$O_2^{-\circ}$ production by PMN

$O_2^{-\circ}$ production was measured by using a flow cytometric assay derived from the HE oxidation technique described by Rothe and Valet (37). Whole-blood samples (1 ml) were preincubated for 15 min with HE (1500 ng/ml) in a water bath with gentle horizontal agitation at 37°C. (HE diffuses into the cells and, during the PMN oxidative burst, nonfluorescent intracellular HE is oxidized by $O_2^{-\circ}$ to highly fluorescent ethidium that is trapped in the nucleus by intercalation into DNA, leading to an enhancement of fluorescence.) Samples were then incubated with TNF (100 U/ml) for various times (0, 5, 10, 15, 30, 45, or 60 min); PBS or IL-10 (30 ng/ml) was then added for 35 min, followed by fMLP (10^{-6} mol/L) for 5 min. The reaction was stopped and RBC were lysed with FACS solution (BD Biosciences). After one wash (400 $\times$ *g* for 5 min), white cells were suspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry on the same day.

Flow cytometry

We used a Becton Dickinson FACScan (BD Immunocytometry Systems, San Jose, CA) with a 15-mW 488-nm argon laser. Forward and side scatter were used to identify the granulocyte population and to gate out other cells and debris. The purity of the gated cells was assessed by using FITC- or PE-conjugated anti-CD3, CD45, CD14, and CD15 Abs (BD Biosciences). Ten thousand events were counted per sample, and the fluorescence pulses were amplified by 4-decade logarithmic amplifiers. The green fluorescence of FITC-avidin was recorded from 515 to 545. The orange fluorescence of ethidium was recorded from 549 to 601 nm (575 $\pm$ 26). All the results were obtained with a constant photomultiplier gain value. The data were analyzed with LYSIS II software (BD Biosciences, San Jose, CA) and the mean fluorescence intensity (MFI) was used to quantitate the responses. The effect of IL-10 on TNF priming of the PMN oxidative burst was calculated as the percentage inhibition, as follows: (MFI of the TNF-preincubated sample $-$ MFI of the TNF-preincubated sample treated with IL-10)/MFI of the TNF-preincubated sample $\times$ 100%.

Immunocytochemical staining of intracellular IL-10R

Smears of unstimulated blood from healthy donors were air-dried for 24 h and incubated in cold acetone/methanol (1:1) at 4°C for 10 min to fix and permeabilize the membranes. Nonspecific staining was blocked by a 5-min incubation with the blocking reagent from the Dako LSAB kit. The smears were then incubated with a rat anti-human IL-10R mAb (25 μg/ml; DNAX) for 30 min, followed by sequential 10-min incubation steps with a biotinylated goat anti-rat Ig Ab and alkaline phosphatase-labeled streptavidin, as recommended by the manufacturer (Dako LSAB Alkaline Phosphatase kit). Staining was revealed with freshly prepared substrate-chromogen solution. Counterstaining was then performed with hematoxylin and ammonia water. Positive staining developed as a fuchsia-colored reaction product. The negative control consisted of incubating smears with an irrelevant Ab of the same isotype, and the positive control with an anti-CD11b mAb.

Subcellular fractionation of isolated PMN

Human PMN were purified in sterile conditions by 2% dextran sedimentation and centrifugation on Ficoll-Paque cushions (38). Contaminating erythrocytes were removed by hypotonic lysis, and the purified neutrophils (100×10^6/ml) were suspended in PBS and treated with DFP (2.7 mM for 15 min at 4°C), then washed and resuspended in 5 ml of ice-cold relaxation buffer [100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L $ATPNa_2$, 3.5 mmol/L $MgCl_2$, 10 mmol/L PIPES, pH 7.2], until subcellular fractionation. To study translocation under stimulation, DFP-treated PMN (10×10^6/ml) were incubated in Hank's buffer supplemented with 0.05% BSA, in the absence or presence of PMA (100 ng/ml for 10 min at 37°C) or TNF (100 U/ml for 30 min at 37°C). The reaction was stopped by adding ice-cold PBS and subsequent centrifugation at 400 $\times$ *g* for 10 min at 4°C. Then PMN were resuspended in ice-cold relaxation buffer supplemented with antiproteases. Subcellular fractionation was performed as previously described (39). Briefly, PMN were pressurized with N_2 for 20 min at 450 psi with constant stirring in a nitrogen bomb. The cavitas was then collected dropwise into EGTA, sufficient for a final concentration of 1.25 mmol/L. Nuclei and unbroken cells were pelleted by centrifugation of the cavitas at 400 $\times$ *g* for 10 min. The supernatant was decanted, loaded at the top of a two-layer Percoll gradient (1.05/1.12 g/ml) precooled to 4°C, and spun at 4°C for 30 min at 40,000 $\times$ *g*. This resulted in a gradient with three visible bands (from the bottom: a band containing azurophil granules, a band containing specific and gelatinase granules, and a band containing plasma membranes and secretory vesicles). The cytosol remained above the upper band, on top of the Percoll gradient. The different fractions were then collected. The purity of the specific and azurophilic granule fractions was assessed by measuring their respective markers, lactoferrin and myeloperoxidase, in each fraction (azurophil granules, specific granules, and membranes) and in the total cavitas using ELISA methods (R&D Systems).

Electrophoresis and blotting

A cellular equivalent of total cavitas and of each subcellular fraction (cytosol, membranes, specific and azurophilic granules) was added to 2$\times$ Laemmli sample buffer, and proteins were electrophoresed in 9% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Protran BA83; Schleicher & Schuell, Keene, NH) at 100 V for 1 h in 25

mM Tris, pH 8.3, 192 mM glycine, and 20% methanol buffer. The membranes were first blocked overnight at 4°C in TBS-Tween (25 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.2% Tween 20) containing 7.5% BSA, and then incubated (60 min, 37°C) with a rabbit anti-human IL-10R polyclonal Ab (final concentration, 0.2 μg/ml; Santa Cruz Biotechnology). The membranes were then washed and incubated for 45 min at room temperature in TBS-Tween with a HRP-conjugated swine anti-rabbit Ig Ab (1/10,000 dilution). The immunoblots were developed using a chemiluminescence method (ECL; Amersham Life Sciences, Arlington Heights, IL) following the manufacturer guidelines. Mononuclear cells (lymphocytes and monocytes) obtained after separation of peripheral blood on Ficoll-Paque were loaded and blotted in parallel and served as a positive control.

Quantification of lactoferrin release after PMN stimulation

Whole blood collected in sterile lithium heparinate tubes was either kept on ice or incubated at 37°C for 30 min with TNF-α (100 U/ml), GM-CSF (100 U/ml), IL-1β (100 U/ml), IL-8 (50 ng/ml), or LPS (5 μg/ml), or for 5 min with PMA (100 ng/ml). Control samples were incubated with PBS. Samples were then centrifuged at 1500 $\times$ *g* for 15 min at 4°C. Plasma samples were stored at −70°C for no longer than 15 days before the assay. Lactoferrin in plasma was assayed in duplicate by using ELISA methods (R&D Systems) with a detection limit of 1 ng/ml. PMN counts in whole blood were obtained using an automated hemocytometer (H1; Bayer, Elkhart, IN). The measurement of lactoferrin in total cavitas of isolated PMN (as previously described) permitted us to evaluate the percentage of release from the intracellular pool to the extracellular medium.

Statistical analysis

All results are expressed as means ± SEM. Means were compared using Student's *t* test, and *p* values of 0.05 or less were considered significant.

Results

Characteristics of IL-10 binding to the PMN surface

After 30 min of incubation at 4°C, IL-10 barely bound to resting whole-blood PMN. The MFI of the sample incubated with biotinylated-rhIL-10 and revealed with FITC-avidin was moderately, although significantly, increased as compared with samples incubated with the biotinylated control reagent and FITC-avidin (8.3 ± 2.5 vs 5.2 ± 1.2, respectively; $n = 10$, $p = 0.0003$). In contrast, incubation of whole blood with 100 U/ml TNF-α for 30 min at 37°C strongly increased IL-10 binding to the PMN surface, with a MFI ~6-fold higher than that of the sample incubated in the same conditions with PBS (Table I). A slightly increased value of IL-10 binding was observed in the sample incubated with PBS alone at 37°C (10.75 ± 1.1) as compared with the values obtained at 4°C. The effect of TNF was concentration-dependent (Table I). Moreover, when a blocking anti-hIL-10 Ab was added before incubation with TNF, IL-10 binding to the PMN surface was suppressed (MFI: 4.6 ± 0.4), supporting the specificity of the reaction. A kinetic study showed that IL-10 binding induced by TNF began as early as 10 min after TNF treatment and increased in a time-dependent manner, with maximum expression at 30 min (Table II). IL-10 binding decreased 45 and 60 min after TNF treatment. This diminution was reversed by sample preincubation with primaquine, an endocytosis inhibitor (40) (Table II), whereas protease inhibitors had no effect (data not shown), suggesting an internalization of IL-10 receptors.

We then examined the regulation of IL-10 binding to the PMN surface by other proinflammatory mediators that are known to regulate PMN. As shown in Table III, incubation of whole blood with predetermined optimal concentrations of LPS (5 μg/ml for 30 min) or GM-CSF (100 U/ml for 30 min) increased IL-10 binding to the PMN surface. This increase in IL-10 binding was in the same range as that observed with the PKC activator PMA (100 ng/ml for 5 min), a potent stimulus of numerous PMN functions. Indeed, the stimulation index (SI) (ratio of the MFI of agonist-treated cells to that of PBS-treated cells) was ~4–5 in the presence of TNF, GM-CSF, LPS, or PMA. In contrast, in our conditions of stimulation, IL-1 (100 U/ml for 30 min) and IL-8 (50 ng/ml for 30 min) did not significantly modify specific IL-10 binding to the PMN surface, with a SI always <2 (Table III); other times and higher concentrations had no effect either (data not shown). Moreover, IL-1 and IL-8 did not down-regulate TNF-induced IL-10 binding to PMN (data not shown).

TNF increases IL-10 binding to the PMN surface by a degranulation process

The TNF-induced increase in IL-10 binding to the PMN surface was observed very rapidly after addition of the reagent and was maximal after 30 min of incubation. Furthermore, the kinetics of release was similar in the presence of GM-CSF and LPS at optimal concentrations (data not shown). As PMA is also a potent inducer of human neutrophil degranulation (41), these results suggested the translocation, to the PMN surface, of intracellular IL-10R. Thus we investigated whether the degranulation process after stimulation contributed to the increased IL-10R expression.

We tested the effect of PTX, a methylxanthine derivative known to inhibit neutrophil degranulation (42, 43), on IL-10 binding after TNF stimulation. Pretreatment of whole blood with PTX induced concentration-dependent inhibition of the TNF-induced increase in IL-10 binding (Table IV). The effect of TNF was completely reversed by 10 mM PTX.

Table I. *Effect of TNFα on IL-10 binding to the PMN surface*[a]

TNF (U/ml)	MFI
0	10.7 ± 1.1
0.1	9.0 ± 1.7
1	9.3 ± 0.9
10	22.5 ± 2.9[b]
100	61.1 ± 9.0[b]
500	61.8 ± 9.4[b]
1000	74.8 ± 12.3[b]

[a] Whole blood was incubated with TNFα at various concentrations (0.1–1000 U/ml) or without TNF in presence of PBS, for 30 min at 37°C, and then incubated with biotinylated IL-10 for 60 min at 4°C. Flow cytometric analysis of IL-10 binding to the PMN surface was performed with biotinylated IL-10 and revealed by FITC-avidin, as described in *Materials and Methods*. The MFI of the sample incubated at 37°C with PBS and then with the biotinylated irrelevant control was 4.5 ± 0.3, and was not modified by TNF stimulation. Values are means ± SEM ($n = 4$).

[b] Significantly different from sample incubated at 37°C with PBS alone ($p < 0.05$).

Table II. *Time course of IL-10 binding to the PMN surface in response to TNFα stimulation*[a]

	MFI	
Time (min)	TNF	TNF + primaquine
5	14.7 ± 2.1	16.0 ± 2
10	34.2 ± 4.5	32.2 ± 2.8
15	53.8 ± 6.4[b]	52.8 ± 3.1
30	56.9 ± 3.0[b]	56.0 ± 3.0
45	39.0 ± 4.6[b]	56.6 ± 2.9
60	32.4 ± 3.4[b]	58.6 ± 5.2

[a] Whole blood was stimulated with TNFα (100 U/ml) alone or combined with primaquine (250 μg/ml) at 37°C for various times (5–60 min) and then incubated with biotinylated IL-10 for 60 min at 4°C. Flow cytometric analysis of IL-10 binding to the PMN surface was performed by using biotinylated IL-10 and revealed by FITC-avidin, as described in *Materials and Methods*. The MFI of the sample incubated at 37°C with PBS and then with the biotinylated irrelevant control was 4.5 ± 0.3, and was not significantly modified by the incubation period. Values are means ± SEM ($n = 5$).

[b] Significantly different from sample incubated at 37°C with PBS alone ($p < 0.05$).

Table III. *Differential effects of incubation with proinflammatory agents on IL-10 binding to the PMN surface and on lactoferrin release*[a]

Reagents	IL-10 Binding		Lactoferrin Release	
	MFI	SI	ng/ml	SI
PBS	12.6 ± 1.5	1	623 ± 56	1
TNF (100 U/ml)	65.0 ± 9.4[b]	5.3 ± 1.1[b]	2996 ± 341[b]	4.9 ± 0.7[b]
GM-CSF (100 U/ml)	54.7 ± 5.5[b]	4 ± 0.7[b]	2299 ± 214[b]	3.8 ± 0.4[b]
IL-8 (50 ng/ml)	16.0 ± 3.0	1.3 ± 0.1	744 ± 96	1.2 ± 0.1
IL-1 (100 U/ml)	20.0 ± 3.0	1.5 ± 0.2	857 ± 101	1.3 ± 0.1
LPS (5 μg/ml)	53.0 ± 4.6[b]	3.9 ± 0.3[b]	2859 ± 675[b]	4.7 ± 1.2[b]
PMA (100 ng/ml)	57.7 ± 9[b]	5.0 ± 0.3[b]	3501 ± 760[b]	5.8 ± 1.5[b]

[a] Whole blood was incubated at 37°C for 30 min with PBS, TNF (100 U/ml), GM-CSF (100 U/ml), IL-8 (50 ng/ml), or IL-1 (100 U/ml), or for 5 min with PMA (100 ng/ml). IL-10 binding was measured as described in *Materials and Methods*, by using biotinylated IL-10 revealed by avidin-FITC and expressed as MFI. Lactoferrin was assayed with an ELISA method in the supernatants obtained after centrifugation at 1500 × g for 15 min at 4°C as described in *Materials and Methods*. The lactoferrin content, measured in the total cavitas of isolated PMN, was 30,200 ± 2,545 ng/ml in 4.2 ± 0.5 × 10^6 PMN. A SI was calculated as the ratio of agonist-treated versus PBS-treated samples. Values are means ± SEM ($n = 5$).

[b] Significantly different from sample incubated at 37°C with PBS alone ($p < 0.05$).

Furthermore, pretreatment of whole blood with cycloheximide (20 μg/ml) for 15 min did not significantly modify TNF-induced binding of IL-10: the MFI values of samples preincubated with and without cycloheximide were similar (56.7 ± 11.2 vs 57.7 ± 11.7; $n = 3$). This supported the hypothesis that IL-10R up-regulation was due to translocation of a pre-existing intracellular pool rather than de novo protein synthesis.

Resting human PMN contain an intracellular pool of IL-10R

To investigate the existence of an intracellular pool of IL-10R in resting human PMN, we performed intracellular immunostaining after PMN permeabilization, using both flow cytometry and immunocytochemical staining. Flow cytometry, performed after methanol permeabilization, revealed a pool of IL-10R in PMN. The MFI of the sample incubated with a biotinylated mouse anti-IL-10R mAb (R&D Systems) and then with FITC-streptavidin was significantly higher than that of the control (biotinylated isotypic control and FITC-streptavidin) (45 ± 2 vs 25 ± 1, respectively). Furthermore, the presence of intracellular IL-10R in PMN was confirmed by immunocytochemistry: a rat anti-human IL-10R mAb (DNAX) yielded positive staining inside unstimulated PMN, whereas no staining was observed with the negative isotype control (Fig. 1). Nevertheless, the staining was weaker than that observed with the anti-CD11b Ab used as a positive control (data not shown).

Subcellular localization of IL-10R in specific/gelatinase granules

To further localize the human PMN intracellular IL-10R pool, subcellular fractionation of human PMN was performed. The purity of fractionation was assessed by measuring the reference markers of specific and azurophilic granules, lactoferrin and myeloperoxidase, respectively, in each fraction (azurophil granules, specific/gelatinase granules, membranes) and in the total cavitas, using ELISA methods (R&D Systems). As shown in Table V, the measurements showed >95% lactoferrin in the specific/gelatinase fraction and >95% myeloperoxidase in the azurophil fraction, the other fractions containing <5% of each marker. Total cavitas, membranes, cytosol, specific/gelatinase, and azurophilic granule fractions were immunoblotted. Fig. 2 shows the presence of a band of ~90 kDa (migrating between the 77- and 103-kDa markers) in the specific/gelatinase granule fraction as well as in the total PMN cavitas. In contrast, no significant amounts of IL-10R were observed in the cytosol or in the azurophilic granules. Small amounts of IL-10R were found in the membranes (<5% of the specific granule content). The immunoreactive band detected in the total PMN cavitas, specific/gelatinase granules, and membranes migrated to the same region as that obtained with a cavitas of mononuclear cells used as a positive control. Stimulation with PMA (100 ng/ml, 10 min at 37°C) induced a 60% increase of the 90-kDa band located in the membrane fraction as compared with unstimulated cells (Fig. 3) with no detectable decrease in the intensity of the specific granule fraction. The amount of translocation from the specific/gelatinase granules to the membranes after stimulation was ~10% of the total IL-10R content (as measured by scanning of the autoradiography) (Fig. 3). Similar results were obtained with TNF-stimulated PMN (data not shown).

Differential effects of proinflammatory mediators on degranulation of lactoferrin

To explain the difference in the induction of IL-10 binding by the various proinflammatory agents and to confirm their differential mobilizing effects of specific granules to the cell surface, we assayed, in parallel to IL-10-binding, the extracellular release of lactoferrin (a marker of specific granules; Ref. 44). As shown in Table III, significant amounts of lactoferrin were released after 30 min of stimulation with TNF-α, GM-CSF, and LPS as compared with the PBS control, whereas stimulation with IL-1 and IL-8 did not induce significant lactoferrin release. The effects of TNF, GM-CSF, and LPS on lactoferrin release were similar to that of PMA, which is known to induce strong degranulation, in particular of specific granules. Variations of SI paralleled that of IL-10 binding using the different agonists. In addition, the amount of lactoferrin released into the extracellular medium after TNF, GM-CSF, LPS,

Table IV. *Effects of PTX on IL-10 binding to the PMN surface*[a]

Reagents	MFI
PBS alone	11.2 ± 1.3
PBS + TNF	65.0 ± 15.04
PTX 1 μM + TNF	67.25 ± 16.11
PTX 100 μM + TNF	40.75 ± 9.38[b]
PTX 1 mM + TNF	13.50 ± 1.32[b]
PTX 10 mM + TNF	5.0 ± 0.41[b]

[a] Whole blood was preincubated at 37°C with PBS or PTX at various concentrations (1 μM to 10 mM) for 5 min and then with TNF (100 U/ml, 30 min). Flow cytometric analysis of IL-10 binding to the PMN surface was performed using biotinylated IL-10 and revealed by avidin-FITC, as described in *Materials and Methods*. Results are expressed as MFI. Values are means ± SEM ($n = 3$).

[b] Significantly different from samples incubated with PBS + TNF ($p < 0.05$).

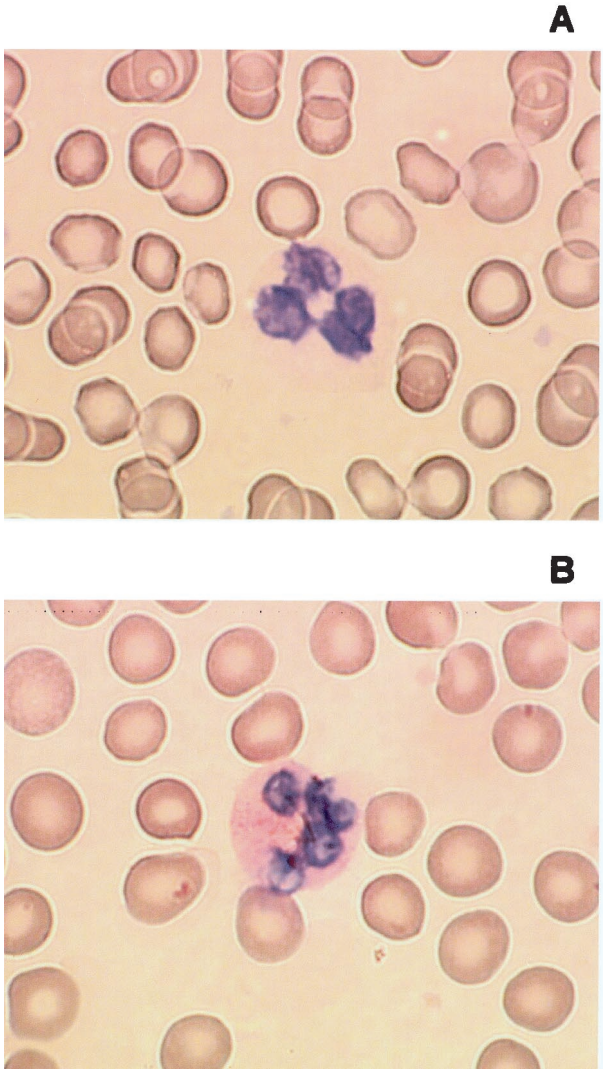

FIGURE 1. Immunocytochemical staining of intracellular IL-10R in whole blood smears. *A*, Negative control: no staining with control Ig. *B*, Intracellular fuchsia staining was observed with the specific monoclonal anti-IL-10R Ab. Smears were examined by light microscopy at ×1200.

and PMA was ~10% of the total content of PMN as calculated from the lactoferrin content measured in the total cavitas of isolated PMN and PMN counts in 1 ml of whole blood (mean ± SEM = 30,200 ± 2545 ng in 4.2 ± 0.5 × 10^6 PMN).

TNF-induced up-regulation of IL-10 binding to the PMN surface was associated with increased inhibitory effect of IL-10 on PMN oxidative burst

To assess the functional importance of the TNF-induced up-regulation of IL-10R, we studied the effect of IL-10 on $O_2^{-\circ}$ production

Table V. *Distribution of lactoferrin and myeloperoxidase among subcellular fractions from nitrogen-cavitated human neutrophils*[a]

Cellular Fractions	Lactoferrin (%)	Myeloperoxidase (%)
Cavitas	100	100
Membrane	<5	<5
Cytosol	<5	<5
Specific granules	>95	<5
Azurophilic granules	<5	>95

[a] Results are expressed as the percentage of the cavitas content of each marker. Data are from a representative experiment.

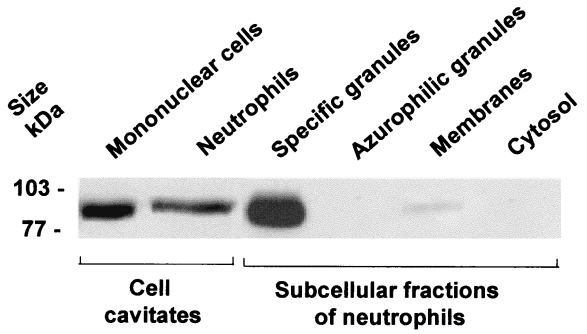

FIGURE 2. Immunoblotting of IL-10R in human neutrophils. A total cavitas, specific and azurophilic granules, membranes, and cytosol were prepared as described in *Materials and Methods*. Western blots were probed with a rabbit anti-IL-10R polyclonal Ab. A positive control (mononuclear cells) was loaded and blotted in parallel. The molecular masses of protein standards are indicated in kilodaltons.

by PMN in response to formyl peptides after TNF preincubation. IL-10 was added after various times of TNF stimulation. Our results showed that the inhibitory effect of IL-10 increased from 5% when IL-10 was added simultaneously with TNF to a maximum of 38 and 36% when IL-10 was added 15 and 30 min, respectively, after TNF treatment and thereafter decreased (Table VI). This IL-10 inhibitory effect on $O_2^{-\circ}$ production paralleled the kinetic of increased IL-10 binding at the PMN surface (Table II).

Discussion

This is the first report of an intracellular pool of IL-10R localized in specific human neutrophil granules, and their differential mobilization to the cell surface in response to inflammatory stimuli. IL-10 binding, which was very low at the surface of resting whole-blood PMN, strongly increased as early as 10 min after preincubation of whole blood with the proinflammatory reagents TNF, GM-CSF, and LPS. In contrast, IL-1 and IL-8 did not significantly modify IL-10 binding to the PMN surface. Given the anti-inflammatory properties of IL-10, this differential modulation of IL-10 binding could play a critical role in balancing beneficial and detrimental PMN activities at inflammatory sites.

This study was performed with whole-blood PMN to minimize cell activation related to isolation procedure (35). The low-level binding of IL-10 to the surface of resting PMN, which confirms data published by Bovolenta et al. (29), may explain the rarity of data on the direct effect of IL-10 on PMN functions (17), whereas IL-10 is widely reported to modulate LPS- and TNF-stimulated functions such as proinflammatory cytokine down-regulation. We clearly show that IL-10 binding to the PMN surface is strongly and

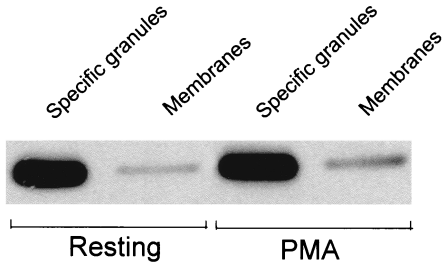

FIGURE 3. Translocation of IL-10R from specific granules to the plasma membrane in PMA-stimulated PMN. PMN were incubated for 10 min in the presence or absence of PMA (100 ng/ml) at 37°C. Specific granules and membranes were prepared as described in *Materials and Methods*. Western blots were probed with a rabbit anti-IL-10R polyclonal Ab.

Table VI. *Inhibitory effect of IL-10 on fMLP-induced O_2°-production by PMN after various times of TNF preincubation*[a]

Time (min) of TNF Preincubation	MFI		% Inhibition by IL-10[b]
	TNF + PBS	TNF + IL-10	
0	133.8 ± 8.1	125.0 ± 7.4	5.2 ± 3.1
5	129.5 ± 8.4	108.8 ± 6.8	16.0 ± 5.7
10	160.0 ± 13.0	125.2 ± 14.2[c]	22.2 ± 2.6
15	149.8 ± 13.5	90.5 ± 5.7[c]	38.5 ± 5.8
30	148.0 ± 46.8	85.8 ± 16.4[c]	36.5 ± 7.0
45	114.3 ± 20.5	96.7 ± 4.4	10.7 ± 3.0
60	103.0 ± 9.7	100.3 ± 3.2	4.7 ± 3.0

[a] After preincubation with HE (1500 ng/ml) for 15 minutes at 37°C, whole blood was treated with TNF (100 U/ml) for various times. Then, Il-10 or PBS was added to 35 min, followed by fMLP (10^{-6} mol/L) for 5 min. The MFI of the samples incubated with TNF, IL-10, or fMLP alone was always <20 and did not differ significantly from that of the sample incubated with PBS alone.

[b] The percentage of IL-10 inhibition was calculated by using the ratio: MFI (TNF) − MFI (TNF + IL-10)/MFI (TNF) × 100%.

[c] Significantly different from samples treated with TNF and PBS.

very rapidly (10 min) up-regulated by TNF, GM-CSF, LPS, and PMA. The diminution of IL-10 binding observed after 45 and 60 min of TNF treatment was reversed by primaquine, an endocytosis inhibitor (40), suggesting an internalization of IL-10 receptors.

This rapid up-regulation of IL-10 binding to the PMN surface, together with the observation that cycloheximide did not inhibit TNF-induced IL-10 binding, strongly suggested TNF-induced translocation of a pre-existing pool of IL-10R instead of de novo synthesis of the receptor by PMN, at least at the early times studied here. This intracellular pool of IL-10R was detected in permeabilized neutrophils by both flow cytometry and immunocytochemistry, using two different Abs. It is noteworthy that these Abs did not reveal IL-10R expression at the PMN surface by flow cytometry, even after optimal stimulation. These results are in keeping with the literature and with a low density of IL-10R expression on hemopoietic cells (19, 20, 24, 26–28). The presence of IL-10R in PMN was confirmed by Western blot analyses using another anti-human IL-10R1 Ab on total extract of PMN disrupted by cavitation, showing a band near the 90 kDa standard. The apparent molecular mass of ~90 kDa is in keeping with previous data obtained with cell lines expressing rhIL-10R, and with mononuclear cells (20, 26).

After subcellular fractionation, Western blotting also showed the same band in the specific granule fraction, but not in cytosol or in the azurophilic granules. A band of <5% the intensity of that observed in specific granules was detected in the membrane fraction of resting PMN. The 60% increase in IL-10R observed in the membrane fraction after stimulation of PMN by PMA is in accordance with data concerning rap proteins, predominantly associated with specific granules, which increase in similar amounts as IL-10R in the plasma membranes following PMA stimulation (45). The low level of translocation from the specific granules to the cell surface (10% of the total specific granule IL-10R pool) is in accordance with previous reports concerning proinflammatory mediator-induced degranulation of other molecules located in specific granules, i.e., lactoferrin and vitamin B-12-binding protein, in contrast to observations for molecules in the more easily mobilizable granules such as secretory vesicles (46, 47). This low level of degranulation of specific granules was confirmed here with TNF, GM-CSF, and LPS, which all induced a release of ~10% of the total lactoferrin content in our experimental conditions (Table III).

To further understand the differences between TNF, GM-CSF, and LPS on the one hand and IL-1 and IL-8 in contrast in the enhancement of IL-10 binding to the PMN surface, we measured in parallel, in the same whole-blood conditions, the extracellular release of lactoferrin. We observed the same differential effects of the proinflammatory mediators on lactoferrin release as on IL-10 binding, with parallel variations in stimulation indices. These data suggest that differences in specific granule mobilization could explain the differences in IL-10 binding.

That TNF, GM-CSF, LPS, and PMA induced increased IL-10 binding is related to translocation from the specific granules to the membranes is further supported by the observation that PTX, which inhibits degranulation, completely reversed the effect of TNF on IL-10 binding to the PMN surface. In addition, we have previously shown that PMA- and TNF-induced vascular endothelial growth factor release from specific granules is inhibited by PTX (42). Lastly, PTX also reduced TNF-induced CD11b up-regulation at the PMN surface (43).

A low level of translocation of different molecules in PMN has been shown to trigger a biological effect, e.g., <10% translocation of p47phox is sufficient to induce NADPH oxidase activity with no detectable decrease in the cytosolic fraction (48). It appears that the low level of IL-10R translocation can indeed account for the increased IL-10 binding and the biological effect of the cytokine. Indeed, we demonstrated that IL-10 significantly inhibited PMN oxidative burst in response to formyl peptides when IL-10 was added 15 or 30 min after TNF treatment. This kinetic pattern matched that of the increase in IL-10 binding during TNF incubation, suggesting that TNF-induced up-regulation of IL-10 binding to the PMN surface may play an important role in the regulation of inflammatory reactions by inhibiting neutrophil activation and thereby limiting tissue injury.

In addition to their role as phagocytic and killer cells, PMN can produce and respond to numerous cytokines that regulate the immune processes. PMN potentiate inflammatory responses by releasing proinflammatory cytokines such as IL-8 and TNF, and generate ROS in response to various stimuli. Some proinflammatory cytokines have been found to prime or activate PMN production of ROS. Thus PMN are a first line of defense against pathogens, and participate in the regulation of inflammatory responses. However, excessive or inappropriate PMN responses can lead to vascular or tissular injury. In acute settings, IL-10 production plays a major role in establishing an anti-inflammatory balance, by down-regulating the inflammatory response and thereby limiting tissue damage (49). Our results demonstrating that functional IL-10R may be rapidly up-regulated by stimuli such as TNF, GM-CSF, and LPS, which are potent inflammatory mediators, points to a potential in vivo role of IL-10R in down-regulating PMN activities and thereby preventing inappropriate inflammatory responses.

In conclusion, this study shows that PMN contain an intracellular pool of IL-10-receptors localized in specific granules. We also observed increased IL-10 binding to the PMN surface after stimulation with LPS and the proinflammatory cytokines TNF and GM-CSF, but not with IL-1 and IL-8. This differential enhancement of IL-10 binding was related to differential mobilization of the relevant specific granules by these agents. Differential modulation of IL-10 binding may play a key role in optimal regulation of inflammatory responses.

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