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Interleukin-10 controls human peripheral PMN activation triggered by lipopolysaccharide

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ABSTRACT

Large amounts of anti-inflammatory mediators, such as interleukin (IL)-10, are produced and found early in the course of sepsis. We explore the role of IL-10 on neutrophil (PMN) activation/function using an *in vitro* model. Isolated human PMN were pre-incubated with lipopolysaccharide (LPS) and/or IL-10 for 18 h. Subsequently, a second LPS exposure was performed and CD11b and CD66b up-regulation, and the reactive oxygen species (ROS) generation were measured 2 h later. We found that IL-10 prevented PMN activation and the secretion of TNF-α and IL-8 induced by the first LPS contact. In the absence of IL-10, a second LPS exposure induced additive effects that were prevented by IL-10. Only ROS generation was highly affected by the blockade of PMN-secreted TNF-α or IL-8. Additionally, IL-10 prevented other possible mechanisms of LPS priming. Therefore, IL-10 modulates PMN activation preventing autocrine activating loops and priming mechanisms, rendering PMN less responsive to a second LPS exposure.

1. Introduction

For a long time, sepsis and other non-infectious Systemic Inflammatory Response Syndromes (SIRSs) have been considered to be the result of an overwhelming proinflammatory state, characterized by the presence of large amounts of systemic proinflammatory mediators. However, large amounts of anti-inflammatory mediators are concomitantly present within the blood compartment. For example, together with the production of proinflammatory cytokines, high levels of Interleukin (IL)-10 and IL-1 receptor antagonist (IL-1Ra) have been found early after surgery, cardiac arrest and at admission in sepsis patients [1–4]. These observations and others led to the postulation that the systemic anti-inflammatory response to stress is not just compensatory, but dominates outside the affected local site (e.g. plasma) to prevent systemic inflammation [5,6]. In line with this hypothesis, treatment with the anti-inflammatory cytokine IL-10 protects mice against endotoxic shock by preventing excessive production of proinflammatory cytokines [7,8].

IL-10 exhibits potent anti-inflammatory activities [9]. The direct effects of IL-10 on different leukocyte populations have been previously investigated. In this sense, the exposure of mononuclear phagocytes or dendritic cells to IL-10 inhibits the synthesis of proinflammatory cytokines, the release of reactive oxygen and nitrogen intermediates, as well as the antigen-presenting capacity of these cells. IL-10 may also suppress proliferative and cytotoxic T cell responses and cytokine production by Th1 cells. However, the role of IL-10 on polymorphonuclear neutrophil (PMN) activation/function has been less explored. In this sense, it has been reported that IL-10 downregulate the delayed production of platelet activating factor and superoxide anions triggered by direct LPS stimulation [10], and markedly inhibited proinflammatory cytokine secretion in PMN from patients with intestinal inflammation [11].

Although PMN are essential for host defense against microbial infections, excessive activation can also be associated with pathological side effects of tissue destruction [12]. PMN-mediated mechanisms involved in tissue injury include the production of toxic reactive oxygen species (ROS), and the proteolytic capacity of enzymes stored in granules [12]. Additionally, cytokine secretion has also been reported upon PMN activation [13–15]. Our hypothesis is that IL-10 from septic plasma may control systemic PMN activation. A two-hit *in vitro* model was used to test this hypothesis. Human peripheral PMN isolated from healthy donors were first pre-incubated with LPS and/or IL-10 for 18 h. After that, a second LPS exposure was performed and different activation parameters were measured 2 h later. With this model we could demonstrate that, in response to a first LPS contact, IL-10 is able to prevent priming mechanisms and secretion of cytokines that autocrinally

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amplify PMN activation, and therefore PMN are rendered less responsive to a second LPS exposure.

2. Materials and methods

2.1. Blood samples

Blood samples were obtained from healthy volunteer donors who had taken no medication for at least 10 days before the day of sampling. Blood was drawn from the forearm vein and was discharged directly into citrated plastic tubes.

2.2. Neutrophil isolation

Neutrophils were isolated by Ficoll–Hypaque gradient centrifugation (Ficoll Pharmacia, Uppsala, Sweden; Hypaque, Wintthrop Products, Buenos Aires, Argentina) and dextran sedimentation as previously described [16]. Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells (96% neutrophils on May-Grünwald-Giemsa-stained Cyto-preps) were suspended in RPMI 1640 supplemented with 10% de human serum, 1% de glutamine and 1% antibiotic/antimycotic.

2.3. Experimental procedure

1×10^6 isolated PMN were incubated in a 48-well plate with medium, LPS (1 ng/ml), IL-10 (20 ng/ml) or LPS + IL-10 for 18 h (first hit). Then, cells were incubated with LPS (100 ng/ml) or lipoteichoic acid (50 μg/ml) for two additional hours (second hit). In other set of experiments, a soluble TNF-α receptor (sTNFR, etanercept, 20 ng/ml) (Enbrel; Wyeth Inc., Collegeville, PA, USA), a IL-8 receptor antagonist (SB225002 was purchase from Sigma, and therefore "(Sigma, St. Louis, USA)", 300 nM), or an anti-human neutralizing polyclonal IL-10 antibody (25 μg/ml, PreproTech, NJ, USA) were added at the beginning of the cultures.

To assess the viability of PMN after the 18-h incubation period, quantification of apoptosis was performed as previously described [17], using the fluorescent DNA-binding dyes acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells. With this method, nonapoptotic cell nuclei show variations in fluorescent intensity that reflect the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. To assess the percentage of cells showing morphologic features of apoptosis, at least 200 cells were scored in each experiment. If more than 30% of apoptotic PMN were observed in any of the treatments, results obtained in that donor were not included in the analysis.

2.4. Flow cytometric analysis

Measurement of the expression of TLR4, CD11b and CD66b on neutrophils was performed by direct immunofluorescence flow cytometry. After incubation neutrophils were stained with anti-human monoclonal antibodies (Abs) for 30 min at 4 °C. The control of isotype-matched Ab was assayed in parallel. Then cells were washed with cold PBS and suspended in 0.3 ml of ISOFLOW. Fluorescence was measured with a Becton Dickinson FACScan. The analysis was made on 10,000 events on each sample using the CellQuest program. Debris were excluded from the analysis.

2.5. ROS generation

PMN were incubated with dihydrorhodamine 123 (DHR, Sigma, St. Louis, USA) (1 μM) at 37 °C in 5% CO_2. Upon ROS generation a brightly fluorescent FL-1 product is produced, which was detected by flow cytometry.

2.6. IL-8 determination

IL-8 determination was performed on 100 μl of the culture supernatant by sandwich ELISA. Briefly, an ELISA 96-well plate (Greiner-Bio One, Frickenhausen, Germany) was covered overnight with 5 μg/ml of anti-IL-8 capture antibody (BD Pharmingen, NJ, USA). Samples were added and incubated at room temperature for 2 h. The biotinylated anti-cytokine detection antibody (2 μg/ml, BD Pharmingen, NJ, USA) was added followed by a 1:10,000 dilution of streptavidin-horseradish peroxidase (Caltag Laboratories, CA, USA) and a solution of 0.4 mg/ml *o*-phenylenediamine (OPD, Sigma, St. Louis, USA) in 0.1 M citric acid, 0.05 M Na_2HPO_4, 3% H_2O_2. The reaction was stopped with H_2SO_4 2 M and the resultant absorbance was read at 492 nm. The amount of IL-8 (pg/ml) was extrapolated using a standard curve constructed over the range of 25–1000 pg/ml of human recombinant IL-8 (BD Pharmingen, NJ, USA).

2.7. TNF-α measurement

The amount of tumor necrosis factor alpha (TNF-α) was determined by a cytotoxicity bioassay using the sensitive fibroblast cell line L929 as previously described [18]. Briefly, 2×10^4 L929 cells per well were seeded into a 96-well tissue culture plate and cultured in RPMI medium supplemented with 5 μg/ml streptomycin, 5 U/ml penicillin, and 10% fetal calf serum. The medium was replaced with 0.1 ml of medium alone (100% viability) or PMN-derived supernatants and 10 μg/ml of actinomycin D (Amersham Biosciences, BA, UK) was added. After 24 h of incubation, the cells were washed with warm PBS, and viable cells were fixed and stained with 0.2% crystal violet in 20% methanol for 20 min at 37 °C. Samples were washed and lysed with 30% acetic acid solution. The resultant absorbance (Abs) was measured with a microtiter plate reader (Organon Tecnika, Argentina) at 550 nm. Bioactive TNF-α was expressed as a percentage of L929 cytotoxicity. This percentage of L929 cytotoxicity was calculated by the following formula:

Percent L929 cytotoxicity = 100 × (Abs of 100% viable cells in control wells – Abs of tested wells)/(Abs of 100% viable cells in control wells).

A commercial ELISA kit (R&D Systems, MN, USA) was also performed following the manufacturer instruction.

2.8. Alkaline phosphatase activity determination

PMN alkaline phosphatase (alkP) was determined measuring the conversion p-nitrophenyl-phosphate to nitrophenol. For these assays, PMN were culture in the same conditions that mention above but RPMI lacked phenol red. Cells were gently scraped from the well, washed and resuspended in PBS 1×. Supernatants or pelleted-PMN were incubated for 30 min at 37 °C with 2 mg/ml of p-nitrophenyl-phosphate (pNPP, Sigma, St. Louis, USA) in 100 mM of 2-amino-2-metil-1-propanol buffer (pH = 10, Sigma, St. Louis, USA) supplemented with 1 mM $MgCl_2$. The reaction was stopped by addition of 2 N NaOH and the resultant absorbance was determined at 405 nm in Asys UVM340 Microplate Reader (Biochrom Ltd., CB, UK). The enzymatic activity was expressed as units (U) of alkP, where 1 U represents the amount of enzyme that dephosphorylates 1 μmol of pNPP/min.

2.9. Statistics

Statistical analysis of the data was performed using the Friedman test (non-parametric, paired), comparing all pairs of columns using the Dunns post test. A $p < 0.05$ was considered significant.

3. Results

3.1. IL-10 attenuates activation of LPS-sensitized PMN in response to a second LPS exposure

Upon PMN activation, mobilization of intracellular granules results in the release of proteolytic enzymes, but also in the up-regulation of adhesion molecules in the plasmatic membrane. As depicted with white bars in Fig. 1, LPS pre-incubation for 18 h (LPS/−) caused a significant increase in the surface expression of both CD11b (Fig. 1A) and CD66b (Fig. 1B). The simultaneous addition of IL-10 (LPS + IL10/−) was able to reverse this up-regulation. After the 18 h period, when a dose of LPS 100 times higher was added for two additional hours (black bars) to untreated PMN (−/LPS), an increased in the surface expression of CD11b and CD66b was found compared to control PMN (−/−), and although the increased tended to be higher with respect to PMN pre-incubated with LPS (LPS/−), the differences between −/LPS and LPS/− groups did not reach statistical significance. Additionally, the 18 h LPS pre-incubation primed PMN to the second LPS incubation (LPS/− compared to LPS/LPS), as additive increases were observed. The 18 h pre-incubation with IL-10 alone was not able to stop up-regulation of the markers caused by the second LPS hit (IL-10/LPS), but annulled the priming effect when the 18 h pre-incubation was performed simultaneously with LPS (LPS + IL-10/LPS).

The respiratory burst was also measured in PMN after the different treatments using DHR (Fig. 1C). This dye becomes fluorescent in the presence of reactive oxygen species (ROS), especially H_2O_2. Similar to what was found for the activation markers, the 18 h pre-incubation with LPS (LPS/−) caused an increase in the percentage of DHR positive PMN, and this was reversed by the

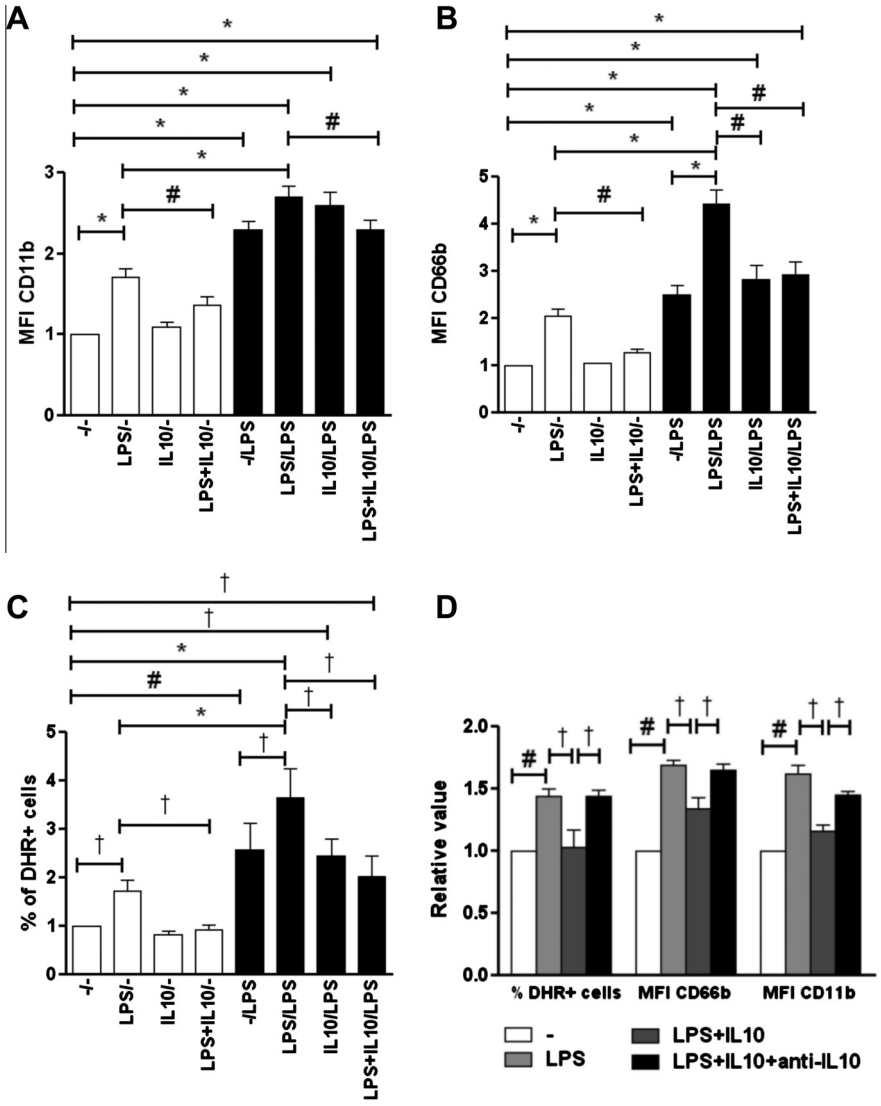

Fig. 1. IL-10 effects on different activation parameters of PMN. Isolated human peripheral PMN (1×10^6) were pre-incubated with LPS (1 ng/ml), IL-10 (20 ng/ml) or LPS + IL-10 for 18 h and then were left untreated (white bars) or were incubated for two additional hours with 100 ng/ml of LPS (black bars). The mean fluorescence intensity (MFI) of CD11b (A), CD66b (B) and the percentage of DHR positive cells (C), were determined by flow cytometry. Results were expressed as mean ± SE, as relative values with respect to untreated PMN (−/−); $n = 11$. $^{\dagger}p < 0.05$, $^{\#}p < 0.01$, $^{*}p < 0.001$. (D) PMN (1×10^6) were incubated with LPS (1 ng/ml), IL-10 (20 ng/ml) or an anti-IL-10 antibody for 18 h and the percentage of DHR positive cells and the MFI of CD66b and CD11b were determined. Results were expressed as mean ± SE as relative values with respect to untreated PMN (-); $n = 3$. $^{\dagger}p < 0.05$, $^{\#}p < 0.01$.

simultaneous addition of IL-10 (LPS + IL10/−). An additive effect was observed in the LPS/LPS group that was annulled when IL-10 was present during the pre-incubation period (LPS + IL-10/LPS).

Lower doses of IL-10 (1 and 10 ng/ml) were also assayed for CD11b and CD66b membrane expression and the % of DHR positive cells. Although the LPS-induced respiratory burst was highly inhibited by lower concentrations of IL-10, the effects on the other parameters were not so evident, and therefore we chose 20 ng/ml to evaluate IL-10 maximal effects for the three parameters assayed (data not shown).

As depicted in Fig. 1D, IL-10 neutralization of LPS-induced activation was abolished by the presence of an anti-IL-10 antibody.

3.2. Short-term activation of PMN by LPS is not affected by IL-10

During a 18 h pre-incubation period, PMN activation may reflect direct LPS interaction and indirect activation caused, for example, by factors released by LPS-activated PMN. Rapid measurement of up-regulation of activation markers and respiratory burst may account for direct LPS-activation, since amplification loops are less probable in short-term incubations. Therefore, the effects of IL-10 on the PMN response to LPS were assayed after 2 h. As shown in Fig. 2, in these conditions IL-10 was not able to stop the up-regulation of CD11b, CD66b or DHR positive cells caused by a short-term incubation with 100 ng/ml of LPS. When we used 1 ng/ml of LPS to stimulate PMN for only 2 h, a very weak response was observed, but similar to what was found for 100 ng/ml, IL-10 did not reverse LPS effects (data not shown). These results suggest that direct LPS activation is not influenced by IL-10, and this cytokine may be regulating the LPS response acting on indirect mechanisms triggered by LPS.

3.3. IL-10 attenuates activation of LPS-sensitized PMN in response to lipoteichoic acid

In order to study if the simultaneous incubation of LPS + IL-10 was also able to reverse the activation triggered by a heterologous stimulus, a second hit of lipoteichoic acid (LA, the main constituent of Gram-positive bacteria) was used to challenge PMN instead of LPS. As shown in Fig. 3, LA caused an up-regulation of CD11b and CD66b and increased the percentage of DHR positive PMN in untreated PMN (−/LA). Additionally, only in the case of CD66b, LA caused an additive effect on LPS-sensitized PMN (LPS/LA, Fig. 3B). The pre-incubation with LPS + IL-10 abolished the stimulatory effects of LA (LPS + IL10/LA) in all parameters. To discard possible LPS contamination in the LA preparation, polymyxin (PMX) was added together with LA. The results obtained for CD11b and CD66b modulation did not change in the presence of PMX (data not shown), ruling out the possible contribution of contaminating LPS in the preparation of LA.

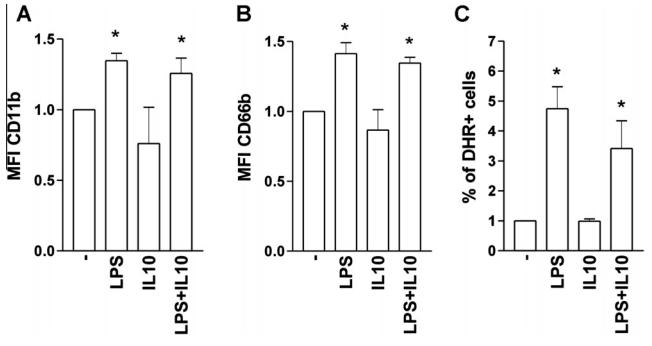

Fig. 2. IL-10 does not prevent LPS-induced short-term activation of PMN. Isolated human peripheral PMN (1×10^6) were incubated with LPS (100 ng/ml), IL-10 (20 ng/ml) or LPS + IL-10 for 2 h, and the mean fluorescence intensity (MFI) of CD11b (A), CD66b (B) and the percentage of DHR positive cells (C), were determined by flow cytometry. Results were expressed as mean ± SE, as relative values with respect to untreated PMN (-); $n = 6$. $^*p < 0.05$ vs. –.

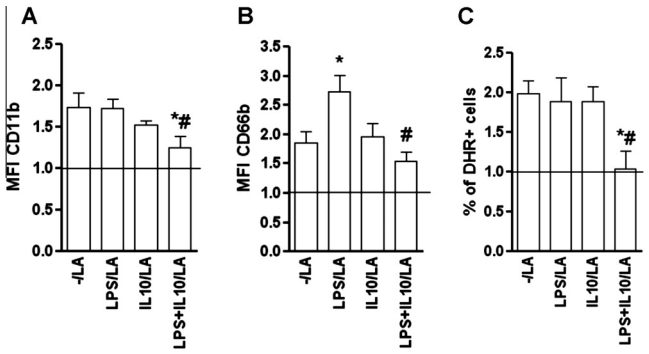

Fig. 3. IL-10 incubation prevents activation triggered by a heterologous stimulus in LPS-sensitized PMN. Isolated human peripheral PMN (1×10^6) were pre-incubated with LPS (1 ng/ml), IL-10 (20 ng/ml) or LPS + IL-10 for 18 h. After this, cells were incubated for two additional hours with lipoteichoic acid (LA, 50 μg/ml) and the mean fluorescence intensity (MFI) of CD11b (A), CD66b (B) and the percentage of DHR positive cells (C), were determined by flow cytometry. Results were expressed as mean ± SE, as relative values with respect to untreated PMN (−/−); $n = 8$. $^*p < 0.05$ vs. −/LA, $^\#p < 0.05$ vs. LPS/LA.

3.4. IL-10 prevents different mechanisms that cause autocrine amplification loops

We then studied different mechanisms by which IL-10 could be interfering with PMN-activation in response to the second exposure to LPS. As shown in Fig. 4A and B, LPS caused an increased secretion of TNF-α and IL-8, whereas the simultaneous addition of IL-10 annulled this secretion. The activation of alkaline phosphatase (alkP), an enzyme found in secretory vesicles of PMN, has been shown to mediate LPS inactivation [19,20]. Therefore, it is possible that LPS priming may be associated with a decreased of this mechanism that limits LPS action. We found a decreased alkP activity in PMN exposed to LPS that was reversed by the concomitant treatment with IL-10 (Fig. 4C). As alkP can be release upon PMN activation, the activity of this enzyme was also measured in the culture supernatants. No alkP activity was observed in these supernatants in any condition assayed (data not shown). Another mechanism that could contribute to LPS priming is the expression of molecules involved in LPS signaling. In this sense, we measured surface expression of TLR4 and found an increased after LPS treatment which, again, was reversed by IL-10 (Fig. 4D). As shown in Fig. 4, the inhibitory effect of IL-10 on cytokine secretion, loss of alkP activity and TLR4 up-regulation induced by LPS were annulled by a anti-IL-10 antibody.

3.5. The blockade of autocrine-produced TNF- or IL-8 prevents ROS generation but has less influence on degranulation of LPS-treated PMN

To establish the contribution of TNF-α and IL-8 on PMN priming and/or activation by LPS, a soluble TNF-α receptor (sTNFR) that blocks TNF-α action or an IL-8 receptor antagonist (SB225002) were added at the beginning of the culture (Fig. 5). First, to evaluate the effectiveness of these blockades, the activity of TNF-α and IL-8 were determined. For TNF-α, the activity of this cytokine was evaluated in supernatants of LPS-treated PMN using the sensitive cell line L929, and no TNF-α activity could be detected in the presence of the sTNFR (data not shown). To evaluate the blockade of IL-8 action, a chemotaxis assay was performed. PMN showed no

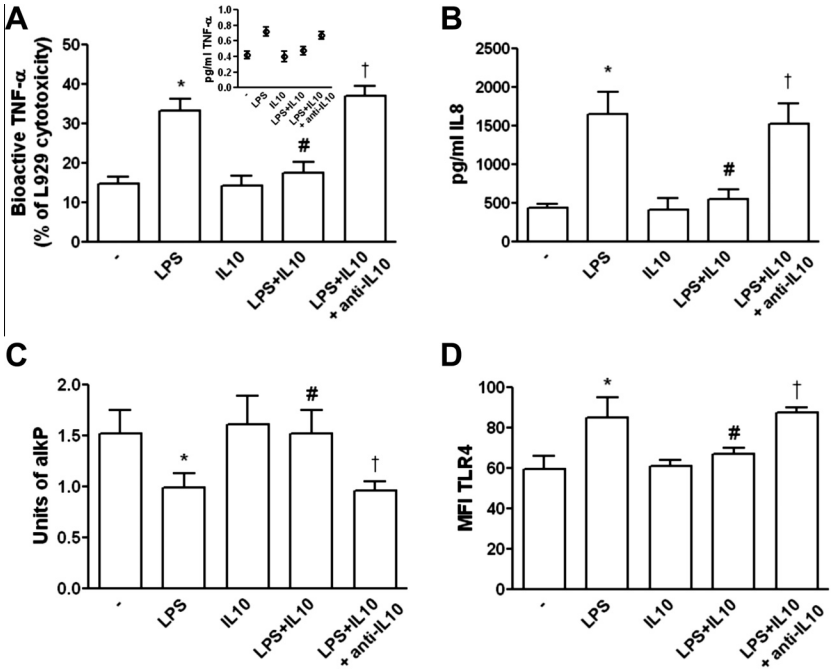

Fig. 4. IL-10 blocks different mechanisms that amplify or prime LPS responses in PMN. (A) TNF-α activity was determined in the culture supernatants by the L929 cytotoxic assay as percent cell cytotoxicity (mean ± SE, $n = 12$). *Inset*: pg/ml of TNF-α determined by ELISA. (B) IL-8 (pg/ml) was determined by ELISA in the culture supernatants (mean ± SE, $n = 12$). (C) The enzymatic activity of alkaline phosphatase (alkP) was measured on PMN by conversion of p-nitrophenyl-phosphate (pNPP) to nitrophenol and expressed as units (U) of alkP, where 1 U represents the amount of enzyme that dephosphorylates 1 μmol of pNPP/min (mean ± SE, $n = 8$). (D) The mean fluorescence intensity (MFI) of the LPS receptor TLR4 was measured on PMN by flow cytometry (mean ± SE, $n = 6$). In all cases an anti-IL-10 antibody was also included ($n = 3$), $^{*}p < 0.05$ vs. –, $^{\#}p < 0.05$ vs. LPS, $^{\dagger}p < 0.05$ vs. LPS + IL10.

chemotaxis towards IL-8 in the presence of SB225002 (data not shown).

When analyzing CD11b, we found that the presence of sTNFR or SB225002 were not able to interfere with the CD11b up-regulation caused by the first or second hit of LPS (LPS/− and −/LPS, respectively) (Fig. 5A). For CD66b (Fig. 5B), the presence of the sTNFR decreased in part the up-regulation of this molecule in the first LPS hit (LPS/−), and in the LPS/LPS, but the SB225002 had no effects (Fig. 5B). On the other hand, the % of DHR positive cells in response to LPS was highly affected by the presence of the sTNFR and SB225002. In this sense, the blockade of TNF-α or IL-8 prevented ROS generation in response to the first LPS exposure (LPS/−) and annulled the additive effect in the LPS/LPS group (Fig. 5C).

4. Discussion

The concept that sepsis is a result of an overwhelming production of proinflammatory mediators, which is lately followed by an anti-inflammatory response, is being review, as both types of mediators can be found early and simultaneously within the blood compartment in septic patients [6]. In this sense, IL-10 is a central modulator of the often lethal overabundant production of proinflammatory cytokines. IL-10 is usually found in septic plasma, and in different animal models of sepsis neutralization of IL-10 results in an exaggerated proinflammatory state and death, whereas administration of recombinant IL-10 confers significant therapeutic protection [7,8]. Taking these observations into account, in this study we explore the effects of IL-10 on peripheral PMN activation triggered by the simultaneous presence of the strongest bacterial stimulator of the inflammatory response, LPS. PMN are essential for host defense against microbial infections but can also be associated with the pathologic side effects of tissue destruction [12,21]. Therefore, it is extremely important to regulate their activation to limit an excessive, harmful response. Potentially PMN-mediated damaging mechanisms include the production of toxic reactive oxygen species (ROS), and the proteolytic capacity of enzymes stored in granules. These storage organelles are not only a source of proteolytic or bactericidal proteins, but are also important reservoirs of membrane proteins that become incorporated into the surface membrane of PMN when these organelles fuse with the plasma membrane and exocytose their content. It is now established that CD11b is mainly found in secretory vesicles and gelatinase granules and CD66b is present in the membrane of specific granules. Therefore, in order to measure PMN activation, we used a functional parameter (ROS generation) and the up-regulation in the plasma membrane of two markers found in different types of PMN granules. Our analysis does not include measurements of mRNA or protein levels expression. Molecules within granule content are synthesize and stored during PMN development in the bone marrow. Since, to our knowledge, *de novo* transcription of CD11b and CD66b in the blood is absence, in a biological and immunological context the most significant data is the modulation of their membrane expression (regardless of their intracellular levels) that allows the interaction/adhesion with the microenvironment.

We set up an *in vitro* two-hit model that allows to determine the response of the same PMN population that are expose to LPS for a first or a second time and the modulation of this response by the simultaneous presence of IL-10. This model can be questioned by the fact that PMN are short-living cells that become apoptotic rapidly in culture. However, some adjustments were performed in order to minimize PMN death, namely cultures were performed in cell culture plates, human serum was used instead of fetal calf serum, and glutamine was added. In these conditions less than 30% of apoptosis was seen at 18 h. Moreover, to consider only the live PMN population during flow cytometry analysis, cell debris with low FSC and/or SSC profiles (death cells) were excluded. Rarely, higher percentages of apoptosis were observed and the results of these donors were excluded.

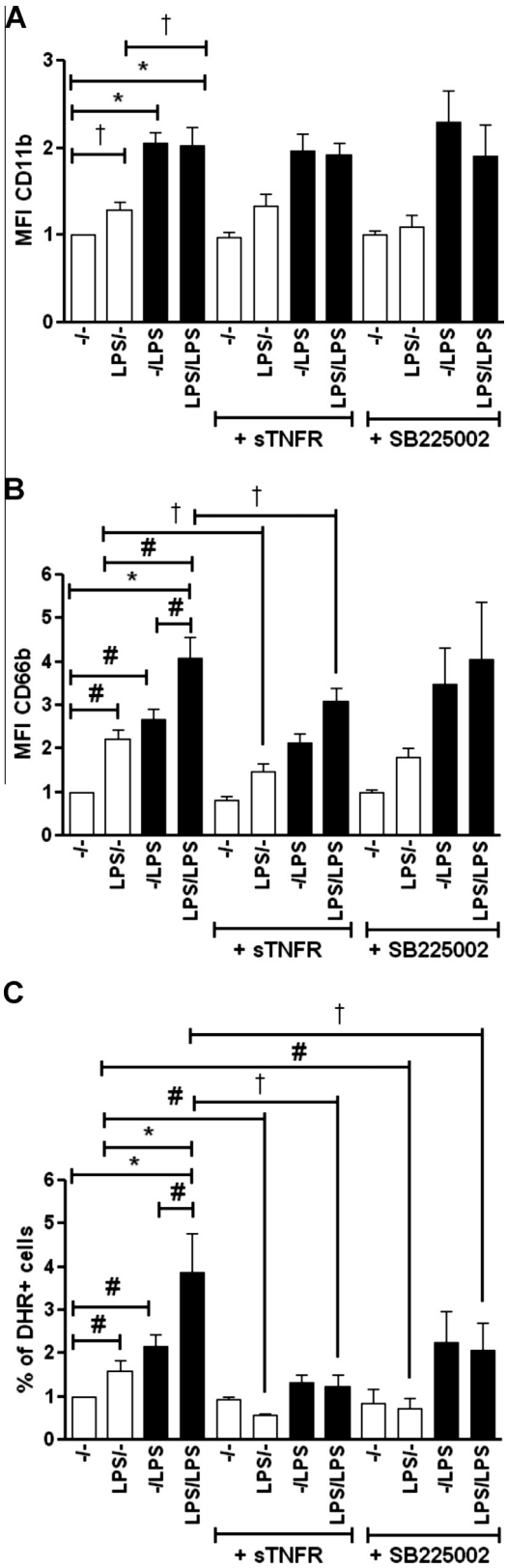

Fig. 5. Effects of blocking the actions of TNF-α and IL-8 on PMN activation induced by LPS. Isolated human peripheral PMN (1×10^6) were pre-incubated with LPS (1 ng/ml), sTNFR (20 ng/ml) or SB225002 (300 nM) or LPS + SB225002 for 18 h and then were left untreated (white bars) or were incubated for two additional hours with 100 ng/ml of LPS (black bars). The mean fluorescence intensity (MFI) of CD11b (A), CD66b (B) and the percentage of DHR positive cells (C), were determined by flow cytometry. Results were expressed as mean ± SE, as relative values with respect to untreated PMN (−/−); $n = 6$. $^{\dagger}p < 0.05$, $^{\#}p < 0.01$, $^{*}p < 0.001$.

We found that IL-10 was able to specifically prevent PMN activation after the first incubation with LPS, preventing the additive response observed after the second hit of LPS. Additionally, desensitization to LPS-derived activation by IL-10 was also observed for an heterologous stimulus, lipoteichoic acid, that interacts with TLR2. However, this is not surprising as signaling through TLR4 and TLR2 share intracellular components [22].

IL-10 did not influence short term activation. This can be explain by previous *in vitro* studies, that have shown that the inhibitory effects of IL-10 on PMN proinflammatory cytokine mRNA expression and production induced by LPS only start after 3–4 h of culture [23,24], coinciding with the moment in which one of the two subunits composing the IL-10 receptor, namely IL-10R1, is expressed [25]. On the other hand, the lack of short term response to IL-10 was an indication that this cytokine could be preventing indirect LPS-triggered mechanisms as well, that could autocrinally amplify PMN activation at long term. In this sense, when we study the release of TNF-α and IL-8, two well-known cytokines that activate PMN, we found that IL-10 was able to specifically annul their LPS-induced secretion. Although we did not analyze the intracellular expression of these cytokines, our data is in agreement with other authors that have described regulation of PMN-derived chemokines expression by IL-10 [26]. Inhibition of TNF-α or IL-8 actions was able to completely prevent ROS generation triggered by LPS, supporting previous observations of Laichalk et al. that described a negative influence of IL-10 on PMN bactericidal activity [27]. However, this inhibition was less effective in stopping degranulation, indicating, on one hand, that IL-10, through the inhibition of TNF-α and IL-8 secretions, was able to modulate the respiratory burst of PMN, and on the other, that activation of PMN encompasses pathways which may be differentially regulated.

Moreover, although both CD11b and CD66b are store in granules, their LPS response and the influence of IL-10 in their up-regulation was sometimes different. IL-10, and both the soluble TNF receptor and IL-8 antagonist, were less efficient in stopping CD11b mobilization in response to LPS, whereas the effects on CD66b were more pronounced. This discrepancy may be explained by their different granule localization. In this sense, there are major differences between the granule subsets regarding the extent to which these are mobilized both *in vitro* and *in vivo* [28,29]. Secretory vesicles are mobilized more readily than gelatinase granules [30], which again are exocytosed more readily than specific granules [31,32]. This hierarchy in granule mobilization and the extent of exocytosis was found to be quantitatively related to the concentration of intracellular calcium, which depends on the stimulus strength [31]. This may also be related to sequential CD11b and CD66b interactions during the process of PMN extravasation to an infected focus, where CD11b regulates leukocyte adhesion to endothelial cells while CD66b is supposed to interact later with extracellular matrix components.

Studies regarding negative regulation of PMN function are not frequently found in the literature. Historically, PMN have been considered as terminally differentiated cells that unselectively spew out their bactericidal armament in response to a pathogenic insult and die consequently. Additionally, as the turn over of PMN is fast and the release of PMN (both of mature and immature forms) from the bone marrow is usually observed after bacterial infections, it is sometimes difficult to establish that the effects of different mediators are acting on the same PMN population. In this sense, our results suggest that the response of an individual PMN can be, in fact, regulated, and that a second exposure to LPS, in the absence of IL-10, increases even more the response of PMN in an additive way. In the context of bacterial infections, the rapid systemic appearance of IL-10 may be crucial, since the encounter of IL-10 with PMN may render them refractory to activation by

bacterial derived products, probably in an attempt to limit PMN-mediated tissue damage. Evidence that IL-10 keeps inflammation under control *in vivo* is support by experimental models using IL-10 knock out mice, where acute lung inflammation triggered by LPS, caused an exacerbated inflammatory reaction, that correlated with increased production of PMN chemoattractants and infiltration at inflammatory sites [33]. Moreover, PMN depletion in IL-10 knock out mice decreased the severity of gastritis in *Helicobacter felis*-infected mice [34].

During sepsis and SIRS, plasma from patients behaves as an immunosuppressive milieu [35]. Our results indicate that IL-10 participates in PMN desensitization, and may be critical against the development of pathologic responses triggered by LPS. Whether desensitization of PMN activation by IL-10 (or the lack of its regulation) plays a role in sepsis or other SIRS-derived states of life-threatening immunosuppression needs to be further established.

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