Downregulation of Mac-1 Expression in Monocytes by Surface-Bound IgG

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Abstract

Physical and functional association between the β2-integrin Mac-1 (CD11b/CD18) and receptors of immunoglobulin G (IgG) (FcγRs) has been previously reported. In this study, we examined the modulation of Mac-1 expression by IgG in different leucocyte populations. Our data show that human monocytes, but not neutrophils, macrophages, dendritic or natural killer cells, downregulate the expression of Mac-1 after overnight exposure to surface-bound IgG. This effect, which requires at least 6 h of incubation, is not associated with a general downmodulation of membrane antigens, and is selectively induced by immobilized IgG (iIgG), as the stimulation of monocytes with *N*-formyl-methionyl-leucyl-phenylalanine, lipopolysaccharide, tumour necrosis factor-α (TNF-α) or soluble IgG did not modify the Mac-1 expression after 18 h in culture. The loss of Mac-1 was completely prevented by blocking antibodies (Abs) directed to FcγRII (CD32) or CD18. On the other hand, the serine protease inhibitor, phenyl methyl sulphonyl fluoride, but not inhibitors of cysteine proteases or neutral endopeptidases, partially prevented the downregulation of Mac-1 by iIgG. Monocytes cultured overnight on iIgG exhibited a dramatic decrease in their capacity to ingest zymosan particles that could be attributed to the reduced expression of Mac-1. However, there was no inhibition of TNF-α production induced by zymosan, suggesting that Mac-1-dependent responses require different levels of its expression to be fully activated.

Introduction

Mac-1 (CD11b/CD18 or CR3) is a member of the β2-integrin family which also includes LFA-1 (CD11a/CD18) and gp150/95 (CD11c/CD18 or CR4). These receptors share a common β-subunit that is noncovalently associated with unique but closely related α-chains [1, 2]. Mac-1, which is expressed by neutrophils, monocytes, macrophages, dendritic and natural killer (NK) cells, plays an important role in cell adhesion through interaction with intercellular adhesion molecule-1 (ICAM-1) (CD54) [3, 4]. In addition, Mac-1 is a receptor of several well-defined ligands, such as fibrinogen, complement fragment C3bi, coagulation factor X and heparin [1, 4]. On activation by ligand binding, Mac-1 is able to initiate signalling, which results in cellular responses, such as phagocytosis, respiratory burst and degranulation [1, 5, 6]. On the other hand, Mac-1 has been recognized as a receptor that cooperates with FcγRs in promoting effector functions [7]. Thus, Mac-1 is required for the optimal phagocytosis via FcγR, as was shown by its impairment with anti-Mac-1 Fab fragments and by the fact that patients lacking β2-integrins exhibit depressed immunoglobulin G (IgG)-dependent effector activity [8–10].

Given the central role of Mac-1 in leucocyte biology, considerable effort has been devoted to elucidate the mechanisms that regulate its surface expression. Our interest has focussed on the modulation of Mac-1 by IgG in leucocytes that coexpress FcγRs. Previous studies of neutrophils demonstrated that Mac-1 interacts both functionally and physically with FcγRIIIB. Direct physical association was first suggested by experiments in which capping of one receptor resulted in the co-capping of the other [11]. Further studies confirmed structural association between a lectin-like binding site present on Mac-1 and a putative carbohydrate moiety on FcγRIIIB [12, 13]. In contrast to what was observed in neutrophils, physical linkage between FcγR and Mac-1 involving lectin-like interactions has not been found in mononuclear

phagocytes [14]. Nevertheless, the treatment of monocytes with aggregated IgG concomitantly reduces the expression of both receptors [15], while this effect could not be observed in murine macrophages treated with surface-bound IgG [16].

The aim of the present study was to examine the modulation of Mac-1 expression by IgG in different leucocyte populations. The data presented show that monocytes, but not macrophages, dendritic cells, NK cells or neutrophils, downregulate the expression of Mac-1 after prolonged exposure to immobilized IgG (iIgG). This effect, which requires the interaction of both FcγRII and Mac-1 with IgG-coated surface, is dependent on microfilament assembly and the activation of serine proteases.

Materials and methods

Antibodies and reagents. The following monoclonal antibodies (MoAbs) were obtained from Coulter-Immunotech (Marseille, France): anti-CD1a (clone BL6, IgG1), anti-CD11a (clone 25.3, IgG1), anti-CD11b (clone Bear 1, IgG1), anti-CD11c (clone BU15, IgG1), anti-CD18 (clone 7E4, IgG1), anti-ICAM-1 (clone 84H10, IgG1), anti-very late activation antigen-4 (anti-VLA-4) (clone HP2/1), anti-CD14 (clone RM052, IgG2a) and anti-CD56 (clone C218, IgG1). The following MoAbs were purchased from Medarex (Annandale, NJ, USA): $F(ab')_2$ fragments of anti-FcγRI (clone 197, IgG2a) and Fab fragments of anti-FcγRII (clone IV.3, IgG2b). Anti-CD18 (clone TS 1/18) was obtained from the American Type Culture Collection (Rockville, MD, USA). Rabbit IgG anti-ovalbumin (anti-OVA) was prepared as previously described [17]. $F(ab')_2$ fragments of anti-CD18 (TS 1/18) and anti-OVA IgG were prepared in our laboratory by pepsin digestion and further purified by Protein A Sepharose, according to standard techniques [18]. For blocking studies, monocytes were preincubated with the corresponding MoAb for 15 min at 4 °C. Concentrations of MoAb three- to five-fold higher than those needed to saturate all binding sites (1–10 μg/ml), as determined by fluorescence-activated cell sorter (FACS) analysis, were employed in these studies.

Ficoll, Dextran T-500 and Percoll were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); Hypaque was obtained from Winthrop Products (Buenos Aires, Argentina). Culture medium RPMI-1640 was purchased from HyClone Logan, UT, USA). Fetal calf serum (FCS), penicillin/streptomycin and Trypsin–EDTA (ethylenediaminetetraacetic acid) were from Life Technologies (Grand Island, NY, USA). OVA, herbimycin A, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), lipopolysaccharide (LPS) (from *Escherichia coli* 0111 : B4), phenyl methyl sulphonyl fluoride (PMSF), phosphoramidon and leupeptin were obtained from Sigma (St Louis, MO, USA). Human recombinant interleukin-4 (IL-4) and granulocyte/macrophage-colony stimulating factor (GM-CSF) were obtained from PeproTech (Rocky Hill, NJ, USA). Human recombinant tumour necrosis factor-α (TNF-α) was purchased from Sigma.

The IgG aggregates (aIgG) were prepared by heating human IgG at a concentration of 5 mg/ml for 12 min at 63 °C. Then, aIgG was centrifuged at $10{,}000 \times g$ for 5 min and the precipitate discarded.

To prepare Zy-fluorescein isothiocyanate (Zy-FITC) particles, Zy A from *Saccharomyces cerevisiae* (Sigma) was suspended at a concentration of 10 mg/ml in phosphate-buffered saline (PBS). FITC at 1 mg/ml was dissolved in carbonate buffer, pH 9.8, and incubated with Zy suspension at a concentration of 100 μg/ml, pH 7.2, for 2 h at room temperature on a rotating shaker. Unbound FITC was removed by extensive washings.

Cell purification. Peripheral blood mononuclear cells (PBMCs) and neutrophils were obtained from heparinized samples of healthy donors. PBMCs were isolated by centrifugation on Ficoll-Hypaque, washed twice and resuspended in RPMI-1640, supplemented with 10% FCS and antibiotics (complete medium). Cell viability was >98%, as determined by Trypan blue exclusion.

Monocytes were purified by centrifugation on a discontinuous Percoll gradient, with modifications of a previously described method [19]. Briefly, PBMCs were suspended in Ca^{++}, Mg^{++}-free Tyrode's solution supplemented with 0.2% EDTA and incubated for 30 min at 37 °C. During this incubation, the osmolarity of the medium was gradually increased from 290 to 360 mosmol by the addition of 9% NaCl. Three different Percoll fractions were layered in polypropylene tubes: 50% at the bottom, followed by 46 and 40%. PBMCs ($5–10 \times 10^6$/ml) were layered at the top, and they were centrifuged at $400 \times g$ for 20 min at 4 °C. Monocytes were recovered at 50–46% interface. The purity was checked by FACS analysis using anti-CD14 MoAbs and was found to be >90%. Viability was consistently >85%.

Neutrophils were isolated by centrifugation on Ficoll-Hypaque and Dextran sedimentation, as previously described [20]. Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells (>92% neutrophils on May–Grünwald–Giemsa-stained Cytopreps, >95% viable cells) were resuspended at desired concentration in complete medium.

To induce the *in vitro* differentiation to macrophages, monocytes were cultured for 6 days at a cell density of 1×10^6 cells/ml in complete medium supplemented with 40 ng/ml of GM-CSF. At the end of the incubation, macrophages were gently scraped off with a rubber policeman and adjusted to $1–2 \times 10^6$ cells/ml in complete medium. To ensure that this recovery procedure did not result in an impairment of cell responsiveness, the capacity of fMPL (10^{-7} M) to induce calcium mobilization was assessed by flow cytometry using Fluo 3-AM [21]. More than 60% of the recovered macrophages were able to mediate high-calcium transient responses (data not shown).

Immature dendritic cells were prepared from purified monocytes as described previously [22]. Briefly, purified monocytes were cultured for 6 days at a cell density of 1×10^6 cells/ml in complete medium containing 1000 U/ml of IL-4 and 40 ng/ml of GM-CSF. Floating and loosely adherent cells were harvested, and their dendritic cell phenotype was checked by FACS analysis using anti-CD1a MoAb.

Cell culture conditions. To prepare surface-bound IgG (iIgG), plastic tissue culture microplates (96- or 24-well) were incubated overnight with monomeric human IgG (200 μg/ml in saline) at 37 °C. Unbound IgG was removed by extensive washing. In some experiments, immobilized immune complexes (iICs) were used. Towards this aim, microplates coated with 100 μg/ml of OVA in PBS were incubated for 2 h at room temperature, washed and further incubated overnight with 10–20 μg/ml of rabbit anti-OVA IgG. Microplates incubated with OVA alone or OVA plus $F(ab')_2$ fragments of anti-OVA served as controls.

Cell populations suspended at $1–2 \times 10^6$/ml in complete medium were added to control or IgG-coated microplates and cultured for different times at 37 °C in a 5% CO_2 atmosphere. To perform flow cytometric analysis, leucocytes were harvested by vigorous pipetting with warm 0.05% Trypsin−0.5 mM EDTA in saline for 1–2 min. All cell populations (adherent and nonadherent cells) were subjected to this procedure, which enabled us to easily recover the cells attached to surface-bound IgG without affecting their viability. To ensure that this treatment did not alter the epitopes recognized by the MoAbs employed, in some experiments, monocytes were harvested by scraping with a rubber policeman. Comparable results were obtained by measuring CD18, CD11b, CD11a and CD11c expression on monocytes harvested by the two procedures.

Flow cytometric analysis. To evaluate the expression of membrane molecules, direct immunofluorescence staining was performed. Towards this aim, single cell suspensions (5×10^5 cells) were incubated with a saturating concentration of MoAb or an equivalent concentration of isotype-matched control MoAb at 4 °C for 30 min. Cells were washed twice with PBS supplemented with 1% FCS and immediately analysed on a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA). For each sample, a minimum of 5000 events were acquired. Dead cells were excluded by gating on forward scatter (FSC) and side scatter (SSC). To analyse the expression of CD11b on NK cells, double immunofluorescence staining of PBMCs using FITC anti-CD56 and phycoerythrin anti-CD11b was performed.

Binding and phagocytosis assays. The interaction (binding and ingestion) between Zy-FITC and monocytes were assessed by flow cytometry. Monocytes cultured overnight on surface-bound IgG or control wells were incubated with 100 μg/ml of Zy-FITC in complete medium for 30 min at 37 °C. Then, the cells were washed to remove unbound particles, gently scraped with a rubber policeman and subjected to flow cytometry. The fluorescence of free Zy-FITC particles was removed by gating using FSC and SSC. A minimum of 5000 monocytes were scored for each sample.

To discriminate between binding and ingestion of Zy-FITC, cells were analysed by fluorescence microscopy. In some experiments, Trypan blue was added to cell suspension to quench the fluorescence of extracellular particles [23]. A total of 100 monocytes were scored for ingested Zy-FITC particles.

TNF-α assay. TNF-α-sensitive L-929 cell line was used to measure TNF-α activity in culture supernatants, as previously described [24]. Briefly, serial dilutions of supernatants were added to cell monolayers of L-929 in 96-well flat-bottom culture plates. After 20 h of incubation in the presence of actinomycin D (1 μg/ml), dead cells were washed out and viable adherent cells fixed and stained with 0.5% crystal violet in 20% methanol. After washing and solubilization with 0.1% Triton X-100, absorbance was read at 550 nm. TNF-α activity in each supernatant was expressed as the reciprocal of dilution capable of exerting a 50% cytotoxicity.

Statistical analysis. Student's paired *t*-test was used to determine the significance of differences between means, and $P < 0.05$ was taken as indicating statistical significance.

Results

Surface-bound IgG downregulates Mac-1 expression on monocytes

To determine whether exposure to surface-bound IgG was able to modify Mac-1 expression in leucocyte populations, we cultured neutrophils, monocytes and lymphoid cells, freshly isolated from human peripheral blood on, iIC (OVA IgG anti-OVA) or iIgG. Macrophages and dendritic cells differentiated *in vitro* from human monocytes, as described in *Materials and methods*, were treated in the same way. Expression of Mac-1 was analysed by flow cytometry after 18 h of incubation. Data from Fig. 1 indicate that, among leucocytes coexpressing FcγR and Mac-1, monocytes were the only population in which long-term incubation on surface-bound IgG induced a marked downregulation of CD11b expression. To confirm these results, we performed additional experiments using a MoAb directed to CD18. We found that monocytes (Fig. 2), but not the other leucocyte subsets tested (neutrophils, macrophages, NK or dendritic cells; data not shown), expressed lower levels of CD18 after overnight incubation on iIgG. Data in Fig. 2 also indicate that there was no inhibition of CD18 expression when monocytes were cultured on plates coated with OVA alone or with

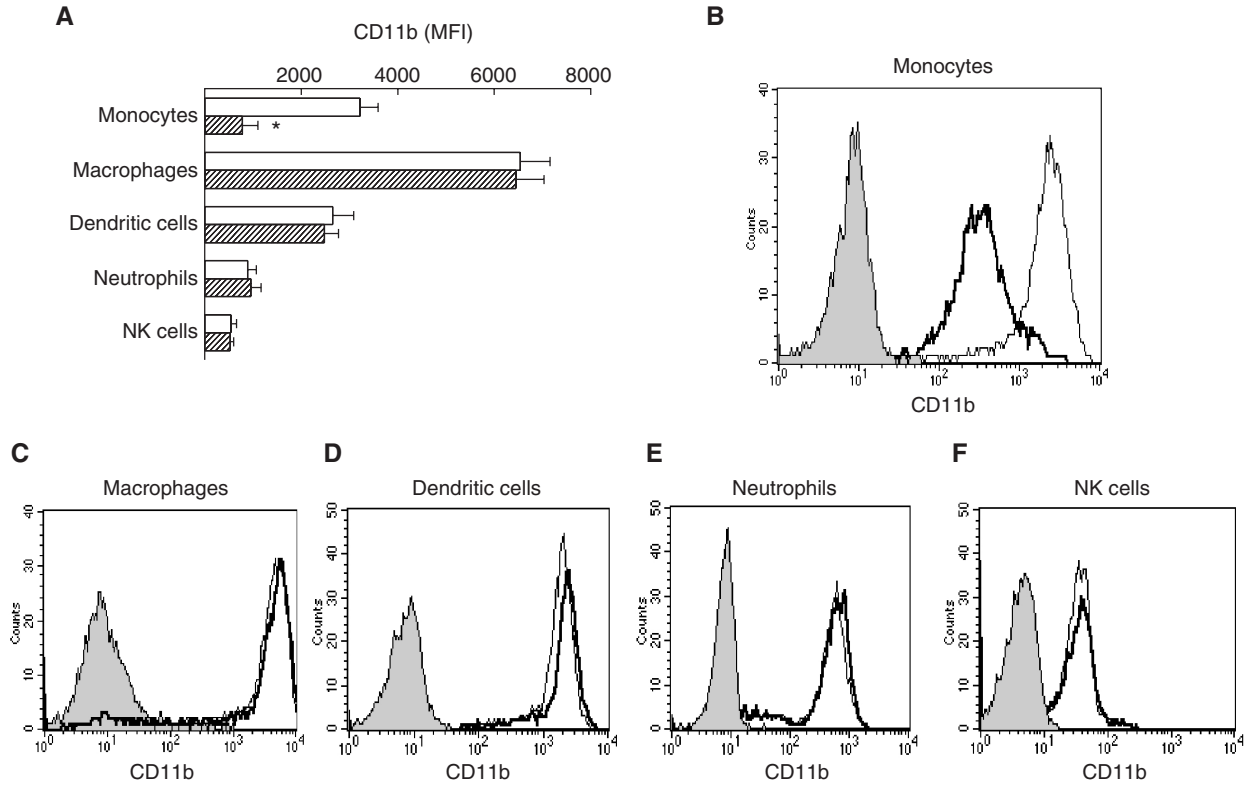

Figure 1 Effect of immobilized immunoglobulin G (IgG) on CD11b expression in different leucocyte populations. (A) Flow cytometric analysis of CD11b expression on purified monocytes, macrophages, dendritic cells, neutrophils and CD56-positive peripheral blood mononuclear cells (natural killer cells) after overnight incubation at 37 °C on untreated (open bars) or IgG-coated microplates (dashed bars). (B–F) Representative histograms from five to eight experiments performed, showing CD11b expression on cells incubated on untreated (thin line) or IgG-coated (bold line) microplates. Fluorescence from isotype control monoclonal antibody is shown in grey. MFI, mean fluorescence intensity. *, Statistical significance ($P < 0.05$) compared with control.

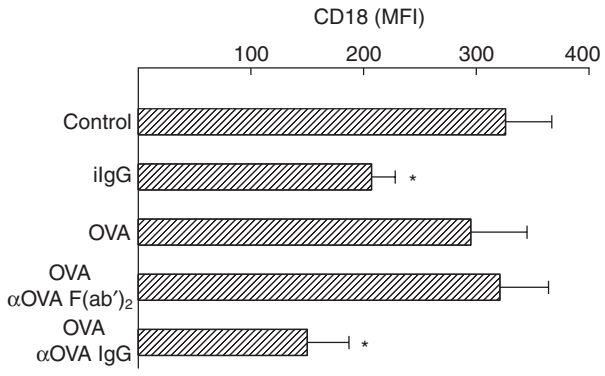

Figure 2 Effect of surface-bound immunoglobulin G (IgG) on CD18 expression in monocytes. Monocytes ($1–2 \times 10^6$/ml) were incubated overnight on microplates coated with human IgG, ovalbumin (OVA) or immobilized immune complexes prepared with OVA–anti-OVA IgG or OVA–anti-OVA $F(ab')_2$, as described in *Materials and methods*. Expression of CD18 was analysed by flow cytometry. Results are expressed as the mean ± standard error of the mean of five to seven experiments. MFI, mean fluorescence intensity; iIgG, immobilized immunoglobulin G. *Statistical significance ($P < 0.05$), compared with monocytes cultured on untreated microplates (control).

iIC formed with OVA and $F(ab')_2$ fragments of IgG anti-OVA, which indicates the requirement of interaction between Fc portion of IgG and FcγR on monocytes. Time-course analysis in Fig. 3 shows that Mac-1 modulation by surface-bound IgG required at least 6 h of incubation and was a long-lasting phenomenon, as it could still be observed even in monocytes that had been in culture for 4 days.

We next evaluated whether the expression of CD11a and CD11c, the α-chains of the other two β2-integrins, as well as the β1-integrin VLA-4 and Mac-1 counter-receptor ICAM-1 was also modulated by iIgG. Results from Fig. 4 indicate that none of these adhesion molecules, nor human leucocyte antigen-DR (HLA-DR) and CD14 (not shown), was affected by iIgG treatment, indicating that a general downmodulation of cell-surface components was unlikely.

In order to determine whether other inflammatory stimuli were also able to downregulate the expression of Mac-1 in monocytes, the effect of fMPL, LPS, TNF-α and aIgG was assessed. The data obtained are depicted in Fig. 5. In agreement with previous reports [25–28], we found that all these stimuli, as well as iIgG, induced a

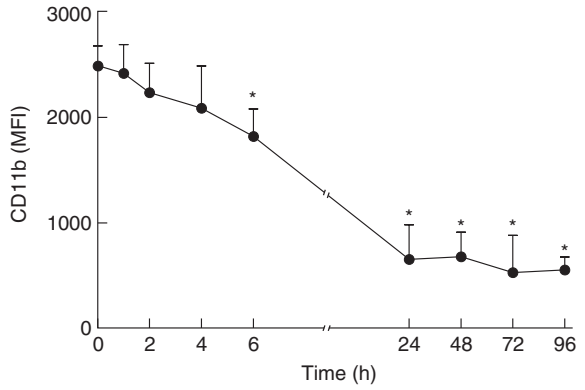

Figure 3 Time-course analysis of CD11b expression in immobilized immunoglobulin G (IgG)-treated monocytes. Monocytes ($1–2 \times 10^6$/ml) were cultured on Ig-coated microplates and harvested at the indicated times to analyse the expression of CD11b by flow cytometry. Results are expressed as the mean ± standard error of the mean of four to eight experiments. MFI, mean fluorescence intensity. *Statistical significance ($P < 0.05$), compared with cells cultured on untreated microplates.

rapid and transient upregulation of Mac-1. By contrast, only iIgG was able to downmodulate Mac-1 expression.

Downregulation of Mac-1 depends on the interaction of FcR type II and CD18 with IgG-coated surface

Peripheral blood monocytes express two classes of FcγR capable of interacting with iIgG: FcγRI (CD64) and FcγRII (CD32) [29]. In order to determine the involvement of each of these receptors in the modulation of Mac-1 by surface-bound IgG, we used Fab/F(ab′)$_2$ fragments of blocking Abs directed to one or another FcγR. We found that the blockade of FcγRII almost completely abrogated the downregulation of CD11b (Fig. 6) and CD18 (not shown) by iIgG, whereas anti-FcγRI had no significant effect. The cytoplasmic domain of FcγRIIa, the predominant FcγRII isoform expressed on monocytes, contains the Y–x–x–L tyrosine activation motif, which becomes phosphorylated upon activation and is essential for receptor function [30, 31]. We observed that the treatment of monocytes with the selective tyrosine kinase inhibitor herbimycin A (10 μM) abolished the downregulation of CD11b by iIgG: mean fluorescence intensity (MFI) of untreated cells, 2455 ± 280; MFI of iIgG-treated cells, 970 ± 135; MFI of iIgG-treated cells in the presence of herbimycin A, 2235 ± 190 (mean ± standard error of the mean (SEM), $n = 3$, $P < 0.05$). This result suggests that signalling through FcγRIIa is necessary for the modulation of Mac-1 by surface-bound IgG. On the other hand, pretreatment of monocytes with saturating amounts of F(ab′)$_2$ fragments of a blocking MoAb directed to CD18 also abrogated the decrease in CD11b expression induced by iIgG (Fig. 6).

Downregulation of Mac-1 by surface-bound IgG depends on serine protease activity and microfilament assembly

Next, we examined whether proteolytic degradation, which has been previously accounted for the loss of different surface receptors in myeloid cells [32–34], was involved in the downregulation of Mac-1 by surface-bound IgG. Towards this aim, we incubated monocytes on untreated or IgG-coated microplates in the presence of three different protease inhibitors and analysed CD11b

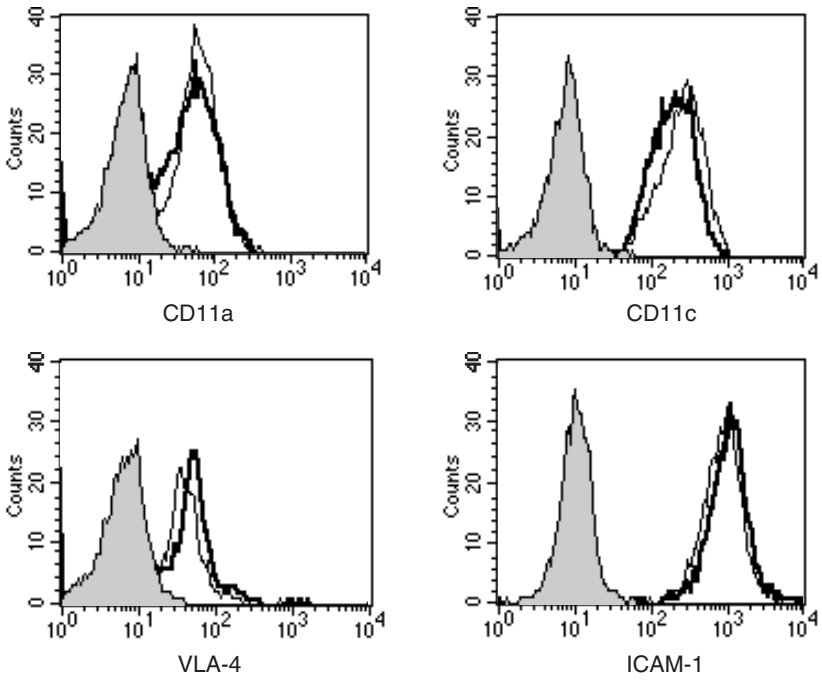

Figure 4 Incubation of monocytes on immobilized immunoglobulin G (IgG) does not modify the expression of CD11a, CD11c, very late activation antigen-4 and intercellular adhesion molecule-1. Monocytes ($1–2 \times 10^6$/ml) were incubated overnight at 37 °C on untreated (thin line) or IgG-coated (bold line) microplates. Expression of adhesion molecules was analysed by flow cytometry. Representative histograms from five different donors are shown. Fluorescence from isotype control monoclonal antibody is shown in grey. VLA-4, very late activation antigen-4; ICAM-1, intercellular adhesion molecule-1.

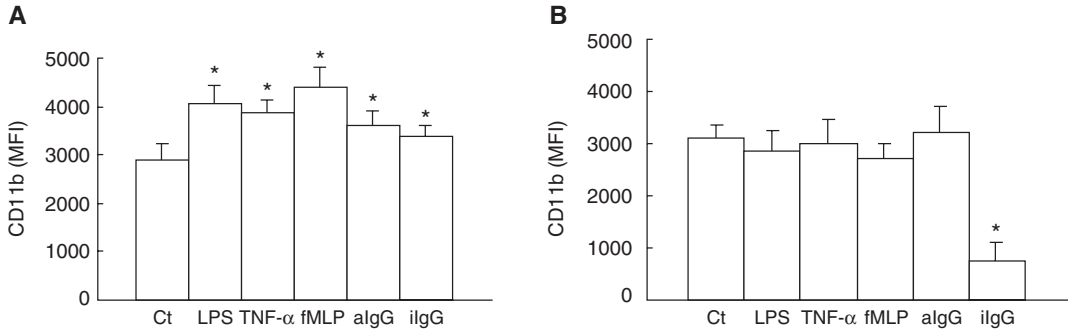

Figure 5 Effect of different stimuli on CD11b expression after short- and long-time incubations. Monocytes ($1–2 \times 10^6$/ml) were stimulated with lipopolysaccharide (5 μg/ml), tumour necrosis factor-α (10 ng/ml), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (10^{-8} M), immunoglobulin G aggregate (aIgG) (100 μg/ml) or exposure to immobilized IgG. The expression of CD11b was analysed by flow cytometry after incubation for 20 min (A) or 18 h (B). Results are expressed as the mean ± standard error of the mean of five experiments. Ct, control; LPS, lipopolysaccharide; iIgG, immobilized IgG. *Statistical significance ($P < 0.05$), compared with cells cultured on untreated microplates.

expression 18 h later. Table 1 shows that neither the cysteine protease inhibitor, leupeptin, nor the neutral endopeptidase inhibitor, phosphoramidon, had any effect on CD11b expression in both control and iIgG-treated monocytes. By contrast, PMSF (1 mM) partially prevented the downmodulation of CD11b induced by iIgG, supporting the involvement of serine proteases in this effect. On the other hand, we found that supernatants from monocytes cultured overnight on iIgG were unable to modify CD11b expression on untreated cells (MFI of untreated monocytes versus monocytes incubated with 75% supernatants from iIgG cultures: 2564 ± 448 versus 2378 ± 398, respectively; mean ± SEM, $n = 3$). These data suggest that proteases involved in the downregulation of Mac-1 expression are cell associated.

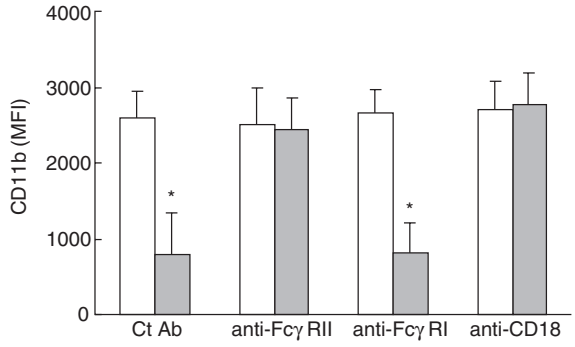

Figure 6 Effect of blocking antibodies (Abs) to FcγRI, FcγRII and CD18 on downregulation of CD11b expression by immobilized IgG. Monocytes ($1–2 \times 10^6$/ml) were cultured for 30 min at 4 °C with saturating concentrations of IV.3 Fab (anti-FcγRII), 197 $F(ab')_2$ (anti-FcγRI) or TS 1/18 $F(ab')_2$ (anti-CD18). $F(ab')_2$ fragments of anti-CD3 (clon UCHT1) were used as control Ab (Ct Ab). Then, monocytes were placed on untreated (open bars) or immunoglobulin G-coated (filled bars) microplates and incubated overnight at 37 °C. The expression of CD11b was analysed by flow cytometry. Results are expressed as the mean ± standard error of the mean of four experiments. *Statistical significance ($P < 0.05$), compared with untreated cells.

Findings in neutrophils show that Mac-1 plays an essential role in neutrophil spreading on surface-bound IgG by mediating redistribution of F-actin [35, 36]. Taking these data into account, we next analysed whether cytoskeletal integrity was necessary for the downregulation of Mac-1 by iIgG. Towards this aim, cultures were carried out in the presence of cytochalasin B (1 μg/ml), an inhibitor of microfilament assembly [37]. Results in Table 1 indicate that cytochalasin B completely prevented the downregulation of Mac-1, confirming the involvement of cytoskeleton in this effect.

Downregulation of Mac-1 impairs phagocytosis of Zy

Mac-1 plays a critical role in the binding and ingestion of unopsonized Zy by monocytes/macrophages [38, 39]. In fact, although mannose/fucose receptor and β-glucan receptor also participate in Zy uptake, we found that $F(ab')_2$ fragments of anti-CD18 antibodies (Abs) were able to substantially block the phagocytosis of Zy (the percentage of monocytes with ingested Zy-FITC evaluated by fluorescence microscopy: control, $82 \pm 5\%$; anti-CD18 $F(ab')_2$, $25 \pm 4\%$; $n = 5$, $P < 0.01$). Therefore, we asked ourselves if the reduced expression of Mac-1 on monocyte surface would affect its capacity to bind and ingest Zy. Towards this aim, monocytes cultured overnight on control or IgG-coated microplates were incubated with Zy-FITC for 30 min at 37 °C. Then, the cells were washed to remove unbound particles and harvested to assess fluorescence staining (binding plus phagocytosis). Results from a representative experiment are depicted in Fig. 7A. It was found that a major proportion of control monocytes were highly stained as a consequence of interaction with Zy-FITC. By contrast, iIgG-treated monocytes exhibited a dramatic decrease in fluorescence intensity, which indicates an impairment to recognize Zy particles. To discriminate between the binding and phagocytosis of Zy-FITC,

Table 1 Effect of protease and microfilament assembly inhibitors on the downregulation of CD11b expression by immobilized immunoglobulin G*

	CD11b (MFI)	
Treatment	Control	iIgG
None	2571 ± 203	1150 ± 135
Leupeptin (50 μg/ml)	2524 ± 252	1222 ± 194
PMSF (1 mM)	2734 ± 280	1731 ± 223†
Phosphoramidon (10 μM)	2493 ± 271	1072 ± 218
Cytochalasin B (1 μM)	2671 ± 199	2598 ± 257†

*Monocytes ($1–2 \times 10^6$/ml) were incubated with different inhibitors for 15 min at 37 °C, placed on untreated or IgG-coated microplates and incubated overnight at 37 °C. The expression of CD11b was analysed by flow cytometry. Results are expressed as the mean ± standard error of the mean of four experiments. MFI, mean fluorescence intensity; PMSF, phenyl methyl sulphonyl fluoride.
†Statistical significance ($P < 0.05$), compared with untreated cells cultured on iIgG.

we quantified the percentage of monocytes with ingested particles by employing fluorescence microscopy. As shown in Fig. 7B, long-term incubation on surface-bound IgG provokes a significant decrease in the number of monocytes that phagocytosed Zy particles. This effect might be attributed to the reduced expression of Mac-1.

It has been previously shown that unopsonized Zy is able to stimulate TNF-α production by human monocytes via CD11b/CD18 molecules [40]. We asked ourselves whether the decreased levels of Mac-1 upon interaction with surface-bound IgG would impair the capacity of monocytes to release TNF-α in response to Zy. Towards this aim, cells were cultured overnight on untreated or IgG-coated microplates, harvested, washed twice and re-incubated with or without Zy (100 μg/ml). Eighteen hours later, cells and supernatants were collected. We found that monocytes which had been exposed to iIgG during the first 24 h of culture expressed lower levels of CD11b than monocytes harvested from untreated plates (MFI of untreated cells, 2396 ± 340; MFI of iIgG-treated

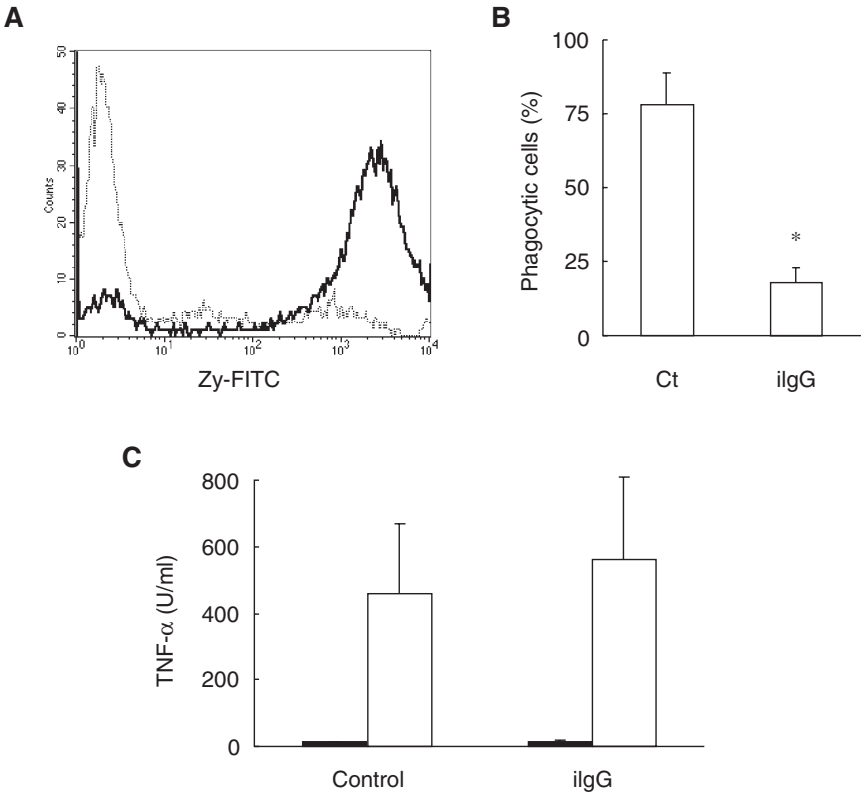

Figure 7 Incubation of monocytes on immobilized immunoglobulin G (IgG) impairs phagocytosis of unopsonized zymosan without affecting tumour necrosis factor (TNF) release. Monocytes ($1–2 \times 10^6$/ml) were cultured overnight on untreated or IgG-coated microplates. To evaluate Mac-1-dependent binding and phagocytosis, monocytes were incubated with Zy-fluorescein isothiocyanate (Zy-FITC) (100 μg/ml) for 30 min at 37 °C, washed and harvested as described in *Materials and methods*. (A) Binding plus uptake of Zy-FITC by untreated (bold line) or immobilized IgG (iIgG)-treated (thin line) monocytes, as detected by flow cytometric analysis. Representative histograms from six independent experiments are shown. (B) Phagocytosis of Zy-FITC particles analysed by fluorescence microscopy. Results are expressed as the mean ± standard error of the mean of six experiments. To evaluate Mac-1-dependent release of TNF, monocytes cultured overnight on untreated (control, Ct) or IgG-coated microplates (iIgG) were harvested, washed and incubated with Zy (100). (C) TNF levels were measured 24 h later in supernatants from unstimulated and Zy-stimulated cultures. *Statistical significance ($P < 0.05$), compared with cells cultured on untreated microplates.

cells, 1184 ± 278; mean $\pm$ SEM, $n = 4$, $P < 0.05$). Despite this, iIgG-treated cells were able to produce high levels of TNF-α in response to Zy stimulation, comparable with those produced by control monocytes (Fig. 7C). Taken together, our data indicate that, although the downregulation of Mac-1 by surface-bound IgG impaired the phagocytosis of Zy, residual levels of the receptor expression were sufficient to activate TNF-α release.

Discussion

The results presented here demonstrate that the exposure of monocytes to surface-bound IgG induces a selective downregulation of Mac-1 expression, which could not be observed in other leucocyte populations that coexpress FcγR and Mac-1: neutrophils, macrophages, dendritic and NK cells. This decrease in Mac-1 expression on monocytes was not associated with a general downmodulation of surface antigens, as the expression of other molecules, such as CD11a, CD11c, VLA-4, ICAM-1, HLA-DR and CD14, was not reduced after iIgG treatment. Of note, the downregulation of Mac-1 expression in monocytes results in an impaired capacity to ingest Zy particles, but does not affect TNF-α release induced by Zy, suggesting that Mac-1-dependent responses require different levels of Mac-1 expression to be fully activated.

Mac-1 and FcγR appear to interact in multiple ways. A large body of evidence indicates that Mac-1 is able to form membrane complexes with neutrophil FcγRIIIb, providing a transmembrane signalling mechanism for this glycosylphosphatidylinositol-anchored receptor that allows it to mediate cytoskeleton-dependent adhesion or phagocytosis and degranulation [8–11, 13, 36, 41]. Physical linkage between Mac-1 and FcγRIIIb involves extracellular interactions which can be disrupted by certain saccharides, such as *N*-acetyl-D-glucosamine and D-mannose [12]. While these lectin-like interactions are restricted to neutrophil FcγRIIIb and Mac-1, the latter can also cooperate with FcγRIIa [14, 42]. In fact, Mac-1 restores IgG-dependent phagocytosis in transfectants with a FcγRIIa molecule lacking a cytoplasmic domain [43]. Although the mechanism(s) responsible for this cooperation has not been elucidated yet, most evidence suggests that the site mediating Mac-1 association with FcγRIIa resides in its intracellular domain and that successful cooperation requires rearrangement of the cytoskeleton.

While numerous reports have focussed on physical linkage and functional cooperation between Mac-1 and FcγR, less attention has been paid to the modulation of these receptors upon activation. In neutrophils, cross-linking of FcγRIIIb with aIgG [11] or $F(ab')_2$ fragments of anti-FcγRIIIb [12] induces co-capping of Mac-1. In these cells, comodulation of both receptors is a rapid phenomenon, as it is completed within 60 min after the addition of a ligand. With regard to the comodulation of Mac-1 and FcγR in mononuclear phagocytes, contrasting results have been obtained, depending on the population evaluated. Thus, adherence of thioglycollate-elicited murine macrophages to IgG immune complexes does not modify the expression of complement receptors [16]. In agreement with this finding, we here show that human macrophages differentiated *in vitro* with GM-CSF lack the ability to downregulate Mac-1 expression in response to iIgG. On the other hand, Mannhalter *et al.* [15] using freshly isolated monocytes have found that soluble aIgG reduces the expression of Mac-1. This is in conflict with our present results, as we observed that iIgG, but not soluble aIgG, downregulates Mac-1 expression. The differences in aggregation status of IgG may explain these contrasting results.

The loss of surface receptors mainly occurs through endocytosis or proteolytic shedding. Proteolytic degradation of Mac-1 has been recently reported in neutrophils, both *in vivo* [34] and *in vitro* [33]. In these papers, loss of Mac-1 was observed within 1 h after a strong stimulation of neutrophils with phorbol 12-myristate 13-acetate or opsonized Zy, and it was inhibited by the serine protease inhibitor, PMSF. We here show that PMSF, but not inhibitors of cysteine proteases or neutral endopeptidase, partially prevents the loss of Mac-1 expression induced by iIgG, supporting the involvement of serine proteases in this process. The fact that supernatants from monocytes cultured overnight on iIgG were unable to modify Mac-1 expression on untreated cells suggests that these proteases are associated with cell membranes.

Our data, showing that Fab/$F(ab')_2$-blocking Abs directed to either FcγRIIa or CD18 completely prevent the downregulation of Mac-1 expression as well as monocyte spreading on surface-bound IgG (unpublished results), strongly suggest that these processes are dependent on the activity of both receptors. We hypothesize that the downregulation of Mac-1 is a two-step process requiring intracellular signal(s) delivered through FcγRIIa and the recognition of an unknown ligand by Mac-1, which may be present at the surface coated with iIgG or at the cell membrane of activated monocytes. However, other possibilities should also be considered. For example, interaction of anti-CD18 MoAb with Mac-1 might interfere with its proteolytic cleavage by serine proteases. On the other hand, the Ab might disrupt the association of Mac-1 with another cell-surface protein, preventing the downregulation of Mac-1. Although we cannot discard the fact that this protein was FcγRIIa itself, previous evidence has suggested that cooperation between Mac-1 and FcγRIIa does not involve extracellular, but rather intracellular, association. We here show that the modulation of Mac-1 by iIgG was almost completely abrogated by the microfilament-disrupting agent, cytochalasin B, supporting the importance of cytoskeleton integrity for the cooperation of both receptors.

Most populations of resident macrophages, such as those in the lung or kidney, do not express Mac-1, while others (i.e. Kupffer cells in the liver, macrophages of the lamina propia in the gut) express low levels of this integrin [44, 45]. In contrast, high levels of Mac-1 expression are retained in macrophages that differentiate from monocytes recruited to tissues in response to inflammatory signals. The results presented here indicate that surface-bound IgG, but not other pro-inflammatory agents, like LPS, TNF-α or fMLP, downmodulates Mac-1 expression in monocytes, suggesting that at the sites of immune complex deposition, at least some of the Mac-1-dependent functions mediated by mononuclear phagocytes might be impaired.

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