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ARTICLES

Effect of Cytokines and Lipopolysaccharide on CD14 Antigen Expression in Human Monocytes and Macrophages

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Abstract The 52 kD myeloid membrane glycoprotein CD14 represents the receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein (LBP); it is involved in LPS induced tumor necrosis factor-alpha production. Expression of CD14 increases in monocytes differentiating into macrophages, and it is reduced by rIFNg in monocytes in vitro. In the present study CD14 membrane antigen expression was investigated in cultures of human mononuclear leucocytes (PBL), in elutriated, purified monocytes, and in blood monocyte derived Teflon cultured macrophages. Cells were incubated for 15 or 45 h with rIL-1, rIL-2, rIL-3, rIL-4, rIL-5, rIL-6, rTNFa, rGM-CSF, rM-CSF, rTGFb1, rIFNa, lipopolysaccharide (LPS), and, as a control, rIFNg. The monoclonal antibodies Leu-M3 and MEM 18 were used for labelling of CD14 antigen by indirect immunofluorescence and FACS analysis of scatter gated monocytes or macrophages. IFNg concentrations were determined in PBL culture supernatants by ELISA. rIFNa and rIL-2 reduced CD14 in 15 and 45 h PBL cultures, an effect mediated by endogenous IFNg, since it was abolished by simultaneous addition of an anti-IFNg antibody. rIFNa and rIL-2 were ineffective in purified monocytes or macrophages. rIL-4 strongly reduced CD14 in PBL and purified monocytes after 45 h, whereas in macrophages the decrease was weak, although measurable after 15 h. The other cytokines investigated did not change CD14 antigen expression. Cycloheximide alone reduced CD14, but when added in combination with rIFNg the effect on CD14 downregulation was more pronounced. The effect of rIFNg on CD14 in PBL cultures was dose-dependently inhibited by rIL-4 and this inhibition is probably due to an IL-4 mediated blockade of IFNg secretion. LPS at a low dose increased CD14, at a high dose it produced a variable decrease of CD14 in PBL, which was probably due to LPS induced IFNg secretion. LPS strongly enhanced CD14 in 45 h cultures of purified monocytes. The results, showing that CD14 antigen expression is upregulated by LPS and downregulated by rIFNg and rIL-4, suggest that the LPS-LBP receptor is involved in the feedback response of IFNg and IL-4 to LPS stimulation.

Key words: cytokines, lipopolysaccharide, CD14 antigen, human monocytes, macrophages

CD14 is a 52 kD glycoprotein which exists in both phosphoinositol anchored membrane bound and soluble forms [1–5]. Ninety percent of circulating monocytes [6] and the vast majority of tissue macrophages [7] express membrane CD14; soluble CD14 is found in monocyte culture supernatants and in normal human plasma [8,9]. CD14 has been carefully studied at the protein and DNA level [8,10,11]. It has been widely used as a marker for myeloid cells in normal blood or tissue and in leukemia [12,13]. The function of membrane CD14 was not known until recently, when it was shown to be the receptor for a complex formed of circulating lipopolysaccharide binding protein (LBP) [14,15] and lipopolysaccharide (LPS) [16,17]. Accordingly, CD14 mediates alone or together with another molecule, LPS induced TNF production [17]. The signal transduction pathways which are activated upon occupation of CD14 include an increase in intracellular Ca^{2+} [18]. CD14 has also been shown to participate in lymphocyte function-associated antigen (LFA-1) dependent homotypic monocyte adhesion; protein kinase plays a role in the signalling pathways which couple CD14 to LFA-1 [19].

In previous studies we have described the regulation of CD14 membrane expression by recombinant interferon-gamma (rIFNg) [6]. We found that in vivo therapy with this cytokine causes an upregulation of membrane CD14 in

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monocytes of cancer patients; on the other hand membrane CD14 is downregulated when rIFNg is applied to monocytes of patients and healthy subjects in vitro [20]. This discrepancy was tentatively explained by a difference in rIFNg induced CD14 modulation of young versus aging monocytes. Accordingly, monocytes maturating in vitro and young cells entering the circulation should respond to rIFNg by a diminished and an enhanced CD14 membrane expression, respectively.

Another possibility is that in vivo, rIFNg induces other cytokines, which in turn may be responsible for the upregulation of membrane CD14 seen during rIFNg therapy [20]. To test this hypothesis, the present study investigated cytokines and growth factors with respect to their in vitro effects on CD14 membrane antigen expression in monocytes and macrophages. In addition, in view of the facts that LPS induces IFNg production [21] and that CD14 is involved in LPS binding [16,17], the response of monocyte membrane CD14 to LPS was investigated.

It is shown herein that monocyte membrane CD14 is selectively downregulated by IL-4 and IFNg and upregulated by LPS.

MATERIALS AND METHODS

Media

The following media were used for cell preparation, culture and flow cytometry: Phosphate buffered saline without Ca^{++} and Mg^{++}, pH 7.4; RPMI 1640 (Gibco) supplemented with sodium pyruvate (1mM, Seromed), nonessential amino acids (10 ml/l, Gibco), sodium bicarbonate (0.2%, Gibco), Hepes (15 mM, Gibco), gentamycin (15 mg/l, Sigma), glutamine (2 mM, Gibco) and adjusted to pH 7.4 with 1N NaOH. For culture either 5% heat-inactivated (56°C, 30 min) fetal calf serum (FCS, Gibco) or heat-inactivated AB serum (ABS pool from 5 healthy individuals), was added. Glucose phosphate buffer (GPB) contained $Na_2HPO_4 \cdot 2H_2O$ (2.4 g/l), KH_2PO_4 (0.45 g/l), KCl (0.4 g/l), glucose (1 g/l), penicillin (100 U/ml), streptomycin (100 mg/ml, Gibco), and FCS (1%), pH 7.35. Balanced salt solution (BSS) was supplemented with 4% FCS and 10 mM NaN_3, pH 7.4. PBS, RPMI, and GPB were made up in pyrogen-free H_2O filtered through a 0.22 μm and a 10000 PM Amicon filter. The concentration of endotoxin was below 0.01 ng/ml as tested by the limulus lysate assay (Skan AG, Basel).

Reagents

Recombinant interferon-gamma (rIFNg; CGP 34264 E, specific activity 2×10^7 U/mg) and recombinant IL-2 (rIL-2) were from Ciba-Geigy AG, Basel, Switzerland; recombinant interferon-alpha (rIFNa; Roferon-A) was from F. Hoffmann La Roche, Basel, Switzerland); monoclonal anti-IFNg (clones 69 B + 127 A) and anti-IFNa (clones LI8B1 + LI9) were kind gifts of Dr. H. Gallati, F. Hoffmann La Roche, Basel, Switzerland; recombinant IL-4 (rIL-4; expressed in CHO cells) and anti-IL-4 (monoclonal antibody 8F12, specific for and neutralizing IL-4) were kindly provided by Dr. Ch. Heusser, Ciba-Geigy; recombinant IL-1 beta (rIL-1b) was a kind gift of Dr. K. Vosbeck, Ciba-Geigy; recombinant IL-3 (rIL-3, expressed in CHO cells, 3×10^6 U/mg) and recombinant IL-5 (rIL-5) were kind gifts of Prof. C. Nissen, University Hospital, Basel; recombinant IL-6 (rIL-6, 2.25×10^6 U/ml) was from Genzyme, Boston, MA; recombinant transforming growth Factor beta (rTGFb) was from AMS Biotechnology, Zurich, Switzerland; recombinant tumor necrosis factor alpha (rTNFa, specific activity 8.1×10^6 U/mg) was kindly provided by BASF, Knoll, Ludwigshafen, Federal Republic of Germany; recombinant granulocyte-macrophage colony stimulating factor (rGM-CSF, 108 mg/ml) was from Sandoz, Basel, Switzerland, recombinant macrophage colony stimulating factor (rM-CSF) was from Genzyme, Boston, MA. Lipopolysaccharide (LPS) from salmonella typhimurium was from Sigma and cycloheximide from Fluka. The IFNg concentration in culture supernatant was determined by a sandwich immunoenzyme assay [22]. The antibodies for this test were kind gifts from Dr. H. Gallati, F. Hoffmann La Roche.

Antibodies for Surface Staining

The following antibodies were used: Leu-M3 (10 μl, mouse IgG 2b, Becton Dickinson [BD], Mountain View, CA); MY4 (1 μl, mouse IgGl, Coulter, Hialeah, FL); Mo2 (10 μl, mouse IgM, Coulter); MEM 18 (0.03 μl, mouse IgGl, gift of V. Bazil, Prague); FMC 32 (20 μl, mouse IgGl, Bio-Science Products, Emmenbrücke, Switzerland); UCHMl (10 μl, mouse IgG2a, Sera-Lab, GB); 63D3 (10 μl, mouse IgGl, ATCC, Maryland); Max.1 (3 μl, mouse IgGl, hybridoma supernatant, gift of Prof. F. Emmrich, Erlangen, Federal Republic of Germany); goat anti-mouse Ig FITC (6 μl, BD); FITC labelled $F(ab')_2$ fragment

of goat anti-mouse IgG (1.7 μl, Jackson, PA). Doses stated were appropriate for a sample containing 10^6 cells. All reagents were centrifuged at 100,000*g* for 10 min prior to use.

Cell Preparation

Peripheral mononuclear leucocytes (PBL) were isolated from heparinized blood of healthy volunteers by Ficoll (d = 1.077) density gradient centrifugation. After washing with PBS, the cells were resuspended either in RPMI for culture or in elutriation medium (GPB) for further purification. The PBL were loaded into a Curamé 3000 centrifuge (Heraeus) at a flow rate of 11 ml/min and elutriation was performed at a constant flow rate of 18 ml/min. The initial centrifugal force of 2,900 rpm was reduced stepwise by 100 rpm every five min down to 2,300. Lymphocytes were found in fractions collected at 2,800 to 2,600 rpm. In fractions 2,500 to 2,300 rpm monocytes and lymphocytes were mixed. The fraction collected after stopping the centrifuge contained 99% pure monocytes as determined by Giemsa and non-specific esterase staining and scatter analysis. The purified monocytes were washed and resuspended in RPMI.

Culture With Cytokines

Cells at a concentration of 2×10^6/ml in RPMI were incubated with or without (control) cytokines and/or anti-cytokines/inhibitors in either Costar (PBL, Cambridge MA) or hydrophobic teflon plates (purified monocytes; Petriperm, Heraeus, Hanau) for 15 and 45 h in the presence of 5% FCS in a humidified 5% CO_2 atmosphere at 37°C. Elutriation purified monocytes were additionally cultured in 5% ABS supplemented RPMI for 7 days to generate monocyte derived macrophages. At the end of this period macrophages were collected, washed, counted, and resuspended at a concentration of 2×10^6/ml for 15 h in medium supplemented with 5% FCS with or without cytokines. Maturation to macrophages was controlled by the expression of the Max.1 antigen [23]. Cultured cells were harvested by gentle pipetting after incubation on ice (2×15 min), washed, and resuspended in BSS at a concentration of 1×10^7/ml for surface staining.

Surface Staining

10^6 cells/sample in 100 μl BSS were pretreated for 10 min at 20°C with 5 μl ABS before incubation (30 min, 4°C) with the monoclonal anti CD14 antibodies (50 μl, diluted in BSS), or Max.1. After washing with BSS the cells were stained depending on the monoclonal antibodies isotype, with either goat anti-mouse IgG $F(ab')_2$ FITC or goat anti-mouse Ig FITC for 30 min at 4°C. Nonspecific staining was assessed by incubating the cells with the second step antibody or an irrelevant hybridoma culture supernatant. After washing, the cells were resuspended in 400 μl BSS for further analysis.

Flow Cytometry Analysis

The analysis was performed with an ORTHO cytofluorograph 50 HH (argon laser 500 mW; 488 nm). 50,000 cells/sample were acquired with the ORTHO 2150 computer in list mode. Fluorescence was analyzed from scatter gated viable monocytes. Median fluorescence intensity (FI) of 4,000 monocytes was measured with a constant linear gain setting during one experiment lasting over 45 h or one week. Results are expressed as the percentage change of median FI in treated cells relative to FI in untreated cells (100%) at a given time point.

RESULTS

Change of CD14 in Monocytes Differentiating Into Macrophages

As described previously [20], expression of monocyte membrane CD14 increased during culture in medium alone. Between 15 and 45 h, median FI increased 112% in PBL cultures (mean value from 16 experiments) and 127% in cultures of purified monocytes (5 experiments).

In macrophages which had been harvested on day 7 and recultured for an additional 15 h, median FI was not further increased (+ 103% of the 15 h value for freshly cultured monocytes). As illustrated in Figure 1, the cell size (FSC) and optical density (SSC) increased with macrophage maturation. The fluorescence spectrum of CD14 was narrow and fluorescence intensity was weak in fresh monocytes. During differentiation the CD14 spectrum became progressively broader and the fluorescence intensity stronger. In previous studies it was shown that the increase in median FI was partially due to an increase in monocyte size, and also in part due to a true acquisition of more CD14 molecules per cell [6]. The culture dependent increase of monocyte membrane CD14 occurring between day 1 and 7 was preceded by a transitory decrease that was observable when the cells were

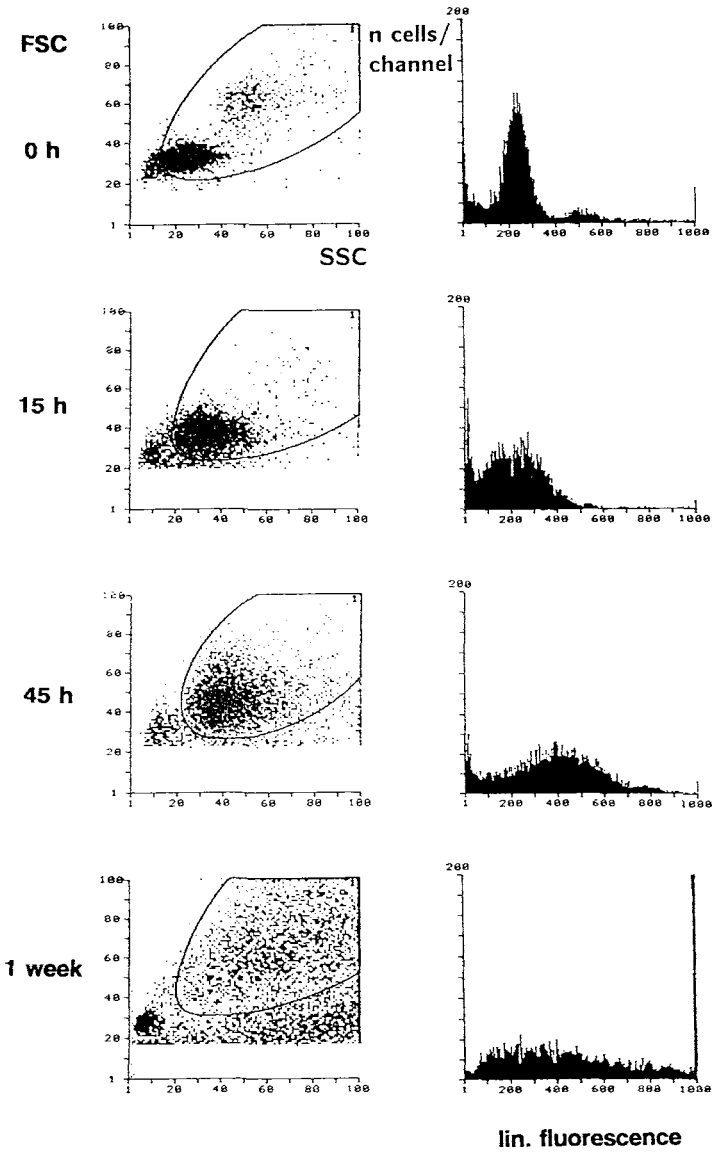

Fig. 1. In vitro maturation of elutriation purified monocytes (0h) to macrophages (1 week). **Left:** Scatter cytograms (forward scatter [FSC], side scatter [SSC]). **Right:** Fluorescence spectra (arbitrary linear units) of Leu-M3 measured in the scatter window at different times. After one week culture cells were washed and reincubated for 15 h, before CD14 analysis.

incubated in medium for only three or six hours (14 and 38% decrease in median FI, respectively, data not shown). When FI of CD14 was compared between monocytes cultured for 15 h and then subsequently reincubated in fresh medium for an additional 3 and 6 h, it was also decreased in the latter cells (by 42 and 40% respectively). It can be concluded from these experiments that handling and washing of monocytes transiently removed a factor necessary for spontaneous CD14 expression.

Effect of Cytokines and Growth Factors on CD14 in Monocytes

As expected [6], addition of rIFNg to cultures of both PBL and purified monocytes decreased monocyte CD14 antigen expression after 15 and 45 h. In both cell preparations the response was dose dependent between 0.5 and 1.25 ng/ml of rIFNg; a higher dose 5 ng/ml of rIFNg elicited no further decrease. The extent of decrease was slightly smaller in monocytes than in PBL (Ta-

TABLE I. Cytokine Effects on Monocyte CD14 Antigen Expression in Cultures of Mononuclear Leucocytes (PBL) and Elutriation Purified Monocytes

		Monocytes in PBL[a]				Elutriation purified monocytes[a]			
Cytokine	Dose	15 h	n	45 h	n	15 h	n	45 h	n
rIFNg	0.5 ng/ml	−48.7 ± 15.1[b]	10[a]	−65.2 ± 24	9	−41	1	−27	1
	1.25	−63	2	−80	1	−49.5 ± 9.9	5	−42.8 ± 21.6	4
	5	−54.7 ± 17.5	3	−76.0 ± 11.1	3	−53	1	−69.0	1
rIFNa	10 U/ml	−36.7 ± 11.2	3	−9.3 ± 10.1	4	−12	2	+7.5	2
	100	−38.1 ± 13.8	8	−36.5 ± 24.1	6	−15.7 ± 21.1	3	+23.0 ± 40.1	3
	1000	−57.3 ± 16.3	3	−81.3 ± 6.7	3	−27.3 ± 24	3	+23.0 ± 60.0	3
rIL2	1 U/ml	−0.7 ± 18.0	3	−19.5	2	+2.5	2	+29.5	2
	10	−31.5 ± 11.7	6	−57.2 ± 20.9	5	+0.7 ± 12.3	3	+60.3 ± 48.9	3
	100	−51.0 ± 7.5	3	−78.0 ± 7.0	3	+4.7 ± 4.5	3	+35.3 ± 25.7	3
rIL4	100 U/ml	+22.5	2	−22.5	2	−15.0 ± 20.2	3	−0.3 ± 13.6	3
	300	+3.6 ± 18.4	5	−45.8 ± 11.3	4	+6.2 ± 41.6	5	−45.7 ± 30.7	3
	1000	+11.7 ± 28.6	10	−67.6 ± 5.8	8	−3.2 ± 39.7	4	−47.8 ± 28.3	4

[a]PBL were cultured in costar dishes, elutriation purified monocytes in teflon wells.
[b]Results are expressed as percentage change of median FI (LeuM3 staining) in relation to cells cultured in medium alone, where median FI at each given time was arbitrarily taken to be 100%.
[c]Mean ± s.d. if ≥ 2 experiments.

TABLE II. Cytokine Effects (15-h Incubation) on CD14 Antigen Expression in Blood Monocyte Derived Macrophages (7-Days Culture on Teflon) Measured With Leu-M3 and MEM 18

Cytokine	Dose	Leu-M3	n	MEM 18	n
rIFNg	1.25 ng/ml	−19.5 ± 12.8[a]	6	−23.0 ± 17.6	4
rIFNa	10 U/ml	−10.0	1	−1.0	1
	100	−5.0	2	−5.0	2
	1,000	−13.5	2	−8.5	2
rIL2	1 U/ml	−13.0	1	−7.0	1
	10	−0.5	2	−0.5	2
	100	+5.0	2	+0.5	2
rIL4	100 U/ml	−18.0	1	−17.0	1
	300	−24.3 ± 2.9	3	−24.3 ± 9.1	3
	1,000	−32.5	2	−42.0	2

[a]Results are expressed as percentage change in median FI relative to that (median FI = 100%) of cells cultured in medium alone. Mean values ± s.d. if n > 2 experiments.

ble I, and summary in Table III). Detection of CD14 with either Leu-M3 (Table I) or other CD14 specific monoclonal antibodies, such as MEM 18, 63D3, UCHM1, MO2, MY4, or FMC32 (data not shown), yielded quantitatively consistent results with respect to this effect of rIFNg. The rIFNg related reduction in CD14 was detectable within 15 min of incubation. After washing, CD14 did not reappear on the monocyte surface for another 24 h (data not shown). It is known that IFNg upregulates its own gene expression in lymphocytes [24]; accordingly rIFNg induced its own production in PBL cultures, since the addition of 0.5 ng/ml resulted in the accumulation of 1.5 and 11.1 ng/ml IFNg in the culture supernatants after 15 and 45 h, respectively.

Both rIFNa and rIL-2 reduced CD14 antigen expression when added to PBL and their effects were dose dependent (Table I and summary in Table III). The effects of the highest doses of rIFNa (1,000 U/ml) and rIL-2 (100 U/ml) were stronger after 45 h than 15 h of incubation. In contrast, in cultures of purified monocytes neither rIFNa nor rIL-2 effected a significant change in median FI of CD14; with rIL-2, even a slight tendency toward an increase in CD14 FI occurred after 45 h (2 of 3 experiments, Table I). rIL-2 dose-dependently induced endogenous

TABLE III. Summary of Cytokine and LPS Effects on Membrane CD14*

	Monocytes in PBL		Purified monocytes		Macrophages
Culture time	15 h	45 h	15 h	45 h	15 h
Treatment					
rIFNg	↓ ↓	↓ ↓ ↓	↓ ↓	↓ ↓	↓
rIFNg + anti-IFNg	nc				
rIFNa	↓ ↓	↓ ↓ ↓	nc	nc	nc
rIFNa + anti-IFNg	↓				
rIL-2	↓ ↓	↓ ↓ ↓	nc	nc	nc
rIL-2 + anti-IFNg	nc				
rIL-4	nc	↓ ↓	nc	↓ ↓	↓
rIL4 + anti-IFNg	nc	↓ ↓			
LPS	↓	↓	↓	↑ ↑	nc
LPS + anti-IFNg	↑	↑			

* ↓ and ↑ indicate a decrease and an increase respectively in median FI in relation to cells cultured in medium alone; nc indicates no change in median FI in relation to untreated cells.

IFNg production and at 1, 10, and 100 U/rIL-2/ml, IFNg levels were, respectively, 0.22, 1.34, and 4.8 ng/ml after 15 h and 0.73, 4.5, and 13.24 ng/ml after 45 h. rIFNa also slightly induced IFNg production; and at 10, 100, and 1,000 U/rIFNa/ml IFNg concentrations in PBL supernatants were, respectively, 0.09, 0.15, and 0.22 ng/ml after 15 h and 0.18, 0.26, and 0.43 ng/ml after 45 h.

rIL-4, in contrast to the previously described cytokines, did not modulate CD14 in either PBL or monocytes after 15 h of incubation. However, after 45 h of incubation, rIL-4 did decrease the expression of this antigen in a dose dependent manner in both PBL and monocytes cultures (Table I and summary in Table III).

The response of CD14 to rIFNa, rIL-2, and rIL-4 was similar for PBL cultured either in costar dishes or in teflon wells. However, CD14 antigen reduction by rIFNg was more marked in adherent monocytes than in teflon-well suspension cultures. The changes in median FI after 15 h and 45 h were, respectively, -17.3 ± 7.3 and $-43 \pm 20\%$ ($n = 3$) for monocyte suspensions in teflon wells, as compared with $-48.7 \pm 15.1\%$ and $-65.2 \pm 24\%$ ($n = 3$) for adherent monocytes.

rIL-1b (30-200 pg/ml), rIL-3 (10-300 U/ml), rIL-5 (0.5-50 μl/ml), rIL-6 (300-3,000 U/ml), and rTNFa (300 pg–3 ng/ml) were all without effect on CD14 FI in either PBL or monocyte cultures at either 15 h or 45 h (data not shown). Similarly the growth factors rGM-CSF (40–2000 U/ml), rM-CSF (5–500 U/ml), and rTGFb1 (0.01–1 ng/ml) did not modify CD14 FI (data not shown).

Modulation of Cytokine Effects by Anti-IFNg-Antibodies

The rIFNg induced reduction of CD14 expression in PBL was antagonized by 1 μg/ml anti-IFNg, which by itself did not modify the expression of this antigen (Fig. 2A). rIFNa elicited a progressively smaller effect on CD14 when applied simultaneously to PBL in increasing doses (0.1–0.3 μg/ml) of anti-IFNg antibodies (Fig. 2B). However, even in the presence of 3 μg/ml of anti-IFNg antibodies, this cytokine still retained its capacity, albeit weakened to reduce CD14 (Fig. 2B). CD14 reduction by rIL-2 was completely due to the induction of endogenous IFNg by this cytokine, since its effect on CD14 was abolished in the presence of anti-IFNg antibodies (Fig. 2C). In contrast, the influence of rIL-4 on CD14 expression was not affected by anti-IFNg antibodies (Fig. 2D). The rIL-4 effect could, however, be completely blocked by 1 μg/ml anti-IL-4 (data not shown).

CD14 Response to Combinations of rIL-4 and rIFNg

As shown in Table I and Figure 3, rIL-4 did not significantly effect a change in PBL-CD14 expression after 15 h, whereas rIFNg (at 0.5 and 5 ng/ml) elicited a 70% reduction in expression of this antigen. On the other hand, rIL-4 was able to dose-dependently inhibit the effect of rIFNg (Fig. 3). In the presence of 100 U/ml rIL-4, 0.5 ng/ml rIFNg reduced CD14 expression by only 27% (vs. 70% in the absence of rIL-4). At a dose of 5 ng/ml rIFNg, 1,000 U/ml of rIL-4 were necessary to obtain the same de-

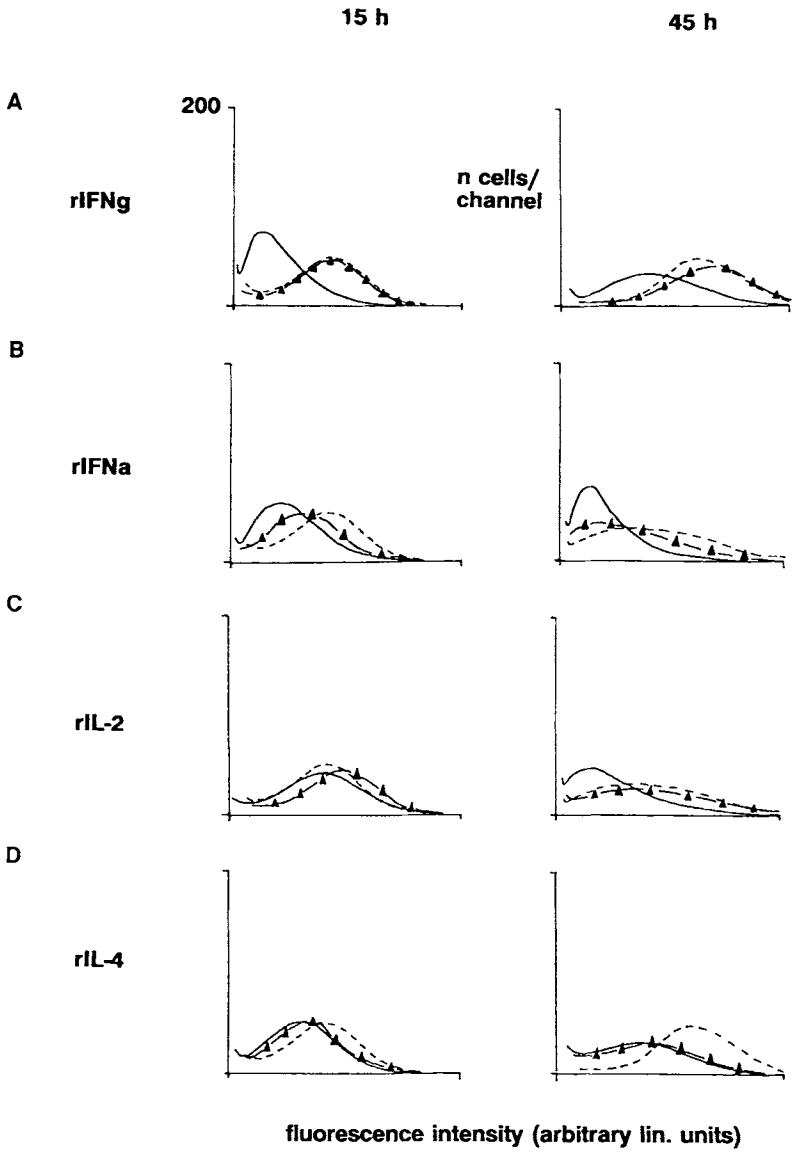

Fig. 2. CD14 antigen expression (measurement of Leu-M3 in scatter gated monocyte cluster) in 15- and 45-h cultures of mononuclear leucocytes. Effect of cytokines alone. **A:** rIFNg 1.25 ng/ml, **B:** rIFNa 10 U/ml, **C:** rIL-2 10 U/ml, **D:** rIL-4 300 U/ml (—) or in combination with anti IFNg antibody (▲—▲—▲) 1 μg/ml (A, C, D) or 3 μg/ml (B). Control cultures with medium alone (– – – –).

gree of inhibition. While both rIL-4 and rIFNg alone were able to reduce CD14, the combination of low doses of rIL-4 (100 U/ml) and rIFNg (0.5 ng/ml) blunted the reductive effects of rIFNg on CD14 after 45 h (Fig. 3). However, higher concentrations of rIL-4 (300 and 1,000 U/ml) combined with 0.5 ng/ml rIFNg resulted in a CD14 decrease which approximated that produced by rIL-4 alone. The CD14 decrease elicited by the higher rIFNg dose (5 ng/ml) was not modified by rIL-4 at 100 to 1,000 U/ml.

IFNg concentrations were determined in PBL culture supernatants. In the presence of 0.5 ng/ml rIFNg, IFNg increased from baseline values of 0.066 and 0.213 ng/ml at 15 and 45 h, respectively, to 1.482 at 15 h and 11.112 ng/ml at 45 h. IFNg was undetectable in the supernatants of PBL cultures treated with rIL-4 for 15 or 45 h. When IL-4 (1,000 U/ml) and rIFNg (0.5 ng/ml) were simultaneously added to PBL, the levels of IFNg in culture supernatants were 0.301 ng/ml and 1.89 ng/ml after 15 h and 45 h,

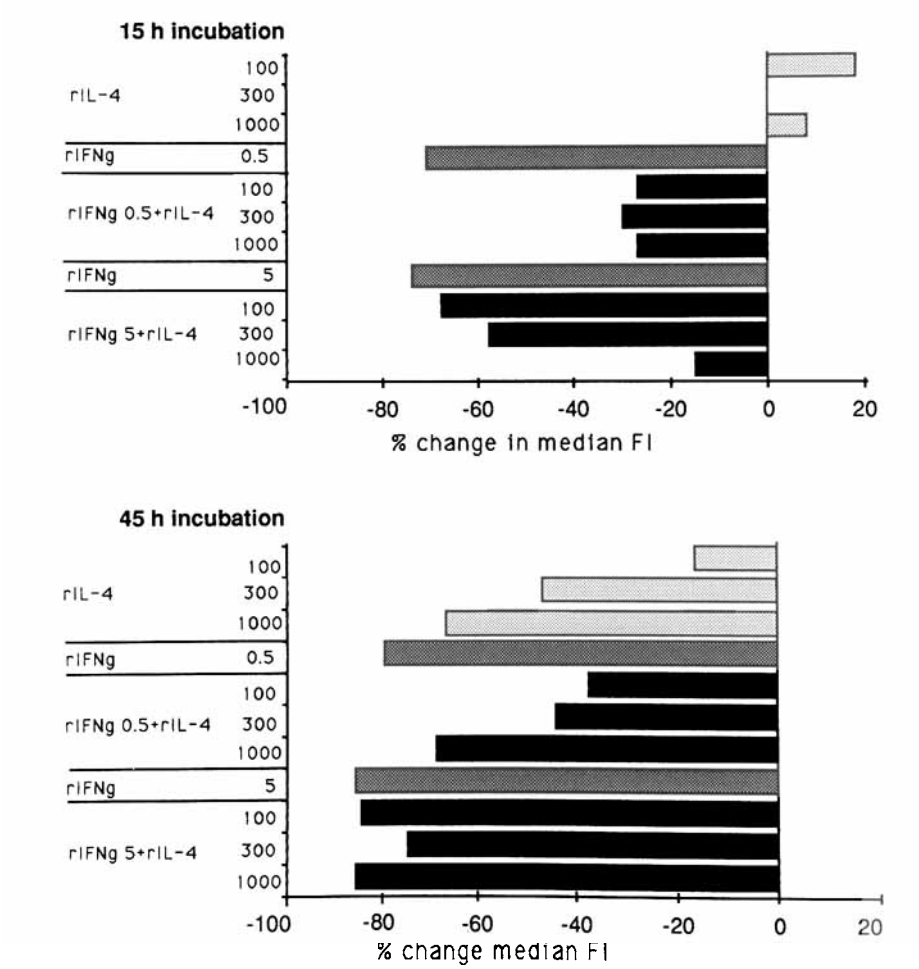

Fig. 3. Effect of rIFNg (0.5 and 5 ng/ml) and rIL-4 (100 to 1,000 U/ml) alone or in combination on CD14 antigen expression in 15- and 45-h cultures of mononuclear leucocytes. Percentage changes in median FI from baseline (cells cultured in medium alone) are indicated.

respectively. These data show that rIL-4 inhibits the ability of rIFNg to induce IFNg production by PBL and that this inhibition may relate to the decreased ability of rIFNg to downregulate CD14 in the presence of rIL-4.

Effects of Lipopolysaccharide and Cycloheximide

Upon 15 and 45 h culture of PBL with LPS, monocyte CD14 expression was found increased when 0.1 and 1 ng/ml LPS were applied, it did not change under the effect of 10 ng/ml LPS and it variably decreased under 100 ng/ml LPS (change in median FI $-24 \pm 25\%$ and $-11 \pm 23\%$ at 15 and 45 h, respectively, mean of 7 experiments, Fig. 4). Simultaneous incubation of the cells with LPS and 1 μg/ml anti-IFNg-antibodies revealed that LPS per se caused an increase in CD14 expression, which was antagonized at higher doses by the progressively higher LPS induced endogenous IFNg secretion (Fig. 4). In purified monocytes CD14 was reduced by LPS after 15 h (-29.6 and -26.7% at 10 and 100 ng/ml, respectively), but markedly increased after 45 h (by $+77.9$ and 66.4%, respectively). In 7-day-old macrophages, 15 h of incubation with a high dose of 100 ng/ml LPS did not produce any change of membrane CD14 (-7%).

Cycloheximide at a low non-toxic concentration of 0.3 μg/ml reduced CD14 in PBL cultured for 15 and 45 h in medium alone (Fig. 5), indicating that spontaneous expression of CD14 in PBL cultures is dependent on protein synthesis. The simultaneous addition of rIFNg and cycloheximide to PBL had a greater effect on reduction of CD14 expression than when either each agent was applied singly (Fig. 5).

Effects of Cytokines on CD14 in Macrophages

rIFNg and rIL-4 caused a 20% reduction of CD14 expression in 7-day cultured macrophages

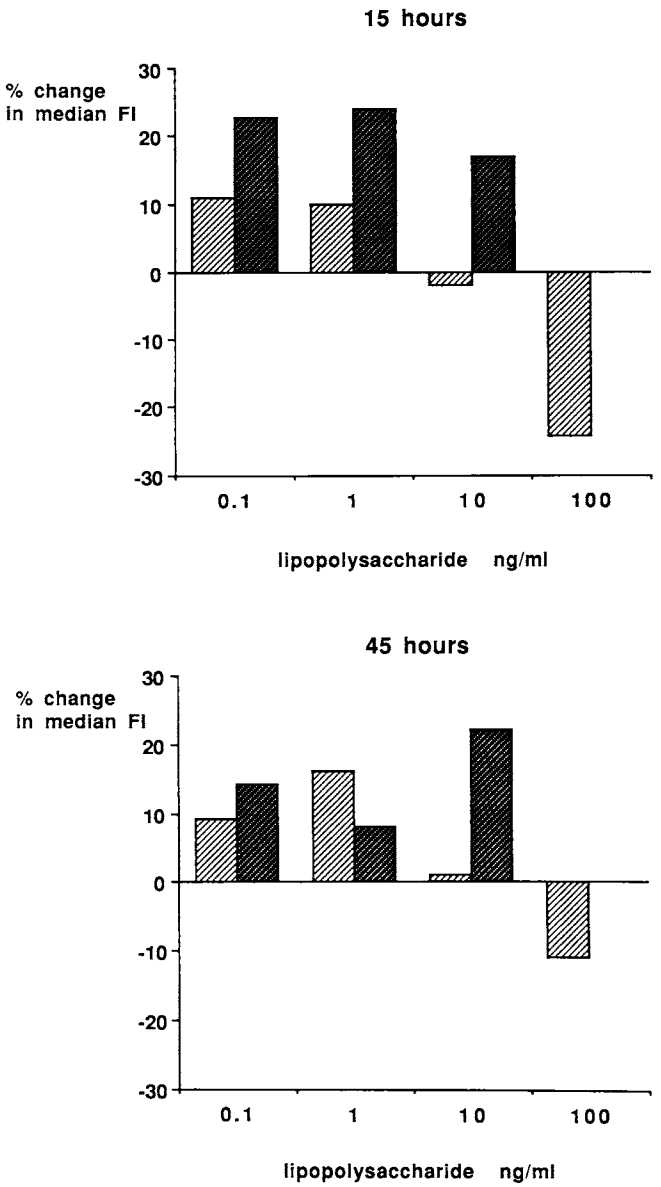

Fig. 4. Effect of lipopolysaccharide (0.1 to 100 ng/ml) on monocyte CD14 antigen expression in 15- and 45-h cultures of mononuclear leucocytes in the absence (▨) or presence (□) of 1 μg/ml anti-IFNg antibodies. Percentage changes in median fluorescence intensity (FI) from baseline (cells cultured in medium alone) are indicated.

(Table II). Thus, both cytokines exerted a smaller effect on CD14 in the differentiated macrophages, which in contrast expressed higher spontaneous levels of CD14 than circulating monocytes (Fig. 1). rIL-2 caused negligible changes of CD14 in macrophages (Table II). Cytokine induced CD14 antigen changes were similar when measured with the antibodies Leu-M3 or MEM 18 (Table II).

DISCUSSION

The present study of in vitro regulation of CD14 membrane expression revealed three major findings. First, among the many cytokines and growth factors tested, only IFNg (as previously described) [6] and IL-4 decreased the expression of CD14 on the surface of monocytes and macrophages. Second, treatment of PBL with IFNa or IL-2 led to an induction of endogenous IFNg production, which may explain the downregulation of membrane CD14 observed after IFNa and IL-2 treatment. Third, LPS was the only substance found to increase CD14 expression on the monocyte surface.

The IL-4 and IFNg effects on monocytes, although qualitatively similar, exhibited differing

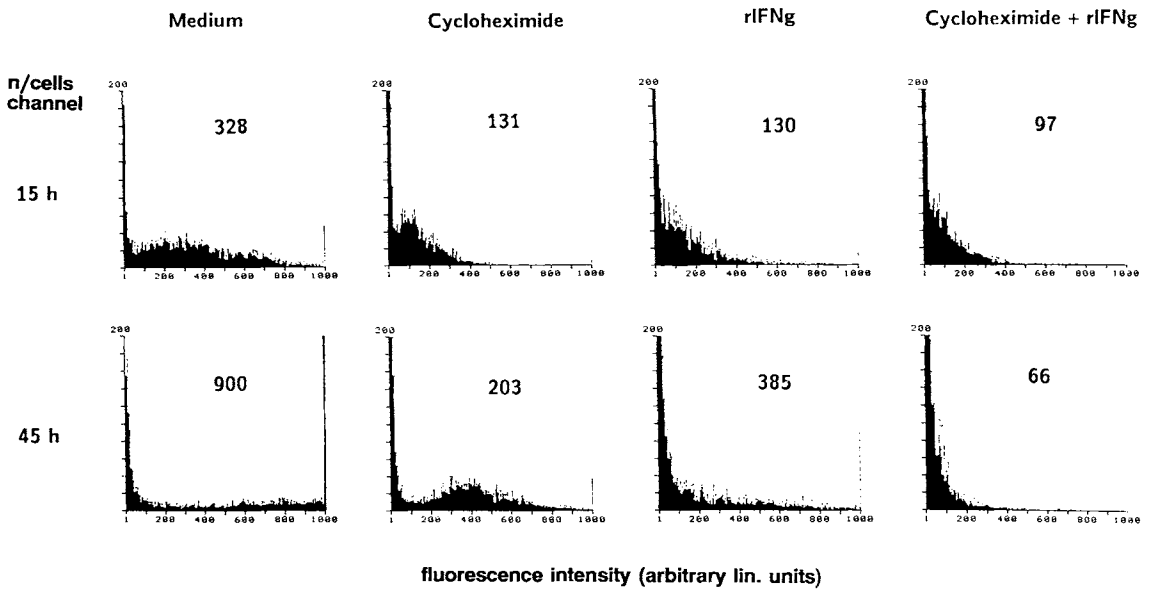

Fig. 5. CD14 antigen expression (measurement of Leu-M3 in scatter gated monocyte cluster) in 15- and 45-h cultures of mononuclear leucocytes. Effect of cycloheximide (0.3 μg/ml) and rIFNg (1.25 ng/ml) alone or in combination. Numbers indicate median fluorescence intensity.

potencies and kinetics. The IFNg effect on CD14 was very potent and we have previously described that it is detectable with 0.02 ng/ml *IFNg [20]. CD* 14 downregulation was evident as early as 15 min after initiation of the incubation and became larger when the monocytes were further treated for one to three days (present study and [6]), probably because IFNg induced its own production in PBL. Our data are in agreement with the observation that IFNg upregulates its own gene expression in lymphocytes [24]. This self-induction may explain why IFNg reduced CD14 to a greater extent in PBL than in purified monocytes which do not secrete IFNg. Adherence, which slightly activates monocytes potentiated the rIFNg effect in PBL cultures.

The IL-4 effect on monocyte CD14 was less pronounced, and a decrease of CD14 occurred only at doses higher than 50 U/ml, which is considered optimal for induction of monocyte markers [25]. Additionally, the ability of IL-4 to downregulate CD14 was observed only after 45 h of culture of fresh monocytes. IL-4 decreases monocyte Fc gamma receptor expression with similar slow kinetics [26]. This may indicate an indirect effect of IL-4 on the surface antigens via induction of an intermediate protein. In contrast to the differential influence of IL-4 and IFNg on monocytes, their effects on macrophage membrane CD14 were quantitatively and kinetically comparable.

IFNg is known to upregulate monocyte antigens such as HLA class I and II [27,28], Fc-gamma receptors [29], and integrins like LFA-1 [30]. Apart from CD14, the only other human monocyte molecules known to be downregulated by IFNg belong to the complement receptor family. There is still controversy as to whether or not the number of complement receptors 1 (CR1) is decreased following IFNg treatment [31,32]. For CR3 it has been shown that rIFNg depresses C3bi ligand binding to CR3 without modulating receptor numbers [32]. CR3 is known to bind LPS [33], and interestingly, CD14 also binds LPS via the intermediate of LBP [17]. Moreover, CD14 is involved in LPS induced TNF production [17,34]. The fact that CR3 and CD14 both share LPS binding properties and are downregulated by IFNg suggests that in the case of monocyte activation by LPS, IFNg may prevent excessive stimulation by decreasing LPS receptors. The mechanism of signal transduction subsequent to IFNg interaction with its receptor is still discussed. For molecules which are induced by IFNg, protein kinase C and Ca^{++} calmodulin kinase activation appear to be involved [35,36] and IFNg is known to enhance gene transcription [27]. However, IFNg is also known to suppress gene transcription [37]. The

present study has shown that the effect of IFNg on CD14 is more pronounced when protein synthesis necessary for maintenance of spontaneous CD14 expression is blocked by cyclohexiimide. IFNg may inhibit CD14 gene expression by decreasing the stability of mRNA or the rate of transcription. This effect may be enhanced in the presence of the translation blocker cyclohexiimide, because proteins required for CD14 transcription are lacking [38]. Another important feature of the IFNg effect is its irreversibility beyond 15 min of incubation of PBL with the cytokine.

In monocytes IL-4 enhances the expression of Fc-epsilon [39] and CR3 receptors and of p 150/95 [25]. Interestingly, it decreases Fc-gamma receptors, which mediate clearance of immune complexes, phagocytosis, and ADCC [26]. Moreover, IL-4 decreases LPS induced gene expression and release of TNFa and IL-1 [25,40]. The IL-4 related inhibition of the LPS-LBP receptor CD14 may be regarded as an anti-inflammatory signal analogous to the IL-4 related blockade of IL-1 and TNF secretion [41].

As for B cells [42], IL-4 and IFNg act on monocytes in an antagonistic way. IL-4 blocks IFNg induced Fc-gamma receptor expression and H_2O_2 production [26,43]. An antagonism between IL-4 and IFNg can also be demonstrated with respect to modulation of CD14. In the present study, IL-4 was found to inhibit the IFNg-promoted decrease in CD14 at a time when IL-4 itself was without any effects on CD14. This inhibitory effect of IL-4 is probably related to its ability to prevent IFNg synthesis [44]. During prolonged treatment with combinations of IL-4 and IFNg, CD14 expression was variously modulated, and the predominance of an effect by one or the other cytokine appeared to be a function of dosage and a resultant differential influence on the endogenous IFNg level. Such findings suggest that IL-4 and IFNg change membrane CD14 by two different pathways, although the mechanisms by which they regulate CD14 is presently unknown.

Of all substances tested, only LPS was found to enhance CD14 expression. The stimulatory effect, however, was observed only in purified monocytes which had been cultured in the presence of LPS for 45 h. In PBL cultures, endogenous IFNg and IL-4 apparently counterbalanced the upregulatory effect of LPS. Although LPS was applied in medium containing FCS (which is assumed not to contain a human CD14-binding LBP), it still was able to upregulate CD14, and this may be due to the binding of LPS to other monocyte receptors, such as CR3 [33] and the 80 kD receptor recently described by Lei and Morrison [45]. However, it cannot be excluded that LPS binds to CD14 via a LBP derived from fetal calf serum and thereby causes an upregulation of its own receptor. Such self-upregulation has been demonstrated to occur for IL-2 [46] and for IFNg [24]. Experiments in which the effect of LPS on CD14 expression and TNF production can be studied in a medium completely free of LBP and soluble CD14 will help to understand the CD14 antigen change in response to LPS.

The present data on LPS modulation of membrane CD14 in the presence of serum, suggest that CD14 is tied in a positive feedback system linking LPS stimulation to an increase in the LPS-LBP receptor expression and to induction of TNF production. Yet, CD14 has been shown to be a phospho-inositol (PI) anchored membrane protein [4,5]. For PI linked molecules there is no transmembrane domain to interact with cytosolic components and signal transduction pathways are still unknown [47]. Two mechanisms are possible: Upon LPS-LBP binding to CD14, either other signal transducing molecules may cluster and be juxtaposed to CD14 or ligand receptor complexes may be internalized and degraded [47]. Indeed, internalized ligand receptor complexes have been demonstrated after incubation of monocytes with CD14 specific antibodies [48]. It is, however, not yet clear whether or not early activation signals like a rise in Ca^{++} [18] are linked to interaction of CD14 with other molecules or to ligand receptor complex internalization.

According to the present results, the enhancement of membrane CD14 which is observed on monocytes of patients treated with rIFNg [20], cannot be attributed to the indirect effect of rIFNg on a cytokine with CD14 inducing properties. Similar to our in vivo findings, IL-4 and rIFNg have been shown to induce membrane CD14 in myeloid cell lines [49,50]. Therefore, it may be postulated that young cells entering the circulation or cell lines representing precursors of monocytes respond to rIFNg and IL-4 by an increase and differentiating monocytes by a decrease in membrane CD14.

In summary, our data show that monocyte membrane CD14 is under stimulatory control of LPS and under inhibitory control of IFNg and IL4. The results may indicate that CD14 participates *in a feedback* regulatory system. Namely, expression of the LPS-LBP receptor is increased by LPS in the course of a gram-negative infection. The cytokines IFNg and IL4 which are subsequently released may prevent excessive monocyte activation by downregulating CD14.

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