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Granulocyte–macrophage colony-stimulating factor down-regulates CD14 expression on monocytes

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SUMMARY

CD14 is a differentiation-stage-linked glycosyl-phophatidyl-inositol-linked glycoprotein on human peripheral blood monocytes and tissue macrophages, which functions as a receptor for lipopolysaccharide. Here, the effects of granulocyte–macrophage colony-stimulating factor (GM-CSF), a cytokine with proliferation- and differentiation-inducing properties on myeloid lineage cells, were studied on CD14 expression by peripheral blood cells. GM-CSF down-regulated the membrane expression of CD14 on monocytes, while it up-regulated expression on neutrophils. GM-CSF also decreased the spontaneous release of CD14 in monocyte culture supernatants. Down-regulation of CD14 expression and release was accompanied by a decrease in the mRNA transcript for CD14, suggesting that it most likely reflects an effect on the transcriptional level. The functional significance of this phenomenon, and its potential relation to the terminal differentiation of monocytes, are discussed.

INTRODUCTION

CD14, a glycoprotein of 55 000 mw, is expressed on most monocytes and tissue macrophages, and is also found (at low density) on B cells and on granulocytes.[1] A glycosyl-phosphatidyl-inositol moiety binds CD14 to the cell membrane,[2,3] from which it can be cleaved by phospholipase or protease digestion, thus generating a soluble form.[4] CD14 is seen as a differentiation marker for monocytes since its expression is first noticed on promonocytes and thereafter on monocytes and tissue macrophages.[1] Functionally, CD14 acts as a receptor for the complex consisting of lipopolysaccharide (LPS) and LPS-binding protein,[5] and thus mediates responses to LPS, such as the LPS-induced production of the cytokines tumour necrosis factor (TNF), interleukin-6 (IL-6) and IL-8.[6] CD14 also plays a role in monocyte adhesion through lymphocyte function-associated antigen-1/intracellular adhesion molecule-1 (LFA-1/ICAM-1) interactions.[7,8] Studies with anti-CD14 monoclonal antibodies (mAb) indicate that CD14 further delivers a negative signal for monocyte-dependent T-cell proliferation.[9] The signalling pathway after CD14-triggering involves a protein tyrosine kinase activity and the mobilization of calcium.[10,11] Soluble CD14, isolated from urine, directly stimulates cytokine production by monocytes, suggesting that CD14 can act as a soluble mediator, similar to cytokines, and that monocytes (and probably other cell types) express a receptor for CD14.[12] LPS interacts with soluble CD14, and this complex can initiate cellular responses in cells lacking CD14 expression.[13] It is therefore interesting to note that the CD14 gene maps to a region on chromosome 5 that encodes several growth factors and receptors.[14]

We have now studied the effect of granulocyte–macrophage colony-stimulating factor (GM-CSF) on CD14 expression by blood monocytes, and we interestingly found a down-regulation. This was remarkable because GM-CSF induces differentiation of myeloid lineage cells and enhances CD14 expression on promonocytic cell lines cultured with vitamin D_3,[15,16] and because it activates mature macrophages and granulocytes.[17] Moreover, GM-CSF enhanced CD14 expression on neutrophils. The mechanism responsible for this down-regulation of CD14 on monocytes has been explored and its potential significance is discussed.

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Abbreviations: FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte–macrophage colony-stimulating factor; HIV, human immunodeficiency virus; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PE, phycoerythrin; PMA, phorbol myristate acetate; r, recombinant; TNF, tumour necrosis factor.

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MATERIALS AND METHODS

Cytokines

The cytokines used were purified recombinant (r) human gene products. GM-CSF for *in vivo* use was donated by Schering-Plough (Brussels, Belgium). One unit of rGM-CSF corresponds to 40 pg. The endotoxin content of this preparation was less than 0·06 ng/mg. A second rGM-CSF preparation, as well as rIL-2 and recombinant interferon-γ (rIFN-γ) were purchased from Janssen Chimica (Beerse, Belgium). The rIL-4 and rTNF were donated by Dr A. Van de Voorde, Innogenetics (Ghent, Belgium).

Monoclonal antibodies and other reagents

Fluorescein isothiocyanate (FITC)-conjugated 3G8 (anti-CD16) was from Medarex (Annandale, NJ, USA). CD14 expression was detected by staining with phycoerythrin^{PE}-conjugated mAb LeuM3 (mIgG2b; Becton-Dickinson, San Jose, CA). The control mIgG1 and mIgG2a and anti-CD45 were purchased from Becton-Dickinson. A neutralizing mAb to TNF (clone B-C7, mIgG1) was purchased from Innotest, Besançon, France, and used in some of the cultures at a concentration of 2·5 μg/ml (25 ng of this mAb neutralizes 50% of the activity of 1 U of TNF). Phorbol myristate acetate (PMA) and LPS were obtained from Sigma Chemical Co. (St Louis, MO). Herbimycine A and fetal calf serum (FCS) were purchased from Gibco (Paisley, UK). Actinomycin D was bought from Merck, Sharp and Dohme (Research Laboratories, Harlow, Essex, UK). The sCD14 enzyme-linked immunosorbent assay (ELISA) was bought from Medgenix Diagnostics, Fleurus, Belgium.

Peripheral blood leucocyte isolation

Heparinized blood was obtained from healthy volunteers (20–50 years old). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque sedimentation (density 1·077). Enriched monocyte preparations were obtained by either cold agglutination[18] or by E-rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells. The monocytes, used for Northern blotting, were prepared by Dr R. van Schie (University of Nijmegen, the Netherlands) by counterflow centrifugal elutriation, and purity was 88% (viability >95%).[19] To isolate neutrophils, the cell pellets of Ficoll–Hypaque gradients were collected, the red cells were lysed in ammonium chloride, and after extensive washing the cells were resuspended in culture medium.

Cell culture

Peripheral blood mononuclear cells or enriched monocytes were cultured at a concentration of $0{\cdot}5 \times 10^6$ cells/ml in 'culture medium' (RPMI-1640 medium (Gibco, Paisley, UK) without phenol red, supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FCS). The RPMI-1640 and FCS contained less than 0·03 ng endotoxin per ml. The cells were incubated with/without rGM-CSF in different concentrations, in 12 × 75 mm polystyrene or polyethylene tubes (Falcon, Becton Dickinson Labware, NJ). There were no differences in the results obtained with the different types of tubes.

Immunofluorescence analysis

The cells were incubated with FITC- or PE-conjugated mAb at 4° for 30 min in phosphate-buffered saline (PBS), supplemented with 10% normal human serum (NHS). The latter was used to block non-specific binding of mAb via FcR. The cells were washed twice in PBS and fixed in 1 ml of 2% paraformaldehyde. Fluorescence intensity was analysed on a FACScan (Becton-Dickinson), using a Consort 30 or Lysis II program. Scatter characteristics were used to gate the monocytes or neutrophils and 5000 cells were analysed. Per sample the data are expressed as a percentage of positive cells or the mean fluorescence intensity after subtraction of the mean fluorescence intensity of the isotype control (arbitrary units).

Northern blot analysis

Total cellular RNA was isolated from (elutriated) monocytes after 12, 24 and 48 hr of culture with/without GM-CSF or IL-4, using the RNA zol B method (Cinna Biotecx, Friendswood, TX).[20] Ten microgrammes of RNA were fractionated by electrophoresis in 1% agarose/formaldehyde gels, and transferred to nitrocellulose (BA85, Schleicher and Schuell, Dassel, Germany). Hybridization with a 796 base-pair (bp) reverse transcription polymerase chain raction (RT-PCR) fragment of CD14,[21] labelled with (α-^{32}P) dCTP using a random primer labelling kit (BRL, Bethesda, MD), was carried out overnight in 50% formamide, 3 × saline-sodium citrate (SSC), 0·1% sodium dodecyl sulphate (SDS) 10 × Denhardt's solution, supplemented with 50 μg/ml denaturated salmon sperm DNA at 42°. Filters were washed in 0·3 × SSC/0·1% SDS at 42° for 30 min, and exposed to Kodak XAR films.

Statistics

The Wilcoxon-paired test was used for statistic analysis of the data.

RESULTS

Modulation of CD14 expression on human peripheral blood monocytes

In kinetic studies, PBMC or enriched monocytes were kept in culture medium with/without GM-CSF for up to 72 h. Typical results are shown in Fig. 1. When PBMC were cultured in medium alone (Fig. 1a), a spontaneous increase in CD14 expression was always observed. In contrast, enriched monocytes (E-Rosette-negative cells in Fig. 1b) had an initial spontaneous decrease in CD14 expression (24 hr) and then regained their original level of CD14 expression. As also shown in Fig. 1, GM-CSF (1000 U/ml) progressively reduced CD14 expression on monocytes in cultures of PBMC. In cultures of enriched monocytes, GM-CSF inhibited the reappearance of CD14, and lead to an almost complete loss of CD14 after 72 hr of culture.

Dose–response experiments showed that GM-CSF concentrations from 10 000 to 100 U/ml all decreased CD14 expression to a similar extent (not shown). Only when concentrations lower than 100 U/ml were used, could a clear dose–response effect be observed (Fig. 2). Recombinant GM-CSF from a different source was found to have the same effect as that used in the previous experiments (not shown).

The reproducibility of our observation was confirmed on enriched monocyte preparations from 10 different donors.

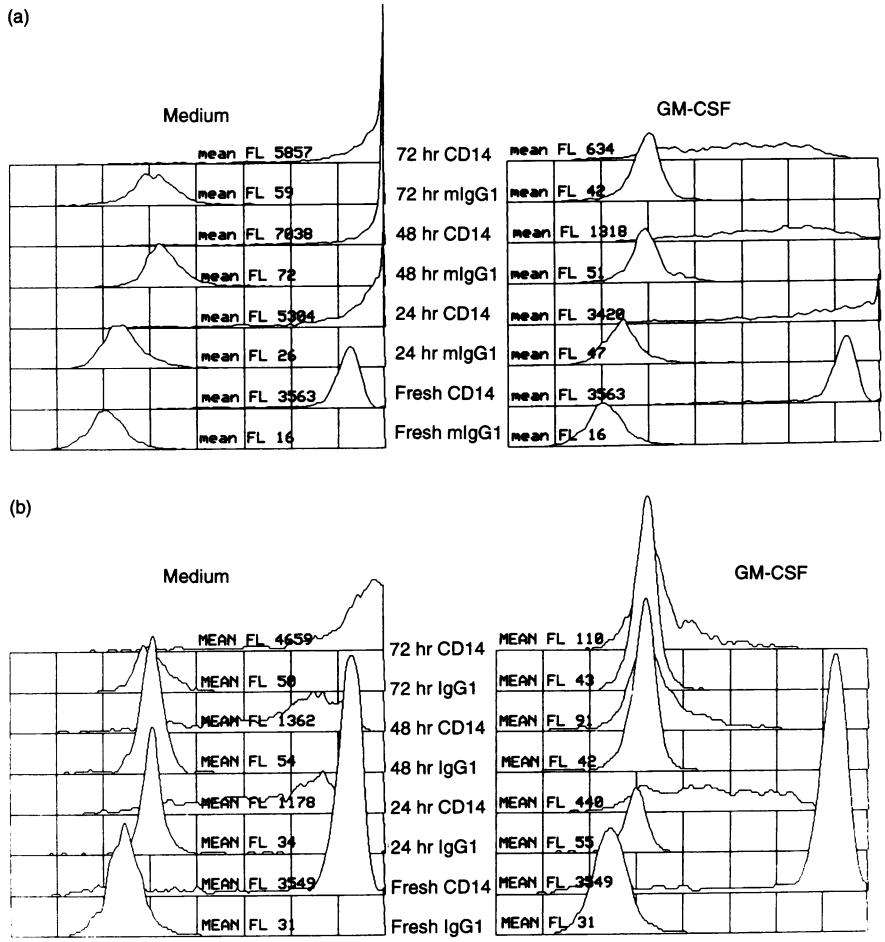

Figure 1. Kinetic studies of CD14 modulation on cultured monocytes. Peripheral blood mononuclear cells (a) at a concentration of 10^6 cells/ml or enriched monocytes (b) at a concentration of $0{\cdot}2 \times 10^6$ cells/ml were cultured for up to 72 hr, with or without GM-CSF (1000 U/ml). The cells were stained with PE-conjugated anti-CD14 mAb (Leu M3) or isotype control mIgG1 and were analysed on a FACScan (Becton-Dickinson). Mean fluorescence intensity (in arbitrary units) is given for each histogram. Cell number is on the y-axis and fluorescence intensity is on the x-axis.

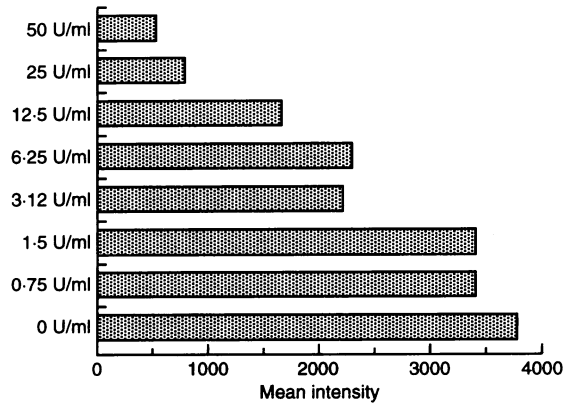

Figure 2. GM-CSF reduces monocyte CD14 expression in a dose-dependent manner. Peripheral blood monocytes ($0{\cdot}5 \times 10^6$ cells/ml) were cultured with different concentrations of GM-CSF for 48 hr at 37°. Monocytes were stained with PE-conjugated anti-CD14 mAb (Leu M3) and were analysed on a FACScan (Becton-Dickinson). Mean intensities of CD14 expression on monocytes cultured with decreasing dosages of GM-CSF are presented (analysis with Consort 30 software). The mean intensity of cells stained with isotype control was subtracted from the mean intensity of cells stained with Leu M3.

Compared to monocytes cultured in medium alone, monocytes cultured with GM-CSF (2 ng/ml) for 48 hr had a significantly lower mean fluorescence intensity of CD14 expression (43% of control, mean of 10 experiments; $P < 0{\cdot}01$) and a reduced percentage of cells positive for CD14 (66% of control; $P < 0{\cdot}01$) (Fig. 3).

In vitro studies have shown that LPS up-regulates the expression of CD14, both *in vivo* and *in vitro*.[22] In contrast, IL-4, IFN-γ, and PMA added *in vitro*, down-regulate the surface expression of CD14 on monocytes.[4,23] We therefore also included these as well as some other agents in our studies on the modulation of CD14 expression. Recombinant IL-4, rIFN-γ and PMA indeed down-regulated CD14 expression, while LPS rapidly increased CD14 expression on monocytes, with maximum effects seen at concentrations of 1 ng/ml or higher after 48 hr of culture (data not shown). At LPS concentrations equal to and above 500 pg/ml, GM-CSF was not able to down-modulate CD14 induction, while at lower LPS concentrations, it did down-modulate CD14 expression. The rIL-2 (100 U/ml) had no effect on CD14 expression (data not shown).

The possibility of an indirect effect of GM-CSF on CD14 expression, through induction of TNF, was studied in detail.

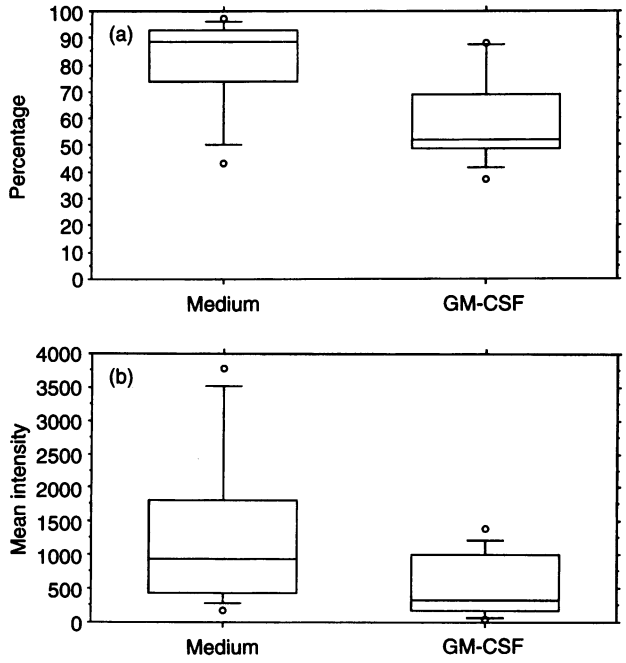

Figure 3. Low concentrations of GM-CSF consistently down-regulate CD14 expression on peripheral blood monocytes. Enriched monocyte suspensions from 10 different donors were incubated at a concentration of $0{\cdot}5 \times 10^6$ cells/ml for 48 hr at 37° with or without GM-CSF (50 U/ml), then stained with a PE-conjugated anti-CD14 mAb (Leu M3) and analysed on a FACScan (Becton-Dickinson) with a scatter gate set to exclude contaminating lymphocytes. Median and the 10, 25, 75 and 90th percentiles are represented by the horizontal lines; ° represents data above and below the 10th and 90th percentile. In (a) calculations are made with the percentages of positive cells and in (b) the mean intensities in arbitrary fluorescence units are used. The mean intensity of cells stained with isotype control was subtracted from the mean intensity after staining with Leu M3. For both (a) and, (b) the differences were statistically significant ($P < 0{\cdot}01$).

PBMC from three donors were cultured for 48 hr with different concentrations of rTNF ranging from 5 ng/ml to 20 pg/ml, and CD14 expression on the monocytes was studied. The concentrations of rTNF are within the range of TNF concentrations in cultures of LPS-stimulated PBMC. No significant effect of rTNF on CD14 expression was found. We have in these experiments also studied the combined effects of GM-CSF (1000 U/ml) and TNF (at different concentrations) on CD14 expression. GM-CSF inhibited CD14 expression, independently from the concentration of TNF in the medium. Finally, we have also studied the effect of a neutralizing anti-TNF mAb on CD14 expression in the presence and absence of GM-CSF during a 48-hr culture. Concentrations of anti-TNF up to 2·5 μg/ml were without effect on CD14 expression, and specifically did not neutralize the inhibitory effect of GM-CSF on CD14 expression. All together, these three sets of data do not support the possibility that the effect of GM-CSF was mediated through TNF induction, and they support the concept that GM-CSF directly affects CD14 expression.

To study CD14 turnover, PBMC were incubated with actinomycin D, a translation inhibitor, for up to 48 hr. Actinomycin D led to a complete disappearance of CD14 expression by monocytes after 24 hr (Fig. 4).

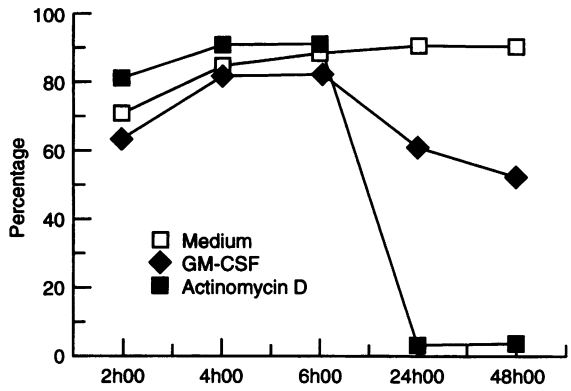

Figure 4. Effect of Actinomycin D on CD14 expression. Peripheral blood monocytes ($0{\cdot}5 \times 10^6$ cells/ml) were incubated in culture medium alone or with GM-CSF (50 U/ml) or actinomycin D (1 μg/ml) for 48 hr. Data from one (out of four) experiment are shown. Monocytes were stained with PE-conjugated anti-CD14 mAb (Leu M3) and were analysed on a FACScan (Becton-Dickinson).

Effect of GM-CSF on the release of CD14 from the monocytes

To investigate whether GM-CSF enhances cleavage of the CD14 molecule from the monocyte surface, an ELISA was used to measure soluble CD14 in the supernatants of cultured monocytes with/without GM-CSF for a period up to 72 hr (Fig. 5). Soluble CD14 was released in the supernatants of cells cultured for 48 or 72 h in medium alone. Release of sCD14 was enhanced by LPS and almost completely blocked by GM-CSF and by actinomycin D. Similar results were also obtained in a second experiment in which enriched monocytes were used (not shown). These data, together with those on the influence of actinomycin D on CD14 expression, suggest a continuous turnover of CD14 synthesis and release by monocytes. We conclude that the down-regulation of membrane CD14 expression by GM-CSF cannot be explained by enhanced release of the CD14 molecule, but more likely results from decreased synthesis.

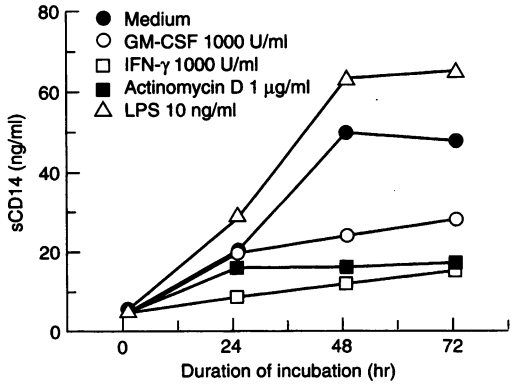

Figure 5. Release of soluble CD14 by monocytes. Peripheral blood mononuclear cells ($0{\cdot}5 \times 10^6$ cells/ml) were incubated in culture medium alone or with GM-CSF (1000 U/ml), IFN-γ (1000 U/ml), actinomycin D (1 μg/ml) or LPS (10 ng/ml). Soluble CD14 was determined by ELISA on the culture supernatants, obtained after 24, 48 and 72 hr of culture.

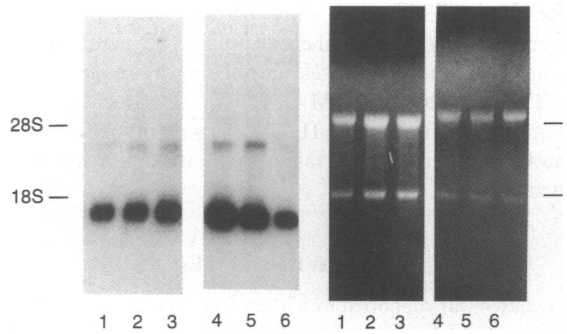

Figure 6. Northern blot analysis of CD14 transcripts. Total cellular RNA was isolated from elutriated monocytes (1×10^7 cells) cultured for 12 or 48 hr in medium (lanes 1,4), medium with GM-CSF (lanes 2,5), or medium with IL-4 (lanes 3,6). Lanes 1–3 represent mRNA obtained at 12 hr, lanes 4–6 at 48 hr. Left pannel, RNA was separated on an agarose/formaldehyde gel, and blotted to nitrocellulose. Blots were probed with ^{32}P-labelled CD14 cDNA. Right panel, ethidium bromide staining patterns for the same samples. Positions of the 28S and 18S ribosomal RNAs are indicated.

The effect of GM-CSF is at the transcriptional level

Total cellular RNA was extracted from (elutriated) monocytes cultured in culture medium alone and with either GM-CSF or IL-4. IL-4 was included in the experiment, because of its known down-regulatory effect on CD14 expression and mRNA levels.[23] At 12 hr a CD14 transcript (with the expected size) was detected. The CD14 transcripts in samples incubated in GM-CSF or IL-4, were stronger than in medium alone. The CD14 transcript was weak in all samples incubated for 24 hr. After 48 hr, the cells in culture medium showed a strong increase in the CD14 transcript level. Both GM-CSF and IL-4 significantly decreased CD14 transcript levels, compared to those in culture medium (Fig. 6). The radioactivity on the blots, as a semi-quantitive evaluation of CD14 transcript, was counted. This revealed that GM-CSF and IL-4 reduced CD14 transcript levels at 48 hr by 36% and 68% respectively. These data support the down-regulation of CD14 expression on peripheral monocytes by GM-CSF and IL-4 to be at the transcriptional level.

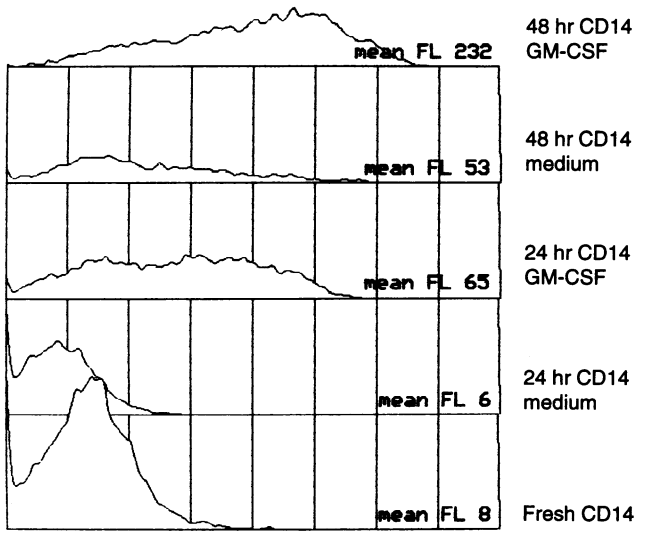

Figure 7. GM-CSF enhances CD14 expression on peripheral blood neutrophils. Isolated neutrophils (2×10^6/ml) were cultured in medium with 10% autologous plasma, without or with GM-CSF (1000 U/ml) and were analysed for CD14 and CD16 expression (using FITC-labelled 3G8 and PE-labelled LeuM3) at 0, 24 and 48 hr after isolation. Neutrophils were gated on the basis of their scatter characteristics and CD16 expression. CD14 expression on fresh neutrophils was negative as compared to isotype control (not shown).

GM-CSF enhances CD14 expression by neutrophils

Although CD14 is a marker for mature monocytes, peripheral blood neutrophils also can express low levels of this molecule. As shown in Fig. 7, we found that culture with GM-CSF up-regulates the expression of CD14 on the neutrophils. This was accompanied by a maintenance of CD16 expression, as compared to neutrophils kept in medium alone (not shown). GM-CSF thus has opposite effects on CD14 expression by either peripheral blood monocytes or neutrophils.

DISCUSSION

The present study focuses on regulation of CD14 expression by GM-CSF. GM-CSF is a member of the family of colony-stimulating factors, involved in proliferation and differentiation of myeloid precursors.[17] The gene for GM-CSF is located on chromosome 5(q21-32), close to the genes encoding CD14 and several other growth factors.[24] Activated T cells, fibroblasts, endothelial cells and macrophages produce GM-CSF.[25] Various haemopoietic (neutrophils, monocytes, eosinophils) and nonhaemopoietic (endothelial and melanoma) cells express receptors for GM-CSF.[26]

We have observed that CD14 expression on monocytes increases during *in vitro* culture. It is unclear whether this represents a physiological phenomenon, or whether manipulation and/or contact activation by plastic are responsible for the increase in CD14 expression. Although LPS up-regulates CD14 expression on blood monocytes,[22] it is unlikely that endotoxins were responsible for the increase in CD14 expression during culture, because the media were carefully selected for their low endotoxin content. Actinomycin D, an antagonist of translation, totally abolishes CD14 expression after 24 hr, which indicates CD14 expression to require continuous *de novo* synthesis. Release of CD14 in culture supernatants of monocytes, as evidenced by ELISA on culture supernatants, further suggest that CD14 is continuously released and resynthesized with a constant turnover of membrane CD14.

We found prominent effects of GM-CSF on CD14 expression. GM-CSF down-regulated the expression of CD14 on the cell surface of cultured monocytes in a dose-dependent manner. It is unlikely that this effect was mediated through induction of TNF, because rTNF had no significant effect on CD14 expression, and because a neutralizing anti-TNF antibody could not inhibit the GM-CSF effect on CD14 expression. The mRNA transcripts for CD14 in the samples incubated with GM-CSF or IL-4 versus medium alone, were significantly decreased, which indicates that the effect is on the transcriptional level (Fig. 6). For IL-4, this effect has previously been reported.[23] Release of CD14 in the culture supernatant was also decreased in the presence of GM-CSF, indicating that a

decrease in synthesis and not an enhanced shedding, were responsible for the loss of CD14 from the membrane. The down-modulation of CD14 on monocytes was remarkable because GM-CSF induces differentiation of myeloid lineage cells, because it enhances CD14 expression on promonocytic cell lines cultured with vitamin D_3,[15,16] and because it activates mature macrophages and granulocytes.[17] The differential effect of GM-CSF on promonocytic cell lines[15,16] versus mature monocytes suggests that GM-CSF supports diverse pathways of differentiation in immature versus mature monocytic cells. Moreover, we found that GM-CSF enhances CD14 expression on neutrophils, an effect which is thus opposite to that on monocytes. GM-CSF has been shown by others to inhibit spontaneous apoptosis of neutrophils,[27] and to maintain CD16 expression,[28] an effect also found in this study.

The importance of CD14 down-regulation on monocytes is not yet clear, but several possibilities deserve some comment. First, CD14 down-regulation might represent a rapid adaptation to aggression. An important implication of the GM-CSF-induced down-regulation of CD14 may indeed be that these altered monocytes become functionally unresponsive to endotoxin *in vivo*. Blockade of TNF by antiserum confers protection of the host against the deleterious effects of endotoxin.[29] Endotoxin-induced production of TNF in septic shock may similarly be modulated by decreasing CD14 expression.[5,6] CD14 expression is also implicated in the endotoxin-induced augmentation of human immunodeficiency virus type 1 (HIV-1) production by infected myeloid cell lines and loss of CD14 expression may confer some protection of the host against acquired immune deficiency syndrome (AIDS).[30] GM-CSF thus may play a role in manipulating the expression of CD14 and thereby modify certain pathophysiologic conditions and clinical pictures.

Alternatively, the down-regulation of CD14 by GM-CSF could represent an initial manifestation of further differentiation of monocytes. Alveolar macrophages have the $CD14^+CD16^+$ phenotype, whereas peritoneal macrophages maintain high levels of CD14 expression ($CD14^{++}$).[31,32] The reason for the difference in phenotype of alveolar versus peritoneal macrophages is unknown, but may be of functional importance. There is also a subset of peripheral blood monocytes that expresses CD14 less intensely, but with an increased expression of CD16 on the cell membrane ($CD14^+CD16^+$).[32,33] This subset also seems to be distinct from regular blood monocytes, since they produce less TNF, IL-1β and IL-6[34] and may represent an early stage of differentiation towards alveolar macrophages. Another possibility is that GM-CSF promotes differentiation of monocytes towards antigen-presenting cells. GM-CSF can indeed induce or enhance the surface expression of B7,[35] CD40 and CD1 on peripheral monocytes,[36,37] which all are important for the antigen-presenting capacity of these cells. GM-CSF and IL-4 together can generate cells with dendritic morphology from cultures of peripheral blood mononuclear cells.[38] These cells have no CD14 expression, but strongly express B7, CD1 and CD40. Further investigation is needed to establish whether these monocyte-derived cells in IL-4/GM-CSF medium represent a subtype of macrophages specialized in antigen-presentation.

In conclusion, GM-CSF down-regulates CD14 expression on peripheral blood monocytes. This down-regulation is on the transcriptional level and may be a first step of further differentiation towards either alveolar-type macrophages or to a subtype of macrophages specializing in antigen presentation. The interaction of GM-CSF with other locally produced cytokines (e.g. TNF and IL-4[38,39]) may influence the final pathway of differentiation taken by mature monocytes at any particular time.

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